Endothelial Tie growth factor receptor provides antigenic marker for assessment of breast cancer angiogenesis

P Salvén¹, H Joensuu¹, P Heikkilä², M-T Matikainen³, V-M Wasenius¹, A Alanko⁴ and K Alitalo²

¹Department of Oncology, Helsinki University Central Hospital, Haartmanink. 4C, 00290 Helsinki, Finland; ²Molecular/Cancer Biology Laboratory and Department of Pathology, Haartman Institute, University of Helsinki, PL 21, 00014 Helsinki, Finland; ³BIO CITY of the University of Turku, 20520 Turku, Finland; ⁴Department of Surgery, Helsinki University Central Hospital, Haartmanink. 4C, 00290 Helsinki, Finland.

Summary: Breast cancer prognosis has previously been linked to the degree of tumour vascularisation. In order to establish additional markers for tumour angiogenesis, we have used monoclonal antibodies against the endothelial Tie receptor tyrosine kinase to study the degree of vascularisation of breast carcinomas and the regulation of Tie expression in the vascular endothelial cells. Antibodies were used for Tie detection and the results were correlated with other prognostic markers. Of four monoclonal antibodies directed against different epitopes of the Tie extracellular domain, two reacted against Tie in unfixed histopathological sections of breast carcinomas. One of these antibodies (clone 7e8) was specific for the endothelial cells whereas the other (clone 10f11) also reacted with basement membranes and occasional carcinoma cells. When Tie expression was studied with the antibody clone 7e8, all 28 carcinomas, two in situ carcinomas, samples of histologically normal breast tissue (n = 16) or normal skin or lymph node tissue (n = 5) showed staining. Microvessel counts were higher in carcinomas (median 14; range 3–27) than in fibroadenomas (median 10; range 5–18) or histologically normal breast tissue (median 7; range 3–15, P = 0.0006). A similar result was obtained using antibodies against the CD31 (PECAM) antigen. Microvessel counts in 7e8 staining were not significantly associated with primary tumour size, axillary nodal status, histological grade or staining for oestrogen receptor, progesterone receptor, Ki-67 proliferation marker or p53 oncoprotein.

Keywords: breast cancer; tumour angiogenesis; Tie; receptor; tyrosine kinase; signal transduction

Receptor tyrosine kinases (RTKs) play key roles in signal transduction across the plasma membrane and thus have a significant role in regulating cellular proliferation and differentiation (van der Geer et al., 1994). Tie (Partanen et al., 1992) and Tek (Dumont et al., 1993) are members of a new subfamily of endothelial cell RTKs whose extracellular domains contain three different types of structural motifs: immunoglobulin (Ig)-like loops, cysteine-rich epidermal growth factor (EGF)-like domains and fibronectin type III (FN III) domains (Maisonpierre et al., 1993; Sato et al., 1993; Ziegler et al., 1993).

The pattern of Tie mRNA distribution in embryonic endothelia and also in some tumours suggests that Tie plays an important role in the development of embryonic vasculature and possibly also in angiogenesis associated with tumorigenesis. Tie mRNA is especially prominent in endothelial cells during embryonic angiogenesis (Sato et al., 1993; Dumont et al., 1993; Korhonen et al., 1992, 1994, 1995). Disruption of the Tie gene loci by targeted mutagenesis was lethal. Tie-deficient embryos survived after the time point when Tie expression normally begins, but the mice died of haemorrhage soon after birth (Puri et al., 1995; Sato et al., 1995). Analysis of these pups indicates that Tie is essential for the formation of microvessels and that this defect is cell-autonomous (Puri et al., 1995). Thus, one could speculate that the inhibition of Tie function could have therapeutic potential in the prevention of proliferation of the microvascular endothelium in human solid tumours.

In adult mice, Tie mRNA has been detected in vascular endothelium of capillaries of the lung, kidney and bone marrow (Korhonen et al., 1994). Tie mRNA has also been detected in endothelia of various tissues of adult rats. (Maisonpierre et al., 1993). These tissues include brain, cerebellum, heart, skeletal muscle, lung, kidney, liver, spleen, thyroid, adrenal gland and ovary. However, the signal for Tie mRNA is reduced in the endothelia of neural tissues of adult rats when compared with the embryonic and neonatal neural tissue. Expression of Tie mRNA has also been detected in human haematopoietic progenitor cells (Batard et al., 1996; Hashiyama et al., 1996), in leukaemia cell lines and in some cell lines from solid tumours (Partanen et al., 1992; Armstrong et al., 1993).

Several studies have pointed out the importance of angiogenesis for tumour growth and progression (Folkman, 1990, 1992). Microvessel density has been shown to be an independent parameter of the severity of tumour disease and a predictor of prognosis of breast cancer patients in most but not all studies addressing this issue (Weidner et al., 1991, 1992; Bosari et al., 1992; Horak et al., 1992; Toi et al., 1993, Axelsson et al., 1995). A similar correlation has also been demonstrated with other human malignancies, including head and neck squamous cell carcinoma (Gasparini et al., 1993), lung adenocarcinoma (Yamazaki et al., 1994), rectal carcinoma (Saclarides et al., 1994), testicular germ cell tumours (Olivarez et al., 1994), prostate cancer (Weidner et al., 1993), tumours of the oral cavity (Williams et al., 1994) and gastric carcinoma (Maeda et al., 1995). Various endothelial cell specific antibodies have been used in immunohistochemistry to quantitate blood vessels within the tumours. Antibens used for this purpose include von Willebrand factor, CD31 (PECAM) and CD34. The purpose of this work was to study the Tie protein in angiogenesis associated with breast cancer. Our aim was also to evaluate the utility of Tie detection as a measure of breast tumour angiogenesis and the association of Tie expression with prognostic factors in breast cancer.
Materials and methods

Tissue sources

Freshly frozen sections of 56 tissue samples in total containing both normal and malignant tissues were retrieved from the histopathological files of the Department of Pathology, University of Helsinki. The tissues examined included 19 infiltrating ductal carcinomas, six infiltrating lobular carcinomas, two infiltrating tubular carcinomas, one ductal carcinoma in situ, one lobular carcinoma in situ, five benign fibroadenomas, one adenosis, 16 samples of normal breast tissue, two normal axillary lymph nodes and three samples of normal breast skin.

Immunostaining

The primary antibodies used were: mouse monoclonal anti-Tie, clones 10F11 and 7e8, used on sections at a concentration of 8 μg ml⁻¹ and mouse anti-CD31, clone HC1/6 (Novocastra Laboratories), used on sections at a concentration of 0.66 μg ml⁻¹. Staining with the primary antibodies was for 1 h at room temperature. Control stainings included irrelevant monoclonal antibodies of the same isotype as well as anti-Tie incubated overnight with a 5-fold molar excess of the Tie extracellular domain expressed in baculovirus (Batard et al., 1996).

Frozen sections (5 μm) on slides were dried at room temperature overnight. Following rehydration in phosphate-buffered saline (PBS) for 5 min the sections were overlaid with normal horse serum for 20 min before incubation with the primary antibody. Subsequent incubation for 30 min in biotinylated anti-mouse serum was followed by a 30 min incubation using reagents of the Vectastain Elite ABC kit (Vector laboratories). Peroxidase activity was developed with 3-amino-9-ethyl carbazole (Sigma) for 10 min. Finally, the sections were stained with haematoxylin for 5 min.

Following the staining procedures, all samples were examined by a trained pathologist. The highest microvessel counts were assessed according to Weidner et al. (1991). After the area of highest amount of stained microvessels (so-called vascular hotspots) was identified by light microscopy, individual stained microvessels were counted using a 400 × magnification field (i.e. 40 × objective lens and 10 × ocular lens). Each count was expressed as the highest number of stained microvessels identified within any high-power field (hpf).

Statistical analysis

Staining count distributions of different groups were analysed using Kruskal–Wallis’s analysis of variance and the Mann–Whitney test.

Results

Tie protein in histologically normal tissue

Tie protein was consistently detected in the microvessel endothelial cells of histologically normal dermis (n = 3), axillary lymph nodes (n = 2) and breast tissue (n = 16) and with anti-Tie antibodies clones 7e8 and 10F11 (Figure 1a and b). Antibody of clone 10F11 stained breast myoepithelial cells in some of the samples, but the staining intensity was weaker than for endothelial cells. Microvessel counts per hpf were

![Figure 1](https://example.com/image.jpg)  
Figure 1  Peroxidase immunostaining of tie in normal and pathological tissues. MAb 7e8 staining of normal skin (a) and breast (b) as well as invasive ductal carcinoma (e). Staining with tie antigen-blocked MAb is also shown (d). Scale bar = 100 μm.
higher when staining was performed using the clone 7E8 antibody (Table I). No specific staining was observed when the sections were incubated with antigen-blocked antibody instead of the primary antibodies, or with the peroxidase-conjugated secondary antibody only.

**Tie protein in breast tumours**

Microvessel counts in fibroadenomas were not significantly higher than those found in normal breast when the anti-Tie antibody clone 7E8 was used (median, 10 per hpf vs 7 respectively, P = 0.12). The microvessel counts were higher in fibroadenomas than in histologically normal breast tissue when anti-CD31 staining was used (median, 10 vs 7 respectively, P = 0.002). The highest microvessel counts were also clearly greater in breast cancer tissue than in normal breast tissue in staining for CD31 (median, 30 vs 15 respectively, P < 0.0001). No specific staining was observed when the tumour sections were incubated with antigen-blocked antibody or normal serum instead of the anti-Tie antibodies (Figure 1d).

In some of the invasive breast cancer samples the anti-Tie antibody clone 10F11 also stained carcinoma cells, basement membranes and myoepithelial cells, whereas the clone 7E8 antibody stained vascular endothelial cells only.

The highest microvessel counts obtained by staining for Tie protein with the two anti-Tie antibodies and for CD31 were correlated with the primary tumour size (<2 cm vs >2 cm), presence of axillary nodal metastases (pN0 vs pN+), histological gradus (well- or moderately differentiated vs poorly differentiated), oestrogen and progesterone receptor status, Ki-67 expression (lower vs higher than the median, 15%), and p53 expression (negative vs positive) among the 27 carcinomas studied, but no significant correlations were found between these parameters and the microvessel counts.

**Discussion**

Several studies have suggested that the Tie protein plays an important role in angiogenesis (Partanen et al., 1992; Sato et al., 1993; Korhonen et al., 1994, 1995). In recent studies high amounts of Tie mRNA and also Tie protein have been detected in human brain tumours in contrast to the less abundant expression of Tie mRNA or protein in the respective normal brain tissue control samples (Kaipainen et al., 1994; Hatva et al., 1995). These studies suggest that a significant difference exists in the expression of Tie when the endothelia of malignant tumours of the CNS are compared with the endothelia of normal adult brain.

Our results show Tie protein in the vascular endothelia of several types of normal human tissues, including normal skin and breast tissue. In a previously published work with human melanoma and normal skin samples using *in situ* hybridisation the Tie probe hybridised very weakly with the vascular endothelium of capillaries of normal skin, except for the endothelium of sweat gland vessels (Kaipainen et al., 1994). In addition, Tie expression appeared to be enhanced in areas of inflammatory reaction around certain skin melanomas. The differences between the results in this *in situ* hybridisation and the present immunohistochemical staining results can be due to a tissue-specific variation in the level of Tie expression, a difference in the sensitivity of detection techniques used or translational regulation of Tie expression. The fact that the 10F11 antibody cross-reacts with an antigen expressed in ductal myoepithelium and some breast carcinomas is not unexpected among monoclonal antibodies, which often detect very small epitopes. Such epitopes may resemble structures in other proteins. However, at least in brain tumours and melanomas Tie sequences as such are only expressed in tumour endothelia and not detected in *in situ* hybridisation of the tumour cells (Hatva et al., 1995; Kaipainen et al., 1994) excluding the possibility that Tie would be aberrantly expressed at least in a significant fraction of myoepithelial or breast carcinoma cells.

In contrast to the expression of CD31 we did not detect Tie expression in all microvessels of normal or malignant breast tissue. Thus the Tie antigen may not be expressed in all endothelial cells. A more likely explanation is, however, that the level of Tie expression is so low that it does not allow immunohistochemical detection. One parameter affecting the staining intensity is obviously the thickness of the histological sections used for staining and another concerns the possible masking of the Tie epitope recognised by the antibodies in tissue sections.

Tumour angiogenesis is essential for tumour growth and metastasis and intratumoral microvessel density correlates with prognosis in breast carcinoma and also in other human tumours. In this study the difference in the Tie-positive microvessel counts between the groups of invasive breast carcinomas and normal breast tissue samples was statistically significant (Mann–Whitney test, P = 0.0002). The difference between these groups was similar to the difference observed when using the anti-CD31 antibody. This result suggests that Tie might have significance as an indicator of tumour angiogenesis and as a prognostic marker for breast cancer patients. Although the microcapillary network of breast carcinomas is known to be unevenly distributed, our results do not suggest that Tie would be up-regulated in breast carcinomas. Whether Tie is enhanced in tumour vessels outside the central nervous system needs further study using more quantitative methods of analysis. Thus we cannot yet exclude scenarios of tumour treatment based on possible differential Tie expression in normal and tumour endothelia.

However, the Tie receptor may appear on the luminal surface of endothelial cells and circulating Tie antigen may be present in the blood. Such findings could preclude the use of anti-Tie antibodies for intravascular injection because of the possible

### Table I

| Antibody | Normal breast (n=16) | Fibroadenoma (n=5) | Breast cancer (n=27) | P       | Kruskal–Wallis test |
|----------|---------------------|-------------------|---------------------|---------|-------------------|
| 7E8      |                     |                   |                     |         |                   |
| Range    | 3–15                | 5–18              | 3–27                | <0.0006 |                   |
| Median   | 7                   | 10                | 14                  |         |                   |
| CD31     |                     |                   |                     |         |                   |
| Range    | 8–30                | 18–28             | 18–40               | <0.0001 |                   |
| Median   | 15                  | 21                | 30                  |         |                   |

Statistical analysis of microvessel counts using the anti-tie antibody clone 7E8 and the anti-CD31 antibody in normal breast tissue, fibroadenoma and invasive breast cancer
formation of immunocomplexes with adverse side-effects. However, it remains possible that inhibition of Tie function by, e.g., interference with Tie-ligand interaction or Tie-specific signal transduction, could prevent tumour angiogenesis.

Acknowledgements
We thank Eola Kukk and Drs Juha Partanen and Riitta Alitalo for collaboration in the generation of anti-Tie monoclonal antibodies. The skilful technical assistance of Ms Päivi Heino, Mrs Päivi Laurila, Ms Mari Helanterä, Mrs Tuula Lindholm is gratefully acknowledged. We thank Dr Vijay Kumar for checking the language. This study was supported by The Finnish Academy, The Idä Montin Foundation, The Finnish Cancer Organizations, The Sigrid Juselius Foundation and the State Technology Development Centre. We thank Dr Vijay Kumar for checking the language.

References

ARMSTRONG E, KORHONEN J, SILVENNOJINEN O, CLEVELAND J, LIEBERMAN MA AND ALITALO R. (1993). Expression of Tie receptor tyrosine kinase in leukemia cell lines. Leukemia, 7, 1585 – 1591.

AXELSSON K, LJUNG B-M, MOORE D, THOR A, CHEW K, EDGE-RTON S, SMITH H AND MAYALL B. (1995). Tumor angiogenesis as a prognostic assay for invasive ductal breast carcinoma. J. Natl Cancer Inst., 13, 997 – 1008.

BATARD P, SANISLIVESTRI P, SCHNEEINCKER C, KNAPP W, DEBILI N, VAINCHENKER W, BÜHRING H-J, MONIER M-N, KUKK E, PARTANEN J, MATIKAINEN M-T, ALITALO R, HATZFIELD J AND ALITALO K. (1996). The Tie receptor tyrosine kinase is expressed by human hematopoietic progenitor cells and by a subset of megakaryocytic cells. Blood, 86, 1729 – 1735.

BEGG J, DELELLIS R, WILEY B, HEATLEY G AND SILVERMAN M. (1992). Microvascular quantitation and prognosis in invasive breast carcinoma. Hum. Pathol., 23, 755 – 761.

DUMONT DJ, GRADWOHL GJ, FONG G-H, AUERBACH R AND BREITMAN ML. (1993). The endothelial-specific receptor tyrosine kinase, tek, is a member of a new subfamily of receptors. Oncogene, 8, 1293 – 1301.

FOLKMAN J. (1990). What is the evidence that tumors are angiogenesis dependent? J. Natl Cancer Inst., 82, 4 – 6.

FOLKMAN J. (1992). The role of angiogenesis in tumor growth. Semin. Cancer Biol., 3, 65 – 71.

GASPARRINI G, WEIDNER N, MALUTA S, POZZA F, BORACCHI P, MEZZETTI M, TESTOLIN A AND BEVILACQUA P. (1993). Intratumoral microvascular density abd p53 protein: correlation with metastasis in head-and-neck squamous-cell carcinoma. Int. J. Cancer, 55, 739 – 744.

HASHIYAMA M, IWAMA A, OSHIRO K, KUROZUMI K, YASUNGA K, SHIMIZU Y, MASUHO Y, MATSUDA I, YAMAGUCHI N AND SUEDA T. (1996). Predominant expression of a receptor tyrosine kinase, Tie, in hematopoietic stem cells and B cells. Blood, 87, 93 – 101.

HATVA E, KAIPAINEN A, JÄÄSKELÄINEN J, HALTIJA M AND ALITALO K. (1995) Endothelial cell-specific receptor tyrosine kinases and growth factors in human gliomas and meningiomas. Ann. J. Pathol., 146, 368 – 378.

HORAK E, LEEK R, KLEINK N, LEJEUNE S, SMITH K, STUART N, GREENALL M, STEPNIEWSKA K AND HARRIS A. (1992). Angiogenesis, assessed lately endothelial cell adhesion molecule antibodies, as indicator of node metastasis and survival in breast cancer. Lancet, 340, 1120 – 1124.

KAIPAINEN A, VLAYKOVTA H, HATVA E, BÖHLING T, JEKUNEN A, PYHRONEN S AND ALITALO K. (1994). Enhanced expression of the Tie receptor tyrosine kinase mRNA in the vascular endothelium of metastatic melanomas. Cancer Res., 54, 6571 – 6577.

KORHONEN J, PARTANEN J, ARMSTRONG E, VAAHTOKARI A, ELENIUS K, JAKLANEN M AND ALITALO K. (1992). Enhanced expression of the Tie receptor tyrosine kinase in endothelial cells during neovascularisation. Blood, 15, 2548 – 2555.

KORHONEN J, POLVI A, PARTANEN J AND ALITALO K. (1994). The mouse Tie receptor tyrosine kinase gene: expression during embryonic angiogenesis. Oncogene, 9, 395 – 403.

KORHONEN J, LAHTINEN I, HÄLMKYTÖ M, ALHONEN L, JÄNNE J, DUMONT D AND ALITALO K. (1995). Endothelial-specific gene expression directed by the Tie gene promoter in vivo. Blood, 5, 1828 – 1835.

MAEDA K, CHUNG Y, TAKATSUKA S, OGAWA Y, SAWADA T, YAMASHITA Y, ONODA N, KATO Y, NIITA A, ARIMOTO Y, KONDO Y AND SOWA M. (1995). Tumor angiogenesis as a predictor of recurrence in gastric carcinoma. J. Clin. Oncol., 13, 477 – 481.

MAISONPIERRE PC, GOLDFARB M, YANCOPoulos GD AND GAQI G. (1993). Distinct rat genes with related profiles of expression define Tie receptor tyrosine kinase family. Oncogene, 8, 1631 – 1637.

OLIVAREZ D, UBRIGHT T, DERIESE W, FOSTER R, REISTER T, EINHORN J, L AND SLEDGE G. (1994). Neovascularization in clinical stage A testicular germ cell tumor: prediction of metastatic disease. Cancer Res., 54, 2800 – 2802.

PARTANEN J, ARMSTRONG E, MÄKELÄ T, KORHONEN J, SANDBERG M, RENKONEN R, KNUUTILA S, HUENBER K AND ALITALO K. (1992). A novel endothelial cell surface receptor tyrosine kinase with extracellular epidermal growth factor homology domains. Mol. Cell. Biol., 12, 1698 – 1707.

PURI M, ROSSANT J, ALITALO K, BERNSTEIN A AND PARTANEN J. (1995). The receptor tyrosine kinase Tie is required for the integrity and survival of vascular endothelial cells. EMBO J., 23, 5884 – 5891.

SACCLARIDES T, SPEZIALE N, DRAB E, SZELOUGA D AND RUBIN D. (1994). Tumor angiogenesis and rectal carcinoma. Dis. Colon. Rectum., 37, 921 – 926.

SATO T, QIN Y, KOZAK CA AND AUDUS K. (1993). Tie-1 and Tie-2 define another class of putative receptor tyrosine kinase genes expressed in early embryonic vascular system. Proc. Natl Acad. Sci. USA, 90, 9335 – 9338.

SATO T, TOZAWA Y, DEUTSCH U, WOLBURG-BCHOLZ K, FUJIWARA Y, GENDRON-MAGUIRE M, GRIDLEY T, WOLBURG H, RISAU W AND QIN Y. (1995). Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. Nature, 376, 70 – 74.

TOI M, KASHITANI J AND TOMINAGA T. (1995). Tumor angiogenesis is an independent prognostic indicator in primary breast carcinoma. Int. J. Cancer, 55, 371 – 374.

VANDER GIER P, HUNTER T AND LINDBERG RA. (1994). Receptor protein-tyrosine kinases and their signal transduction pathways. Annu. Rev. Cell Biol., 10, 251 – 337.

WEIDNER N, CARROLL P, FLAX J, BLUMFELD W AND FOLKMAN J. (1993). Tumor angiogenesis correlates with metastasis in invasive prostate carcinoma. Am. J. Pathol., 143, 401 – 409.

WEIDNER N, FOLKMAN J, POZZA F, BEVILACQUA P, AD RED E, MOORE D, MELI S AND GASPARRINI G. (1992). Tumor angiogenesis: a new significant and independent prognostic indicator in early-stage breast carcinoma. J. Natl Cancer Inst., 82, 1875 – 1887.

WEIDNER N, SEMPLE J, WELCH W AND FOLKMAN J. (1991). Tumor angiogenesis and metastasis—correlation in invasive breast carcinoma. N. Engl. J. Med., 324, 1 – 8.

WILLIAMS J, CARLSON G, COHEN C, DEROSE P, HUNTER S AND JURKIEWICZ M. (1994). Tumor angiogenesis as a prognostic factor in oral cavity tumors. Am. J. Surg., 168, 373 – 380.

YAMAZAKI K, ABE S, TAKEKAWA H, SUKOH N, WATENBANE N, OGURA S, NAKAJIMA I, ISOBIE H, INOUE K AND KAWAKAMI Y. (1994). Tumor angiogenesis in lung adenocarcinoma. Cancer, 74, 2245 – 2250.

ZIEGLER SF, BIRD TA, SCHNERINGER JA, SCHOOLEY KA AND BAUM PR. (1993). Molecular cloning and characterization of a novel receptor protein tyrosine kinase from human placenta. Oncogene, 8, 663 – 670.