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## Original Paper

# Intratumoral Microvessel Density and Expression of ED-A/ED-B Sequences of Fibronectin in Breast Carcinoma

M.C. D'Ovidio,[1] A. Mastracchio,[1] A. Marzullo,[1] M. Ciabatta,[1] B. Pini,[1] S. Uccini,[1] L. Zardi[2] and L.P. Ruco[1]

[1]Dipartimento di Medicina Sperimentale e Patologia, Università La Sapienza, Viale Regina Elena 324, 00161 Rome; and [2]Istituto Nazionale per la Ricerca sul Cancro, Genoa, Italy

**The aim of this study was to examine the correlation between intratumoral microvessel density (iMVD) and the presence of cellular fibronectin isoforms, ED-A and ED-B, in order to identify those tumours with a prominent angiogenic phenotype. 91 cases of invasive ductal breast carcinoma were evaluated for TNM, histological grading, percentage of Ki-67+ cells and receptor hormonal status. iMVD was determined as a single microvessel count in a 200× microscope field from the region of the tumour that appeared to be most densely vascular. When the mean values of iMVD of the various groups were compared, no significant difference was noted (Mann–Whitney test). When tumours were classified as high or low iMVD, based on a cut-off value (99 vessels/0.74 mm$^2$), cases with high iMVD were significantly more numerous in poorly differentiated G3 tumours ($P = 0.01$, Chi-square test), and in tumours with lymph node metastasis (N0 versus N1 + N2; $P = 0.002$). The possibility that high iMVD was the expression of prominent vascular neoformation was explored using ED-A and ED-B isoforms of fibronectin as markers of neoformed vessels. ED-A+ and/or ED-B+ blood vessels were < 10% of total vessels, were detected in approximately 50% of cases independently of iMVD values, and were not more numerous in tumour areas with hot spot vascularisation. Our findings indicate that iMVD and expression of ED-A/ED-B reflect different aspects of tumour-associated angiogenesis.** © **1998 Elsevier Science Ltd. All rights reserved.**

**Key words: angiogenesis, microvessel density, ED-A, ED-B, fibronectin, breast carcinoma, tumour**
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## INTRODUCTION

THE ROLE of intratumoral microvessel density (iMVD) as an indicator of tumour prognosis is controversial [1–15]. It has been suggested that iMVD is an independent prognostic factor for overall survival and for disease-free survival [1–9]. Other authors have not confirmed these observations [10–15], although some established that higher values of iMVD are more often detected in metastatic tumours, in poorly differentiated tumours, and in more aggressive histological subtypes [13, 14].

Neoangiogenesis results in the appearance of neoformed vessels which can be recognised by the presence of specific antigenic trails. Cellular fibronectin is a high molecular mass adhesive glycoprotein present in the extracellular matrix in different isoforms, resulting from the alternative splicing of three regions (IIICS, ED-A, ED-B) of the fibronectin primary transcript, as well as from post-translational modification [16, 17]. It has been found that ED-A and ED-B are two isoforms of cellular fibronectin often associated with blood vessels [18–21]. The observation that ED-B is absent in normal adult tissues, and is associated with blood vessels of gliomas, breast carcinomas, and of inflammatory tissues, has raised the possibility that the molecule could be used as a marker for neoformed vessels [21].

In the present study, we correlated iMVD with the expression of ED-A and ED-B in tumour-associated vessels in the attempt to identify those tumours characterised by a prominent angiogenic phenotype.

## MATERIALS AND METHODS

*Patients and immunohistochemistry*

91 cases of invasive breast carcinoma were studied. All patients were Caucasian females with a mean age of 59 ± 12

Correspondence to L.P. Ruco.
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years; 53 cases were node negative and 38 were node positive (N1 + N2). 29 cases were classified as G1, 41 as G2, and 21 as G3, according to Eltson and Ellis [22]. Fragments of fresh tissue were snap-frozen in liquid nitrogen and cryopreserved at − 80°C until sectioning. Fresh cryopreserved material was used to avoid the possibility of reduced sensitivity of the immunostaining due to the paraffin embedding procedure. Other tumour fragments were formalin fixed, and paraffin embedded for conventional histology. All cases were routinely evaluated using immunohistochemistry for the percentage of proliferating cells (Ki-67 +, Dakopatts, Denmark) and oestrogen and progesterone receptors (Abbott Laboratories, Customer Support Center, North Chicago, U.S.A.).

Cryostat sections were fixed in acetone for 10 min at room temperature. Slides were pre-incubated with normal horse serum to prevent non-specific binding; they were then incubated with an optimal dilution of the primary antibody for 30 min, and sequentially with biotin-conjugated horse anti-mouse immunoglobulin antibody followed by avidin–biotin–peroxidase complex (ABC) (PK 8800; Vector Laboratories, Burlingame, California, U.S.A.). Each incubation step lasted 10 min with 5 min phosphate buffer saline (PBS) washes between each step. The sections were then incubated with 0.03% $H_2O_2$ and 0.06% 3,3′-diaminobenzidine (BDH, DAB; Sigma-Aldrich, St Louis Missouri, U.S.A.) for 3–5 min. Finally, slides were washed for 5 min in running tap water and counterstained with haematoxylin for 5 min. In all experiments, a routine control was included in which the primary antibody was omitted.

Optimal visualisation of blood vessels was achieved on cryostat sections immunostained with a mixture of anti-CD31 (5F4; kindly provided by Dr E. Dejana, Istituto 'Mario Negri', Milan, Italy), and anti-von Willembrand factor (vWF) (Dakopatts, Denmark) monoclonal antibodies (MAbs). iMVD values were determined according to the method of Weidner and colleagues [23]. Briefly, immunostained slides were examined by two investigators for the identification of highly vascularised areas. The number of microvessels present in a microscope field at 200×, corresponding to an area of 0.74 $mm^2$, was considered the value of iMVD. Tumours with iMVD values higher than 99 (the mean value of all the investigated cases) were classified as highly vascularised. Statistical analysis was conducted using the Mann–Whitney test, for evaluating differences in the mean values of immunostained microvessels, and the Chi-square test applied to the percentages of cases with high iMVD [(iMVD value higher than 99/total iMVD cases)×100]. All tests were two-tailed and $P < 0.05$ was accepted as significant.

In a preliminary study, aimed to optimise experimental conditions, tumour sections from 5 cases were immunostained with antibodies directed against six endothelial antigens (vWf, CD31, CD34, Qbend/10; Signet Laboratories, Dedham, Massachusetts, U.S.A.; type IV collagen, Dakopatts, Denmark; endoglin, kindly provided by Dr P.G. Natali, Istituto Tumori 'Regina Elena', Rome, Italy; VE-cadherin, 1B5, kindly provided by Dr E. Dejana, Istituto 'Mario Negri', Milan, Italy), and iMVD values were evaluated independently and blind in each section. Our findings indicated that iMVD values can vary by a 3-fold range in each case, depending on the reagent used; the highest values were obtained with anti-vWf (mean iMVD = 131 ± 67), and the lowest with anti-endoglin (mean iMVD = 45 ± 24). In spite of this variation, the tumour case with the highest degree of vascularisation was recognised independently in six different sections, providing evidence for the reproducibility of the observation.

The presence of the cellular isoforms of fibronectin ED-A (IST-9) and ED-B (BC-1), as well as total fibronectin (IST-4), was investigated using specific mouse MAbs previously described and characterised [20, 21, 24]. The MAb IST-9 is specific for cellular fibronectin and does not react with plasma fibronectin in both radioimmunoassay and immunoblot experiments. The specifity of IST-9 for ED-A was proven using β-galactosidase–fibronectin fusion protein expressed in *Escherichia coli*. IST-9 reacted with the fusion protein pXFN-111 which contains the ED-A sequence, and did not react with the fusion protein pXFN-154 which is identical to pXFN-111 except lacking the ED-A sequence. The MAb BC-1 reacts strongly with a 120 kDa fibronectin fragment corresponding to cell-binding domain 4 containing the ED-B sequence. Expression of ED-A/ED-B in tumour stroma and blood vessels was investigated in 49 cases (18 high iMVD and 31 low iMVD). The number of ED-A + and/or ED-B + vessels was determined in a single field at 200×. Immunostained blood vessels were identified by morphology, and the microscope field with the highest density of positive vessels was chosen for evaluation. The experiment was limited to 49 observations because the results obtained in the two experimental groups were extremely similar.

## RESULTS

iMVD was evaluated following the method of Weidner and colleagues [23] in 91 cases of invasive ductal breast carcinoma in which TNM, histological grading, percentages of Ki-67 + cells and receptor hormonal status were also determined (Table 1). When tumours were grouped according to nodal status, grading or size, no significant difference was observed among the various groups in the mean values of iMVD (Mann–Whitney test). When the same cases were classified as high and low iMVD, it was found that the number of cases with high iMVD was significantly higher in poorly differentiated tumours (G1 versus G3; $P = 0.01$, Chi-square test), and in cases with lymph node metastasis (N0 versus N1 + N2; $P = 0.002$). iMVD could not be correlated with endothelial cell proliferation as determined by Ki-67 immunostaining. In fact, endothelial cells were consistently Ki-67 − even in cases characterised by a high percentage of Ki-67 + tumour cells.

ED-A and ED-B isoforms of cellular fibronectin were used as markers of neoformed vessels in 49 tumours (Table 2). ED-A + and/or ED-B + blood vessels showed a zonal distribution, were present in 40–50% of cases with mean values of 4–8 stained vessels/0.74 $mm^2$ and were equally expressed in cases with high and low iMVD. When serial sections were examined, tumour areas with high iMVD were not characterised by a significantly higher density of ED-A + and/or ED-B + vessels. Staining for ED-A was diffusely present in the connective stroma of 35/49 (71%) tumours (Figure 1), whereas ED-B was detected in only 5/49 (10%) cases (Figure 1). When the 49 cases were stratified for grading and lymph node metastasis, it was found that staining for ED-A/ED-B in the stroma was significantly more frequent in poorly differentiated and metastatic tumours (Table 2). Normal and neoplastic epithelium and the connective stroma of the normal breast were not stained for ED-A/ED-B.

*Table* 1. *Tumour grading (G), tumour size, lymph node metastasis (pN) and intratumoral microvessel density (iMVD)† in* 91 *cases of breast carcinoma*

| | ER + PR + (%) | %Ki-67 + mean ± S.D. (range) | Ki-67 high‡ (%) | iMVD mean ± S.D. (range) | iMVD high‡ (%) |
|---|---|---|---|---|---|
| Grade (*n*) | | | | | |
| G1 (29) | 13/27 (48) | 5 ± 5 (1–22) | 3/29 (10) | 81 ± 32 (26–181) | 5/29 (17) |
| G2 (41) | 12/36 (33) | 13 ± 13 (1–56) | 14/38 (37) | 87 ± 56 (28–330) | 11/41 (27) |
| G3 (21) | 3/21 (14) | 35 ± 22 (0.5–90) | 19/20 (95) | 80 ± 34 (34–135) | 7/21 (33)* |
| Size (*n*) | | | | | |
| < 2 cm (51) | 16/47 (34) | 14 ± 15 (1–60) | 20/49 (41) | 85 ± 48 (28–330) | 12/51 (24) |
| > 2 cm (40) | 12/38 (32) | 18 ± 21 (0.5–90) | 17/38 (45) | 81 ± 48 (26–330) | 11/40 (28) |
| Lymph node status (*n*) | | | | | |
| LN–(53) | 16/49 (33) | 13 ± 19 (0.5–90) | 16/49 (33) | 80 ± 47 (28–330) | 9/53 (17) |
| LN + (38) | 12/36 (33) | 18 ± 17 (1–78) | 21/38 (55) | 88 ± 40 (22–230) | 14/38 (37)* |

LN – versus LN + ($P = 0.002$). All the other differences in percentage of iMVD high were not statistically significant. Chi-square non-parametric test. †The number of vessels (iMVD) was counted in a 200× microscope (0.74 $mm^2$) field of a tumour area showing prominent vascularisation. ‡Ki-67 high = > 10% immunostained tumour cells. iMVD high = > 99 immunostained blood vessels/0.7386 $mm^2$. ER, oestrogen receptor; PR, progesterone receptor; SD, standard deviation. *G1 versus G3 ($P = 0.01$).

## DISCUSSION

The prognostic value of iMVD is still controversial [1–15]. Possible explanations may reside in the different immunostaining procedures, interobserver variability, tumour heterogeneity, patient selection, and perhaps other factors. The relevance of the detection technique for blood vessels is highlighted by the observation that some studies with negative results report iMVD values lower than those obtained in studies describing positive correlations with lymph node metastasis or tumour grading [10–12]. Reduced sensitivity due to paraffin embedding, the type of anti-endothelial antibody, and the quality of the reagents may all profoundly affect the number of immunostained vessels. In the present study, we optimised the immunostaining procedure using fresh cryopreserved material and a mixture of anti-endothelial antibodies. Using this approach, we failed to find any significant difference in the mean values of iMVD, but we confirmed that tumours with high iMVD are significantly more numerous in the poorly differentiated and metastatic groups, as previously reported [1–9]. However, this observation is of a limited biological/clinical relevance, as cases with high iMVD accounted for only 33% of grade III and 37% of node positive tumours.

iMVD was compared with the expression of ED-A/ED-B isoforms of fibronectin in blood vessels in the attempt to improve the identification of those tumours characterised by an angiogenic phenotype. The identification of different splicing products of fibronectin has allowed cellular fibronectin to be distinguished from plasma fibronectin. ED-A and ED-B are two isoforms of cellular fibronectin which can be recognised using specific MAbs. ED-B is considered a marker for neoformed vessels as it is present on the vascular endothelium

*Table* 2. *Immunostaining for ED-A and ED-B in* 49 *cases of breast carcinoma. Tumours were classified according to intratumoral microvessel density (iMVD), size, and presence of lymph node (pN) metastasis†*

| | Tumour stroma | | Blood vessels | |
|---|---|---|---|---|
| | ED-A + (%) | ED-B + (%) | ED-A + | ED-B + |
| iMVD status | | | | |
| iMVD high‡ (*n* = 18; 146 ± 56) | 16/18 (89)¶ | 2/18 (11)¶ | 8 ± 7‖ | 4 ± 3‖ |
| iMVD low§ (*n* = 31; 59 ± 19) | 19/31 (61) | 3/31 (10) | 8 ± 8 | 3 ± 4 |
| Grade | | | | |
| G1 (*n* = 13; 90 ± 42) | 5/13 (38)* | 1/13 (8)** | 9 ± 9 | 4 ± 6 |
| G2 (*n* = 26; 91 ± 10) | 21/26 (81)* | 2/26 (8)** | 6 ± 8 | 2 ± 2 |
| G3 (*n* = 10; 89 ± 34) | 9/10 (90)* | 2/10 (20)** | 6 ± 7 | 3 ± 3 |
| Size | | | | |
| < 2 cm (*n* = 24; 94 ± 64) | 17/24 (71) | 3/24 (13) | 7 ± 8 | 2 ± 4 |
| > 2 cm (*n* = 25; 87 ± 44) | 18/25 (72) | 2/25 (8) | 7 ± 9 | 3 ± 3 |
| Lymph node status | | | | |
| LN – (*n* = 28; 87 ± 65) | 16/28 (57)* | 2/28 (7) | 8 ± 9 | 3 ± 4 |
| LN + (*n* = 21; 95 ± 42) | 19/21 (90)* | 3/21 (14) | 6 ± 7 | 2 ± 3 |

G1 versus G3 ($P < 0.0001$), LN – versus LN + ($P < 0.0001$). **G1 versus G3 ($P = 0.01$), G2 versus G3 ($P = 0.02$). All the other differences in percentages of ED-A + /ED-B + were not statistically significant. Chi-square non-parametric test. †Cryostat sections were immunostained for ED-A (IST-9) or ED-B (BC-1), using mouse monoclonal antibodies and avidin–biotin–peroxidase complex. ‡Cases with high iMVD were as follows: G1 = 5 (28%); G2 = 9 (50%); G3 = 4 (22%); LN – = 7 (39%); LN + = 11 (61%). §Cases with low iMVD were as follows: G1 = 8 (26%); G2 = 17 (55%); G3 = 6 (19%); LN – = 18 (58%); LN + = 13 (42%). ¶Cases with stained tumour stroma/total cases. Immunostaining for ED-A was detected diffusely in tumour stroma. ED-B was focally expressed in a minority of the cases. ‖Mean ± standard deviation (S.D.) of immunostained blood vessels/0.74 $mm^2$ evaluated in the tumour area with the highest density of stained vessels. *G1 versus G2 ($P < 0.0001$)

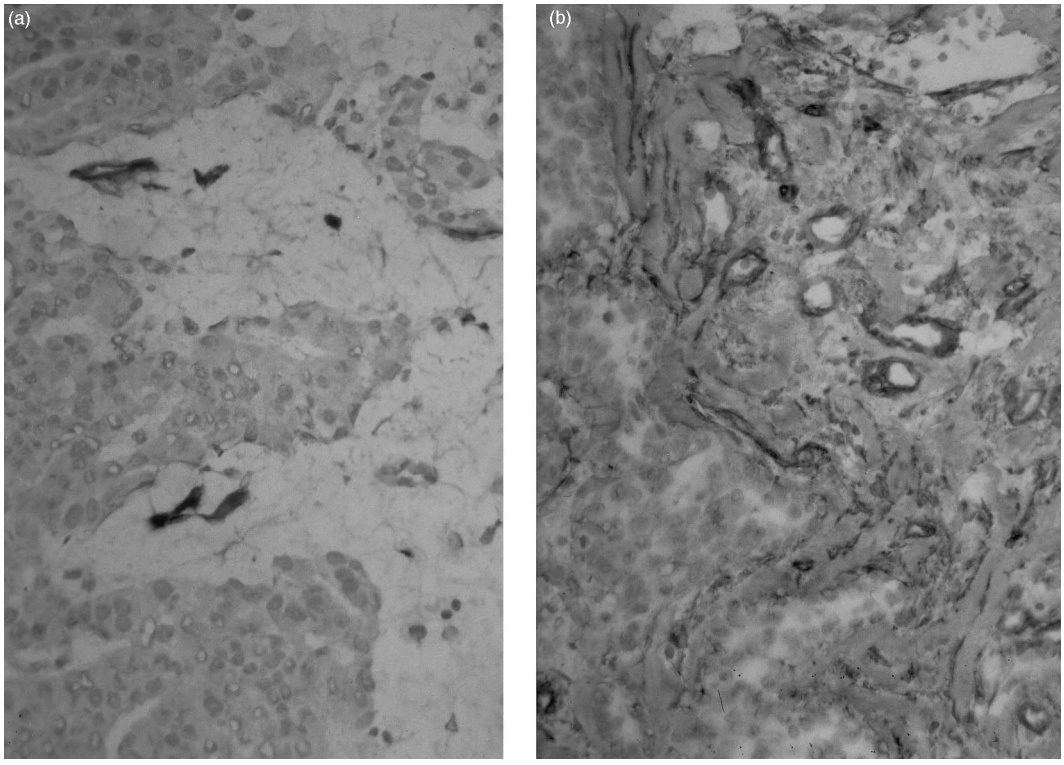


**Figure 1. (a) Cryostat section of an infiltrating ductal carcinoma of the breast immunostained for the ED-B isoform of cytoplasmic fibronectin. ED-B is present in some blood vessels, probably neoformed, whereas the tumour stroma is not stained. (b) Serial section of the same tumour immunostained for the ED-A isoform of fibronectin. Reactivity for ED-A is diffusely present in the stroma and in numerous blood vessels (×250) (avidin–biotin–peroxidase complex (ABC), counterstained with haematoxylin).**

of tumours and inflammatory tissues [21]. ED-B is undetectable in normal tissues, and is produced by a variety of transformed cells [25]. High levels of ED-B have been found in cultures of malignant breast tissue, but not in cultures of normal breast [26]. In tissue sections, reactivity for ED-B has been detected in a high percentage of cases of breast carcinoma, often being associated with endothelial cells [27]. In the present study, we have observed that the number and distribution of ED-A + and/or ED-B + vessels were not spatially associated with hot spots of vascularisation. Our findings may indicate that ED-B is not sensitive and/or specific enough as a marker for visualisation of neoformed vessels. Alternatively, and perhaps most likely, microvessel density is the final result of a multifactorial process, probably discontinuous in time, which is difficult to correlate as a whole with a single event such as the presence of ED-A + and/or ED-B + vessels at a given time in a single section.

Although we were unable to find a correlation between the expression of ED-A/ED-B in blood vessels and the degree of tumour vascularisation, we noted that staining for ED-A/ED-B was significantly more intense and diffuse in the stroma of poorly differentiated and metastatic tumours. Our observations confirm those of Loridon-Rosa and colleagues [28] who also found a significantly higher expression of oncofetal fibronectin in cases of poorly differentiated breast carcinoma. It cannot be ruled out that the presence of oncofetal fibronectin in tumour stroma is one of the events facilitating tumour cell migration and perhaps metastasis. The observation that ED-A was present in 90% of node positive tumours and in only 57% of node negative tumours is consistent with our interpretation, but needs to be confirmed in a larger series.

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