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Immunohistochemical expression of extracellular matrix components tenascin, fibronectin, collagen type IV and laminin in breast cancer: their prognostic value and role in tumour invasion and progression

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Abstract

The immunohistochemical expression of the extracellular matrix (ECM) components tenascin (TN), fibronectin (FN), collagen type IV (Coll) and laminin (LN), and their possible relationships were studied in a series of 134 operable breast cancer cases. Their expression was also compared with the expression of the proteolytic enzyme cathepsin D (CD), the adhesion molecule CD44 standard form (CD44s) and other known factors to clarify the prognostic value and role of these molecules in tumour progression and metastasis. TN expression in the tumour stroma was positively correlated with tumour grade and size, CD44s expression, tumour and stromal CD expression as well as with FN, laminin and Coll expression in the same areas. TN expression was inverse correlated with ER status. Its expression at the invasion front was only positively correlated with the lymph node status. Survival analysis showed an increased mortality risk associated with high levels of TN expression. In multivariate analysis, among the ECM proteins, only TN expression was independently correlated with patients' survival. FN expression was positively correlated with lymph node involvement, with the proliferation-associated index Ki-67 and stromal CD expression. Survival analysis showed an increased mortality risk associated with a high level of FN expression. Coll expression was positively correlated with the tumour size and LN expression. An inverse relationship of Coll expression with ER and PgR receptor status was also found. LN expression was positively correlated with tumour and stromal CD expression, with the proliferation-associated index Ki-67 and inversely with ER receptor status. The observed alterations in the expression of ECM proteins in breast cancer tissue and their correlations with the proteolytic enzyme CD and the adhesion molecule CD44s, suggest an involvement in cancer progression. In addition, overexpression of stromal TN and FN seems to have negative prognostic value in breast cancer patients.

Keywords: Tenascin; Fibronectin; Collagen IV; Laminin; Cathepsin D; CD44s; Breast cancer

1. Introduction

Cancer cells are surrounded by a modified extracellular matrix (ECM) composed of a complex meshwork of collagens, fibrillar glycoproteins and proteoglycans which intercommunicate with the cell interior and thus modulate cell adhesion, proliferation and differentiation [1]. Tenascin (TN) and fibronectin (FN) are glycoprotein components of the ECM which seem to have competitive functions. It can be speculated that this competitive relationship between these molecules is important for cellular functions [2]. TN is a protein of the ECM that contains 14 repeats of the epidermal growth factor (EGF)-like domain. It is produced by stromal fibroblasts and also by epithelial cells of normal and malignant breast tissues and is expressed transiently during embryogenesis, inflammation and malignancy [3]. Recently, a new class of insoluble low affinity matrix-tethered ligand for the epidermal growth factor-receptor (EGFR) was recognised within the EGF-like repeats of tenascin-C [4].

In adult breast tissue, TN expression is menstrual cycle dependent [5] and it has a different pattern of distribution in normal, benign and malignant breast tissue.

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This is variably present in the basement membrane region of breast epithelia and is enhanced periductally next to the basement membrane in intraductal carcinoma of the breast [6–8]. In infiltrating breast carcinoma, TN is expressed in the stroma and appears to be associated with progressive disease and poor prognosis [9–13].

FN is regarded as the major mesenchymal ECM glycoprotein involved in cell–matrix and cell–cell adhesion, cell migration, morphogenesis, differentiation and oncogene transformation [14]. Tissue FN is found in the connective tissue in close apposition to the BMs. Studies of FN in breast carcinomas showed a strong expression and different distribution compared with normal breast parenchyma [15,16].

Laminin and collagen type IV constitute the major intrinsic components of BMs and are involved in cellular adhesion to BMs and the extracellular matrix. Proteolytic enzymes, form a cascade system to facilitate the breakdown of the extracellular matrix, including the BM as the initial event [17,18]. It has been showed that type IV collagen is involved in regulation of mammary cell proliferation, cell attachment and migration [19]. Laminin has been reported to play a role in the adhesion of the cell to the matrix. Invading tumour cells are capable of attachment to the matrix through specific laminin receptors present on epithelial cell membranes and of protease release in the interstitial, promoting BM disruption and cell diffusion [20]. Laminin, like FN, has been shown to play an important role in regulating cell migration, facilitating tumour invasion [21]. Immunohistochemical studies of the distribution of laminin in human breast carcinomas and its role in the process of tumour invasion, have been reported [21,22]. However, cathepsin D (CD) is an ostrogen-induced lysosomal enzyme that can act either directly, by digesting the extracellular matrix, or indirectly by initiating the proteolytic cascade, that may be responsible for the breakdown of BM components [23]. In addition, current evidence suggest that CD44 proteins participate in a large number of related molecular processes, which involve specific adhesions to hyaluronate, collagen and fibronectin [24] and cell migration [25].

In this study, we assessed the interrelationship of the expression of extracellular matrix components TN, FN, collagen type IV and laminin in a series of invading breast cancer in order to elucidate the role of these molecules in tumour expansion. Their expression was also correlated with the expression of proteolytic enzyme Cathepsin D (CD) and with the adhesion molecule CD44s, in order to obtain a better understanding of the role of these molecules in breast tumour invasion and progression. In addition, their expression were correlated with other known potential prognostic factors (ER, PgR, Ki-67 and proliferating cell nuclear antigen (PCNA) and clinicopathological parameters (tumour grade and size, lymph node status, distant metastasis, overall survival) in an attempt to clarify their prognostic importance for clinical implications. In 24 cases, the immunohistochemical expression of extracellular matrix components was also studied in the lymph node involved tissue.

2. Materials and methods

A cohort of 138 patients with primary invasive breast carcinoma who were surgically resected were investigated. For 82 of patients, we had follow-up data and these patients were included in the survival analysis. All patients had a mastectomy with axillary lymph node dissection performed as indicated and were followed-up regularly at the Medical Oncology Department of University Hospital of Ioannina. The patients' ages ranged from 28 to 91 years (mean $\pm$ SD age 55.5 ± 13.8 years). The median follow-up of patients was 5 years with 82 women followed for 3–10 years. Distant metastasis occurred in 34 women.

Archived material was used from formalin-fixed and paraffin-embedded breast carcinoma tissues, including adjacent non-neoplastic tissue or fibrocystic disease. Each specimen was examined histologically on haematoxylin and eosin (H&E)-stained slides. Tumour size was varied from 1 to 17 cm (mean = 3.95 cm). Tumour histotype, lymph node status and the patient's age were recorded for each case. Tumour grade was assessed on H&E stained sections by an experienced pathologist blinded with regard to the results of immunohistochemistry. Tubule formation, nuclear morphology and the mitotic rate were evaluated and scored in the neoplastic cells according to the modified grading scheme of Bloom and Richardson: grades 1, 2 and 3 correspond to well, moderately and poorly differentiated invasive carcinoma of the breast, respectively [26]. The control specimens consisted of normal breast tissue, fibroadenomas and fibrocystic breast disease.

Immunohistochemistry was performed on one or two selected paraffin blocks, on 4 μm tissue sections from each case plated on poly-L-lysine-coated glass slides. In brief, tissue sections were deparaffinised in xylene and dehydrated. For the detection of TN, CD, Coll, LN and FN, slides were pretreated with 1 μl/ml pronase (Dako) for 10 min at room temperature. For the detection of CD44s and Ki-67, slides were immersed in citrate buffer (0.1 m, pH 0.6) in plastic Coplin jars and subjected to microwave irradiation twice for 15 min. Subsequently, all sections were treated for 30 min with 3 μl/ml hydrogen peroxide in methanol to quench endogenous peroxidase activity and then incubated with primary antibodies. We used the method involving the avidin–biotin–peroxidase complex and developed the chromogen with immersion of the slides in a diaminobenzidine–H_2O_2 substrate for 5 min. The slides were counterstained

in Harris' haematoxylin, dehydrated and mounted. To assess the specificity of the reaction, negative controls were included where tumour sections were not incubated with the primary antibody. The antibody sources and dilutions are shown in Table 1. Human intact tenascin is a relatively large glycoprotein (MW approximately 1900 kD) consisting of a pair of trimeric segments linked by disulphide bridges. Its molecular structure is unique in that it contains domains homologous to epidemial growth factor, fibronectin and fibronogen. Fibronectin is the cell-attachment domain of human fibronectin. Collagen type IV and laminin are the major constituent of the basement membranes.

2.1. Immunohistochemical evaluation

Tumours were classified as 'positive' with regard to the immunoreactivity for TN, FN, Coll and LN when there was unequivocal immunostaining of the matrix components in at least one representative area of the tumour. The positive tumours were semi-quantitatively scored as $+$, $++$, and $+++$ corresponding to weak, moderate and extensive immunoreactivity, respectively. TN and FN stains were scored for periductal and stromal expression and were estimated separately in the intratumour stroma and at the periphery of the tumour, in the invasion front. The pattern of staining was also estimated. Nuclear (for Ki-67 and proliferating cell nuclear antigen (PCNA), membrane immunostaining (for CD44s) and cytoplasmatic (for CD) was calculated as the percentage of positive tumour (or stromal for CD expression) cells in relation to the total number of cells encountered in at least 5–10 representative high-power fields (500–1000 epithelial cells). Every stained cell was considered positive, irrespective of the intensity. Each sample was first scanned at low magnification and at least 10 fields were assessed with a high-power magnification. Immunostaining for steroid receptor content was assessed on the basis of the visually estimated percentage of neoplastic cells with positive nuclear staining and the staining intensity. All slides were reviewed and scored in a blind test by two pathologists. Differences in interpretation were reconciled by re-reviewing the slides separately or jointly at a double-headed microscope.

Table 1
Antibodies used

Antibodies	Supplier	Dilution	Incubation time
Tenascin (M0636)[a]	Dako	1:50	1 h
Fibronectin (NCL,FIB)[a]	Novocastra	1:100	1 h
Collagen type IV (MO785)[a]	Dako	1:50	1 h
Laminin[a]	Menarin	1:1000	1 h
CD44s (DF, M7082)[b]	Dako	1:40	Overnight
Cathepsin D (CD13A, A0561)[a]	Dako	1:300	1 h
ER (M7047)	Dako	1:50	1 h
PgR (M3569)	Dako	1:75	1 h
PC-10 (M0879)	Dako	1:50	1 h
Ki-67 (M0722)[b]	Dako	1:10	1 h

ER, oestrogen receptor; PgR, progesterone receptor; CD, Cathepsin D; CD44s, CD44 standard form.
[a] Incubation with pronase.
[b] With antigen retrieval using the microwave oven.

2.2. Statistical analysis

All data were entered into a microcomputer and Chi-square, univariate and multivariate analysis of variance were adopted in a Statistical Package for the Social Sciences (SPSS) 10.0 for windows programme. The prognostic significance of TN, FN, Coll and LN in determining survival, was studied with both univariate (Kaplan–Meier curves, by log-rank test) and multivariate (Cox proportional hazards) analyses, separately for each group of patients. The relationship between the different biological markers was examined using pairwise correlation coefficients (Kendall's Tau-b test). P values $\leqslant 0.05$ were considered as statistically significant.

3. Results

3.1. Tenascin immunostaining

In the normal breast, a thin, focally discontinuous band of TN immunoreactivity was usually noted around ducts and acini; no appreciable reaction was observed beyond the immediate periductal and periacinar stromal regions, except for occasional thin bands around vessels. Intralobular and interlobular stroma was usually negative in fibroadenomas and fibrocystic disease.

In carcinomas, there was an increased TN immunoreactivity in comparison to normal gland that surrounded the cancer nests and in the tumour stroma as well (Fig. 1). In 24/113 (21.2%) cases, TN expression was absent in the tumour stroma. A small amount of

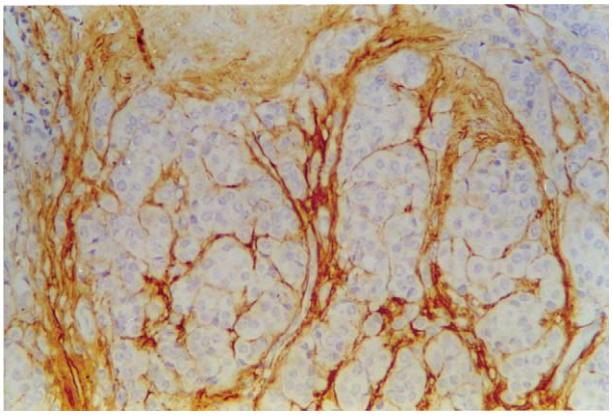

Fig. 1. Infiltrating ductal breast carcinoma displaying extensive Tenascin (TN) immunostaining in the extracellular space (original magnification ABC ×200).

TN expression was detected in 44/113 (38.9%), moderate in 13/113 (11.5%) and strong TN expression was detected in 32/113 (28.3%), of the primary tumours. TN expression was found in 22/24 (91.7%) of the cancer infiltrated lymph nodes. Cytoplasmic staining for TN of tumour cells was found in 9.7% of the primary tumour and in 54.2% of the involved lymph nodes. This type of staining was usually in cases that had weak or absent TN stromal immunoreactivity. Although epithelial TN expression was observed in a small number of cases, this expression was strong inversely correlated with lymph node status ($P=0.0004$). 49/90 (54.4%) cases, in which it was possible to assess TN expression at the invading front of the tumour, were negative for TN expression, 26/90 (28.9%) showed positive and negative areas and 15/90 (16.7%) of the cases showed positive staining in most areas. In 50% of the cases, the TN expression pattern was diffuse in the tumour stroma, in 36.9% it was more continuous around the tumour cell nests (band-like pattern) and 13.1% of the cases showed a mixed distribution. TN expression in the tumour stroma was positively correlated with tumour grade ($P=0.01$) and tumour size ($P=0.042$) (Table 2). The TN-positive group had a

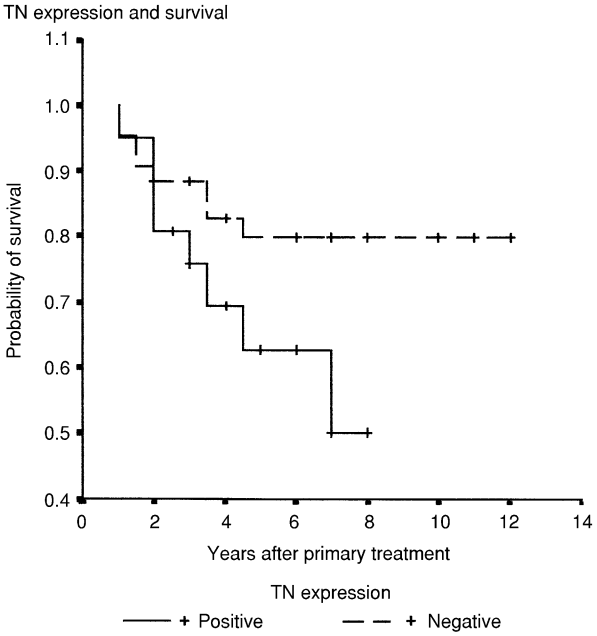

Fig. 2. Survival of patients with breast carcinomas, according to TN staining. Differences between TN-positive and TN-negative patients groups were statistically significant ($P=0.017$).

Table 2
Correlation of stromal TN, FN, Coll and LN expression with clinicopathological features in breast cancer

	TN, FN, Coll, LN expression		*P* value
	−, +	+ +, + + +	
Age (years)			
<45	20, 12, 17, 27	11, 19, 19, 9	
45—55	16, 6, 11, 18	11, 18, 20, 11	NS, NS, NS, NS
>55	27, 15, 35, 48	16, 29, 27, 7	
Tumour size (cm)			
<2	14, 4, 13, 18	3, 13, 4, 2	
2—5	34, 15, 35, 47	18, 34, 38, 18	$P=0.042$, NS, $P=0.004$, NS
>5	12, 9, 10, 19	14, 17, 21, 8	
Type			
Ductal	43, 19, 36, 63	26, 46, 54, 21	
Lobular	12, 9, 16, 19	6, 11, 6, 3	NS, NS, $P=0.02$, $P=0.06$
Mixed	9, 4, 11, 11	6, 11, 7, 4	
Grade			
G1	10, 2, 7, 12	1, 7, 8, 2	
G2	30, 14, 34, 42	15, 29, 24, 11	$P=0.01$, NS, NS, NS
G3	23, 13, 22, 35	18, 30, 30, 14	
Lymph node			
(−)	20, 10, 6, 27	8, 14, 17, 9	NS, $P=0.04$, NS, NS
(+)	32, 15, 34, 49	21, 41, 23, 14	
ER[a]			
<10	8, 5, 15, 12	14, 16, 21, 11	$P=0.017$, NS, $P=0.005$ $P=0.0001$
>10	37, 17, 39, 58	19, 41, 36, 8	
PgR[a]			
<10	12, 7, 10, 22	15, 20, 19, 10	NS, NS, $P=0.061$, NS
>10	28, 14, 30, 44	18, 33, 20, 7	

Numbers represent the no. of cases. ER, oestrogen receptor; PgR, progesterone receptor; TN, tenascin; FN, fibronectin; Coll, collagen type IV; LN, laminin; NS, non significant.

[a] % of stained tumour cells.

Table 3
Correlation of stromal TN, FN, Coll and LN expression with CD44s, CD and proliferation-associated indices

	TN, FN, Coll, LN expression		*P* value
	−, +	++, +++	
CD44s[a]			
<5	12, 4, 7, 12	4, 11, 7, 2	$P=0.034$, NS, NS NS
>5	28, 13, 20, 42	16, 31, 18, 9	
CD tumour[a]			
<10	11, 6, 4, 13	2, 7, 6, 1	$P=0.037$, NS, NS, $P=0.02$
>10	55, 30, 50, 84	44, 67, 51, 29	
CD stromal			
+	42, 26, 34, 58	23, 38, 31, 14	$P=0.05$, $P=0,023$, NS, $P=0.01$
++	13, 4, 11, 16	9, 17, 9, 8	
+++	11, 6, 9, 23	14, 19, 17, 8	
Ki-67[a]			
<10	34, 25, 36, 58	22, 31, 30, 10	NS, $P=0.028$, NS $P=0.005$
>10	22, 6, 16, 28	16, 31, 19, 13	
PCNA[a]			
<50	22, 10, 20, 34	14, 24, 17, 8	NS, NS, NS, NS
>50	30, 17, 26, 47	23, 37, 31, 14	

Numbers represent the no. of cases. TN, tenascin; FN, fibronectin; Coll, collagen type IV; LN, laminin; CD, Cathepsin D; CD44s, CD44 standard form; NS, non significant.
[a] % of stained tumour cells.

significantly worse prognosis than the TN-negative ($P=0.017$). The influence of stromal TN expression on overall survival is shown in Fig. 2. In multivariate analysis, among the ECM proteins, only TN expression was independently correlated with patients survival ($P=0.04$). Stromal TN expression was inversely correlated with ER status ($P=0.017$) (Table 2) and positively correlated with CD44 expression ($P=0.034$), as well as with tumour and stromal CD expression ($P=0.037$ and $P=0.05$, respectively) (Table 3). A strong positive correlation between TN expression in the tumour stroma with FN ($P=0.0041$), laminin ($P=0.007$) and collagen type IV ($P=0.003$) at the same areas was also revealed (Table 4). TN expression at the invasion front was only positively correlated with lymph node status ($P=0.045$). No correlation were found with the patterns of TN expression.

Table 4
Correlation of tenascin (TN) expression with the other extracellular matrix components

	TN expression		*P* value
	−, +	++, +++	
Fibronectin			
−, +	24	11	$P=0.0041$
++, +++	38	34	
Collagen type IV			
−, +	38	10	$P=0.003$
++, +++	19	31	
Laminin			
−, +	39	14	$P=0.007$
++, +++	26	31	

Numbers represent the no. of cases.

3.2. Fibronectin immunostaining

In normal breast, intralobular and interlobular stroma and the epithelial basement membrane were usually negative for FN expression. Fibroadenomas and fibrocystic disease were also usually negative. FN expression was noticed in some of the included vessels.

In carcinomas, 39/113 (34.5%) cases were negative for FN expression in the tumour stroma, 39/113 (34.5%) showed a small amount of FN expression, 33/113 (29.2%) showed moderate expression and 2/113 (1.8%) showed extensive expression.

FN expression at the invasion front was negative in 29/113 (25.7%) of the cases, positive and negative in 52/113 (46.0%) and positive in >50% of the areas in 32/113 (28.3%) of the cases. The pattern of FN expression, was continuous-band-like surrounding tumour nests in 39.6% of the cases and diffuse distribution in the tumour stroma in 60.4% (Fig. 3). Stromal FN expression was found in 23/24 (95.8%) of the involved lymph node tissue. Cytoplasmic staining of the tumour cells for FN was found in 9.9% of the primary tumours and in 50% of the involved lymph nodes. FN expression in the primary tumour stroma was positively correlated with involvement of cancer in the lymph nodes ($P=0.04$)

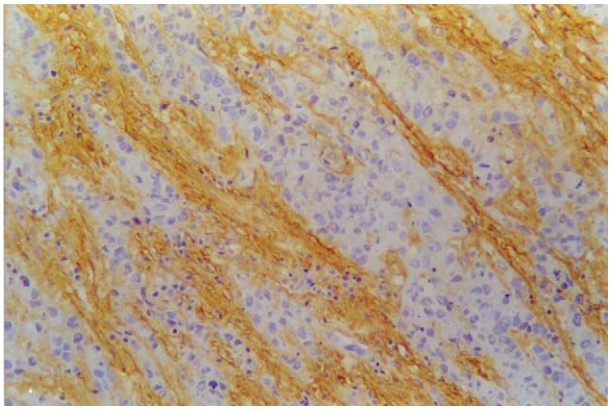

Fig. 3. Strong and diffuse FN immunoreaction in the stroma, in a case of infiltrating ductal breast carcinoma (original magnification ABC ×200).

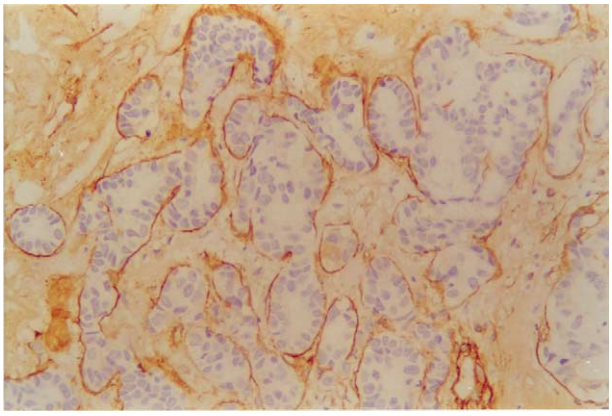

Fig. 5. Collagen type IV-defined basement membranes is seen around the tumour nests in infiltrating ductal breast carcinoma (original magnification ABC ×200).

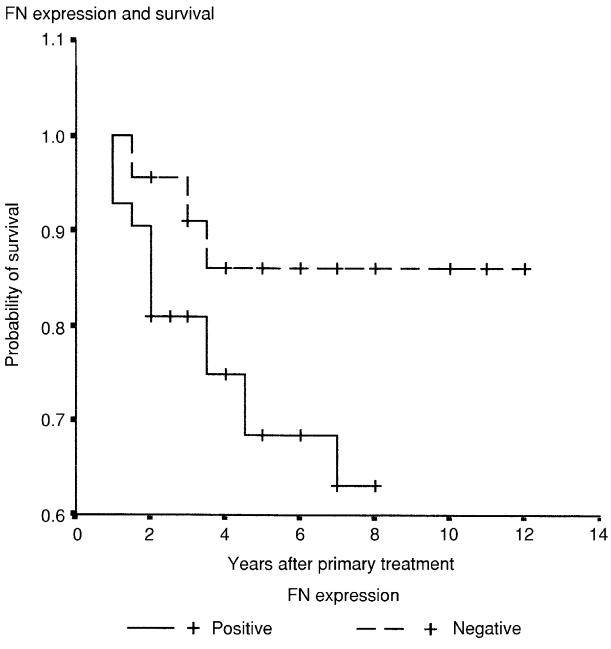

Fig. 4. Survival of patients with breast carcinomas, according to FN staining. Differences between FN-positive and FN-negative patients groups were statistically significant ($P=0.037$).

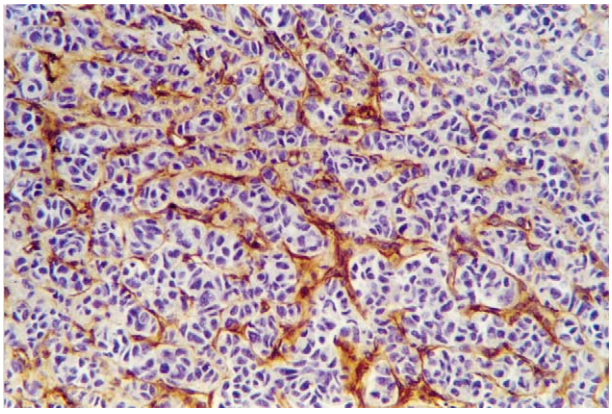

Fig. 6. Infiltrating ductal breast carcinoma showing strong LN immunoreactivity around the tumour nests (original magnification ABC ×200).

(Table 2), with proliferation-associated index MIB1 ($P=0.028$), with stromal CD expression ($P=0.023$) (Table 3) and a trend of positive correlation with laminin expression ($P=0.07$). The FN-positive group had a significantly worse prognosis than the FN-negative ($P=0.037$). The influence of FN expression on overall survival is shown in Fig. 4. FN expression at the periphery of the tumour did not show any correlation with the parameters studied.

3.3. Collagen type IV and laminin immunostaining

There were observed in vascular and epithelial basement membrane. Both displayed a continuous linear pattern in the ducts and ductules and in the adjacent *in situ* component of the invasive carcinomas. This staining pattern was used as an internal control. In carcinomas, different staining patterns of Coll and LN were observed (Figs. 5 and 6). Coll expression around tumour nests was continuous or fragmented in 42.9% of cases, diffuse in the tumour stroma in 35.1% and mixed in 22.1% of the primary carcinomas. In rare cases, cytoplasmic staining for Coll in the primary and in the involved lymph node tissue was also detected. Small amounts of Coll expression was observed in 22/138 (15.9%) of the carcinomas, moderate in 22/138 (15.9%), extensive in 29/138 (21.0%), while 65/138 (47.1%) were negative. Coll expression was found in 20/24 (83.3%) of the involved lymph nodes. Ductal invasive carcinomas displayed higher Coll expression than lobular ones ($P=0.02$) (Table 2). Coll expression was positively correlated with the tumour size ($P=0.004$) and inversely with ER ($P=0.005$) and PgR ($P=0.061$) receptor status (Table 2). A strong positive relationship of Coll and LN expression was also observed ($P<0.0001$). No relationship of Coll expression with patient survival was observed.

Laminin immunostaining showed a continuous or fragmented linear pattern around the tumour nests in 69.5% of the carcinomas. An abnormal multilayered basement membrane (extending beyond the tumour nests–stromal interface) was also detected in some cases. Small amounts of LN expression were observed in 38/134 (28.4%), moderate in 24/134 (17.9%), extensive in 7/134 (5.2%), while 65/134 (48.5%) of the carcinomas were completely negative. LN expression was found in 20/24 (83.3%) of the involved lymph nodes. Cytoplasmic staining for LN was found in a very few cases of the primary tumours and in the involved lymph nodes. The ductal type of carcinomas showed a higher LN expression than the lobular type ($P = 0.06$) (Table 2). LN expression was positively correlated with tumour and stromal CD expression ($P = 0.02$ and $P = 0.01$), with the proliferation-associated index Ki-67 ($P = 0.005$) (Table 3) and inversely with ER receptor status ($P = 0.0001$) (Table 2). No correlation of LN expression with the patient's survival was observed.

In the involved lymph node, the expression of the extracellular matrix components was higher than in the primary tumour and this was statistically significant for FN ($P = 0.02$). Increased cytoplasmic staining for TN, FN and LN was also observed in the involved lymph nodes in comparison to the primary tumours ($P < 0.0001$).

4. Discussion

For a long time, only neoplastic cells were the focus of interest in cancer research, while the stroma was thought to lack any major biological and clinical significance. However, it has now become clear that stromal cells and their products play a significant role in the phenotype of cancer cells [6,7,10,14,20]. Thus, tumours present a complex ecosystem in which interactions between tumour cells and the extracellular matrix, as well as host cells, lead to reciprocal influences resulting in tumour promotion, invasion and metastasis [1–3,14,19,27]. The process of tumour invasion and metastasis requires complex changes in the normal cell–cell and cell–matrix interactions which, in turn, are reflected in variable- up and downregulation of significant molecules. One of these molecules is TN, the expression of which suggests an altered cell matrix interaction that may facilitate epithelial tumour cell invasion during carcinogenesis and tumour progression [2,6,7,14,27]. Breast carcinomas are often characterised by a stromal reaction that consists of modifications in the composition of the extracellular matrix [6,7,14]. Although the expression of TN and other extracellular matrix components has been studied in breast cancer tissue, to our knowledge, there are no reports to date on the relationship between the immunohistochemical TN expression and the major BM components, as well as with the adhesion molecule CD44. The results of TN expression showed a positive correlation with tumour grade in accordance to Iskaros and colleagues [12] and in contrast to the findings of other investigators [8,28]. We also found a positive relationship with tumour size in contrast to the findings of Melis and colleagues [8]. In addition, upregulation of TN expression was correlated with oestrogen-negative tumours, supporting a potential menstrual cycle dependence of TN activity as suggested by Fergusson and colleagues [5]. This finding could also be due to the association of TN expression with progressive disease [9–13]. Furthermore, a negative relationship of TN expression with patient survival, in line with the findings of other investigators, was detected [10–13]. The TN expression at the invasion border was correlated with lymph nodal status, providing useful information on tumour progression. Our data support a significant role for TN expression in tumour progression and provide prognostic information for the management of breast cancer patients.

Cell culture experiments suggest that TN promotes cell growth by augmenting the mitogenic effect of fibroblast growth factor that is a prerequisite for epidermal growth factor-induced proliferation [27]. However, TN has a complex function: an anti-adhesive effect and a strong cell binding capacity stimulating or inhibiting tumour growth [2,27]. In our study, we found no correlation of TN tumour stromal expression with the growth fraction as estimated by proliferation-associated indices according to the findings of Tokes and colleagues [28]. In contrast, other investigators have suggested a growth-promoting activity of TN expression [9–11]. These findings were observed in *in-situ* ductal breast carcinomas [11] and in the invasion border of early breast cancers [9].

Of interest in the present study, was the correlation of TN with CD44s expression. The latter is a widely distributed receptor type protein, which has been found expressed in various types of human cancer, although the prognostic value of the various isoforms have not been recognised as independent predictors of breast cancer outcome [29]. In a previous study concerning the CD44s expression in breast cancer, we found a strong relationship of CD44 expression with lymph node involvement [30].

Tumour production of Cathepsin D (CD), an oestrogen-induced lysosomal enzyme, has been implicated in tumour invasiveness and metastatic dissemination through the breakdown of the extracellular matrix. There are discrepancies and conflicting opinions about the value of CD [15,31]. It has been shown that CD expression may provide independent prognostic information for breast cancer patients [32]. In our study, the tumour and stromal CD expression, showed a relationship with TN expression. This finding was in accordance with the results of a very recent study by Jahkola and

colleagues [33] who showed a positive correlation of TN expression, at the invasion border of early breast cancers, with cancer and stromal cell CD expression. The observed positive correlation of TN expression with CD44, as well as CD, expression suggests these molecules may cooperate in the invasion and progression processes of breast carcinomas.

In our study, a strong relationship between TN and FN expression was found supporting the theory of their competitive functions [2]. An *in situ* hybridisation study of human colon tissues showed a positive correlation between *FN*-mRNA expression and the depth of invasion, as well as the frequency of lymph node metastases, suggesting that FN expression could be important for the remodelling process of neoplastic tissues during cancer development and progression [34]. In addition, cell culture experiments have shown that FN can stimulate cancer cell migration under certain conditions [27]. In our study, FN expression was correlated with lymph node status supporting this data. However, Gorczyca and colleagues [16] found no correlation of FN expression with the lymph node status in breast cancer. In addition, we found that a high level of FN expression correlated with an increased mortality risk of the patients suggesting its expression is of prognostic significance. This is further supported by the positive relationship of its expression with the lymph node status and the proliferation associated index Ki-67. A previous study showed that FN-positive staining was a favourable prognostic factor associated with a low metastatic potential of breast carcinomas [17].

Several studies provide evidence that the deposition of BM material coincides with a better tumour prognosis [16,18]. In the current study, Coll and LN were positively correlated with the ER receptor status. Both also correlated each other and with TN expression. The presence of LN expression in the carcinoma cells in rare cases shows the potential for synthesising of this molecule by the neoplastic cells themselves. The significance of this synthesis is not known, but data on the cell adhesion-promoting properties of LN [20] suggest that LN could be important for the attachment and growth of such tumour cells. In the present study, LN expression was correlated with the proteolytic enzyme CD and the proliferation-associated index Ki-67 suggesting an active role in tumour expansion and development.

Expression of extracellular matrix components was found in most of the involved lymph nodes in variable amounts. An interesting finding was the higher cytoplasmic staining for TN, FN and LN in the involved lymph nodes, suggesting the production or the derivation of these proteins from the neoplastic cells themselves and that they may have a role in the metastatic process. These proteins were not expressed in any stromal cell, but only in the extracellular matrix. In addition, the presence of cytoplasmic expression in cases with only a weak or absent TN stromal immunoreactivity showed it could be synthesised by the neoplastic cells themselves. Furthermore, TN cancer cell expression was usually correlated with a negative lymph node status. Thus, we can hypothesise that, TN expression by the neoplastic cells themselves could be an early event in breast carcinogenesis, followed by stromal TN expression in cases with a more aggressive tumour phenotype.

Taken together, all this information suggests that extracellular matrix components may be involved in the development and progression of breast cancer. In addition, the studied molecules seem to be implicated in the remodelling of stroma of the cancer cells. Among them, TN and FN may prove to be useful markers of poor prognosis in operable breast cancer when considering adjuvant therapy options.

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