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In vivo demonstration of cytoplasmic fibronectin in human breast carcinomas

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Summary. The distribution pattern of fibronectin in 24 invasive human breast carcinomas has been studied using the indirect immunoperoxidase technique. A positive cytoplasmic staining reaction was observed in 16 tumours. Well-differentiated carcinomas showed weak or no staining, whereas all moderately or poorly differentiated carcinomas and one signet ring cell carcinoma contained fibronectin positive tumour cells with moderate or strong staining. The staining intensity was positively correlated to degree of anaplasia with the exception of two moderately differentiated duct carcinomas and the two medullary carcinomas, which were only slightly positive. Non-attached independently growing tumour cells stained more intensely than tumour cells in clusters. Pericellular fibronectin was found in only one carcinoma of medullary type. In normal ducts and glands it was seen at the stromal-epithelial junction corresponding to the basement membrane, around myoepithelial cells and along the luminal border. The results support the findings of several in vitro investigations that breast tumour cells synthesize fibronectin. It also suggests that cytoplasmic fibronectin expression might be an indicator of tissue differentiation in non-solidly growing invasive duct carcinomas of the human mammary gland.

Key words: Fibronectin – Breast carcinoma

Introduction

The matrix glycoprotein fibronectin, FN, has been reported to be synthesized and secreted by a variety of cells in culture such as fibroblasts, myoblasts, chondrocytes, astroglial cells, platelets and certain macrophages (for review see Mosesson and Amrani 1980).

Immunohistochemical studies of epithelial tissues have found FN present in the basal lamina between epithelium and stroma (Couchmann et al. 1979;

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D'Ardenne et al. 1983; Natali et al. 1984; Stenman and Vaheri 1981; Szendröi et al. 1983), and the glycoprotein has been observed on cell surfaces as well (Birembaut et al. 1981; Labat-Robert et al. 1981; Stampfer et al. 1981).

In normal human epithelial breast tissue FN has been seen mainly at the stromal-epithelial junction around ducts and glands and around myoepithelial cells (Asch et al. 1981; Gibert et al. 1982; Natali et al. 1984; Noel et al. 1982; Stenman and Vaheri 1981). FN has, however, also been observed at the luminal surface (Stampfer et al. 1981) or surrounding the duct-lining cells (Birembaut et al. 1981; Labat-Robert et al. 1980).

The absence of cell-associated FN in malignant human epithelial breast carcinomas has been documented by several authors (Asch et al. 1981; Birembaut et al. 1981; Labat-Robert et al. 1980; Noel et al. 1982; Stenman and Vaheri 1981). Only Stampfer et al. (1981) and Natali et al. (1984) found FN positive material around the surface of some tumour cells.

In vitro observations of human breast carcinomas, however, have shown that FN production by the tumour cells occurs (Smith et al. 1979; Stampfer et al. 1981; Taylor-Papadimitriou et al. 1981). Morphologically FN is generally located pericellularly, but cytoplasmic FN or FN situated over the nuclei of tumour cells has been reported (Noel et al. 1982; Stampfer et al. 1981; Taylor-Papadimitriou et al. 1981).

In the present study we describe the distribution of FN in 24 human invasive breast carcinomas, comprising 9 histologically different subtypes.

Material and methods

Tissues. Surgically removed breast tumours from 24 women were included in the study. The tumours were histologically selected and classified according to the WHO criteria (Scharff and Torloni 1968) into 12 invasive ductal carcinomas, 3 invasive lobular, 2 tubular, 2 colloid, 2 papillary, 2 medullary and 1 signet ring cell carcinoma. The invasive ductal carcinomas were grouped according to decreasing degree of differentiation into Grade I, 3 cases, Grade II, 4 cases and Grade III, 4 cases.

Morphologically normal-looking mammary tissue adjacent to the tumour area was used as control material.

Tissue preparation. The tissue samples were fixed overnight at 4° C in ethanol-glacial-acetic acid (99:1, v/v), dehydrated in ethanol and xylene and embedded in Paraplast® at 56° C. All sections were placed on ethanol-cleaned glasses.

Fresh frozen sections of the same or histologically similar tumours were used as positive controls.

Treatment of the tissue with hyaluronidase. Tissue was investigated before and after hyaluronidase treatment as described by Hølund and Clemmensen 1982.

Immunoperoxidase staining. The indirect double layer technique as previously described by Clausen and Thomsen 1978, was used. Sections were deparaffinized, washed with 0.05 M sodium phosphate buffer at pH 7.4 (PBS). In short the steps were as follows: 1) Methanol with 0.5% H_2O_2, 30 min, 2) PBS 15 min, 3) rabbit anti-human fibronectin 1:80, 30 min, 4) peroxidase-conjugated swine anti-rabbit immunoglobin 1:20, 30 min, 5) 0.04% 3 amino-9-ethylcarbazole (Sigma, St Louis, HO, USA) in dimethylformamide with 0.01% H_2O_2 in 0.5 M sodium acetat/acetic acid buffer pH 5, 15 min, 6) mounting in Aquamount® and counterstain-

ing in Mayer's haemalun. All antisera were diluted in PBS. When staining frozen sections step 1 was omitted. Control stainings were performed by replacing rabbit anti-human fibronectin (step 3) with 1) anti-human fibronectin absorbed by fibronectin, 2) IgG fraction from non-immunized rabbits and 3) with PBS.

For routine histological examination paraffin sections were stained with haematoxylin-eosin, van Gieson and Gordon and Sweet argyrophilic stain.

Antisera and control reagents. Human fibronectin was isolated as described by Clemmensen and Andersen 1982. Rabbit antiserum and IgG fraction of antiserum against human fibronectin were produced and purified as described by Harboe and Ingild 1973. Specific IgG against human fibronectin was prepared as described by Hølund et al. 1981. For immunohistochemical staining the specific IgG against human fibronectin was used.

The control IgG was the purified rabbit IgG after removal of specific antifibronectin by affinity chromatography on fibronectin linked Sepharose 4 B. Furthermore, Ig fraction of serum from non-immunized rabbits DAKO, code no. X903, lot 038 B was used.

Evaluation. The staining results were evaluated by two of the authors independently (LC, MN), according to staining intensity and the extent of the staining reaction.

The following scoring system was used: a) staining intensity: 0: none, +: slight, ++: moderate, +++: strong, b) extent of staining: 0: no reacting tumour cells, +: 0–10% of the tumour cells reacting, ++: 10–50% of the tumour cells reacting and +++: 50–100% of the tumour cells reacting.

Results

In morphologically normal breast tissue adjacent to the tumour tissue FN was observed within connective tissue stroma, in basement membrane areas around ducts, glands, vessels and nerves and sometimes as fine granules along the luminal border of secretory cells (Fig. 1). A diffuse cytoplasmic staining was present within some fibroblasts, macrophages and plasma cells.

In situ elements occasionally seen in the periphery of infiltrating tumour areas were FN negative, and the reactivity of the basement membrane areas in general was not consistent enough to assess continuity in precancerous lesions.

The cytoplasmic staining reaction for FN appeared as a diffuse or granular staining of individual tumour cells with a tendency to concentrate peripherally in the cytoplasm. The intensity of the staining reaction varied between tumours of the same histological type and to some extent also within the same tumour. Detached tumour cells tended to stain more intensely than tumour cells in clusters.

The well differentiated carcinomas (Grade I carcinomas) showed a very weak (1 case) or no (3 cases) cytoplasmic staining reaction (Table 1).

The moderately differentiated carcinomas (Grade II carcinomas) all reacted positively for FN. Two tumours with a solid growth pattern contained only scattered slightly stained tumour cells, while the other 2 showed a moderate staining reaction in the majority of tumour cells (Fig. 2).

The poorly differentiated carcinomas (Grade III carcinomas) all contained moderately or strongly FN positive tumour cells. Two carcinomas displayed

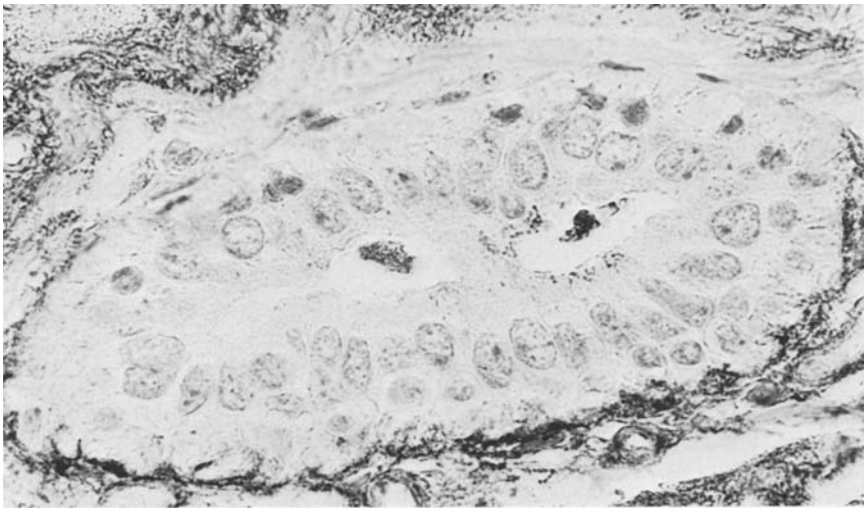


Fig. 1. Immunoperoxidase staining for fibronectin of a normal-looking mammary duct. Apart from fine granules of fibronectin positive material along the luminal border and luminally the epithelium is negative. Note the inconsistant staining at the stromal-epithelial junction. (×138)

Table 1. Semiquantitative estimation of immunoperoxidase staining for fibronectin in 24 primary invasive human breast carcinomas

	Cytoplasmic staining	Amount of reacting tumour cells
Invasive ductal carcinoma		
Grade I	+(1) 0(3)	+(1)
Grade II	+ +(2) +(2)	+ + +(1), +(1) + +(2)
Grade III	+ + +(2) + +(2)	+ + +(2) + +(2)
Invasive lobular carcinoma	+(1) 0(2)	+ +(1)
Colloid carcinoma	+ +(1) 0(1)	+ +(1)
Medullary carcinoma	+(2)	+ +(1), +(1)
Tubular carcinoma	+(1) 0(1)	+ +(1)
Papillary carcinoma	+(1) 0(1)	+(1)
Signet ring cell carcinoma	+ +(1)	+ + +(1)

( ): number of tumours

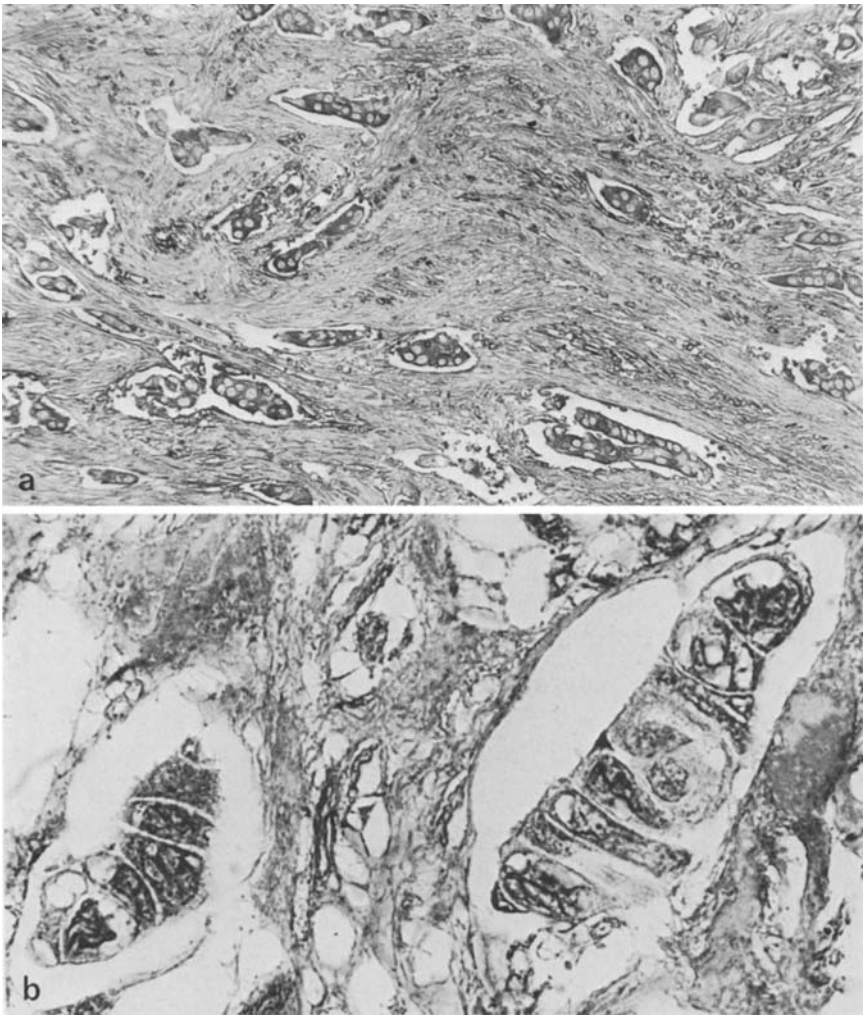


Fig. 2a. A moderately differentiated duct carcinoma presenting clusters of tumour cells with a moderate cytoplasmic staining reaction. Immunoperoxidase staining for fibronectin. (×86) **b** High-power view of the same tumour showing the cytoplasmic granular staining of tumour cells. (×344)

a moderate staining in a minority of the tumour cells, the majority being weakly FN positive. A third tumour contained several intensely stained independently growing tumour cells contrasted by groups of cohesive tumour cells, which stained only slightly or not at all (Fig. 3). The fourth tumour contained a large focus of pseudosarcomatous giant cell pattern, in which almost all tumour cells appeared detached from one another. The majority of these cells showed an intense cytoplasmic staining for FN (Fig. 4).

The invasive lobular carcinomas demonstrated no staining reaction in 3 cases. Only one was weakly FN positive.

The signet ring cell carcinoma, consisted of tumour cells almost all of which displayed a moderately FN positive cytoplasm. The mucus globules were negative (Fig. 5).

One colloid carcinoma did not show any staining reaction. The other exhibited a moderate reaction in about one fifth of the tumour cells.

Both medullary carcinomas contained a few weakly reacting tumour cells. In one of the tumours FN was furthermore observed around most tumour cells creating a pericellular network (Fig. 6)

The two tubular carcinomas and the two papillary carcinomas showed staining pattern of the well-differentiated invasive duct carcinomas. One tumour in each group was totally FN negative, the other two reacted faintly in a minority of the tumour cells.

The stroma of tumour infiltrated areas was more intensely stained than that of normal-looking breast tissue, but with the exception of one medullary carcinoma no distinct pericellular reaction could be identified.

Discussion

One of fibronectin's more important physiological functions seems to be to act as anchoring agent between cells and cell-matrix (Yamada et al. 1978).

This ability, has during the last ten years, attracted attention to the glycoprotein as a possible tool in cancer diagnostics, owing to the well-known dissociation -and spreading behaviour of malignant tumour cells (Ali et al. 1977; Chen et al. 1979; Hynes 1976; Nicolson 1976; Pearlstein et al. 1976; Vaheri and Mosher 1978; Yamada and Olden 1978).

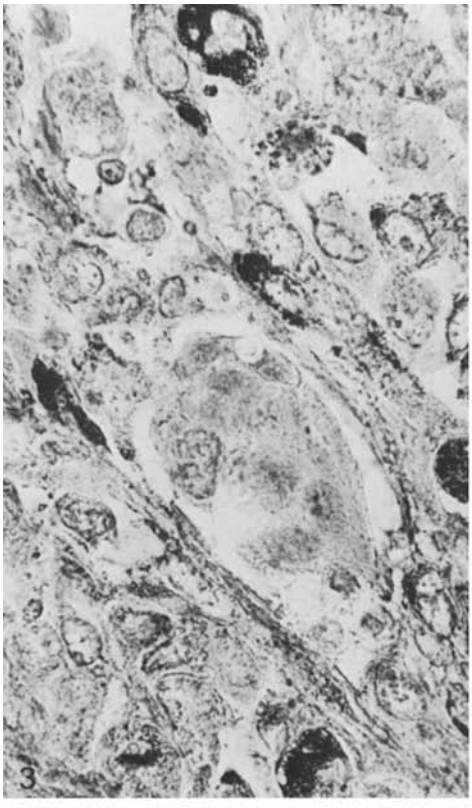
In contrast to similar investigations employing the indirect immunofluorescence technique on fresh frozen material, we utilized the indirect immunoperoxidase technique on ethanol/acetic acid fixed, paraffin embedded sections treated by the Sainte Marie technique (Sainte Marie 1962). The advan-

Fig. 3. A poorly differentiated duct carcinoma. Individually growing tumour cells give a strong staining reaction for fibronectin, whereas cells in clusters (*center*) stain weakly or not at all. (× 344)

Fig. 4. A poorly differentiated duct carcinoma containing a large area of giant tumour cells many showing an intense cytoplasmic staining reaction for fibronectin. (× 86)

Fig. 5. The signet ring cell carcinoma. Most tumour cells exhibit a moderate cytoplasmic staining for fibronectin. The mucus globules are negative. (× 138)

Fig. 6. A medullary carcinoma presenting a distinct fibronectin positive pericellular network. (× 344)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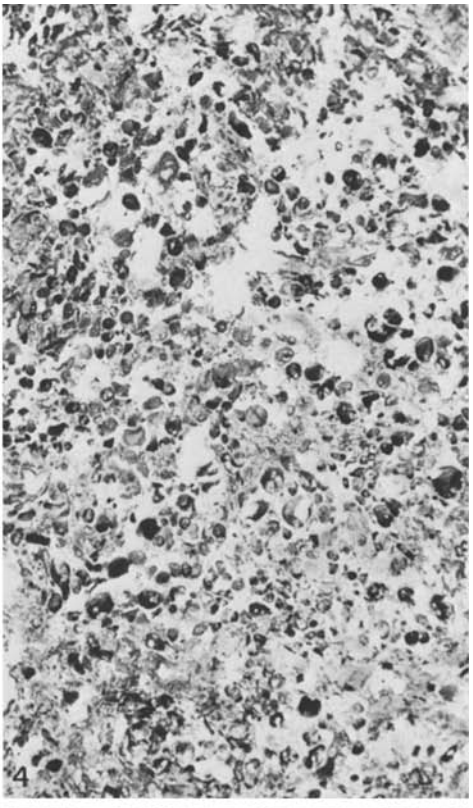
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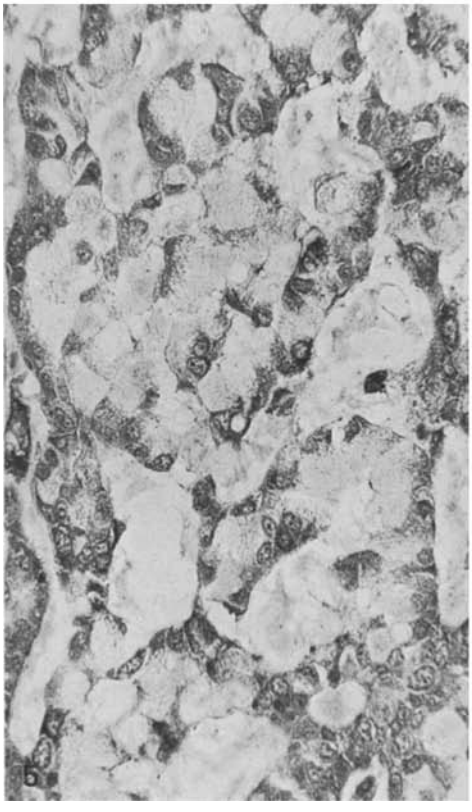
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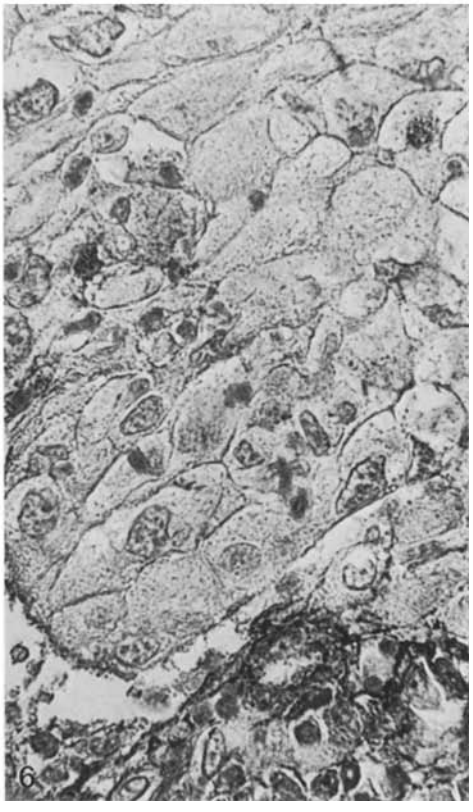
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tage of this procedure is optimally preserved morphology and reproducable results. Unfixed frozen sections of the same or histologically similar tumours served as valuable positive controls for the technical accuracy of the staining method.

The pericellular distribution pattern of FN in morphologically normal breast tissue and its absence in various carcinomas of the present study is in accordance with previous observations (Asch et al. 1981; Birembaut et al. 1981; Labat-Robert et al. 1980; Noel et al. 1982; Stenman and Vaheri 1981). Only one medullary carcinoma presented a distinct pericellular FN positive network, a characteristic of this tumour type also mentioned by others (Birembaut et al. 1981; Labat-Robert et al. 1980; Natali et al. 1984). Interestingly, medullary carcinomas are known to have a considerably better prognostic index than other types of invasive breast carcinomas.

The finding of cytoplasmic FN in all moderately and with an increased intensity all poorly differentiated invasive duct carcinomas and very weak or no cytoplasmic reaction in well-differentiated ductal -and lobular carcinomas suggests that tumour's degree of anaplasia is positively correlated to its intracellular FN concentration. The intense cytoplasmic staining of isolated tumour cells, however, indicates that also the ability these cells to grow unattached may be connected with their cytoplasmic FN expression.

Some of the cultured breast carcinoma cell lines, in which cytoplasmic FN has been observed, were derived from metastatic lesions and differed from well defined primary carcinoma cell lines by being devoid of peri -or extracellular FN (Smith et al. 1979; Taylor-Papadimitriou et al. 1981). Neri et al. 1981, demonstrated in a combined in vivo/in vitro mouse mammary carcinoma cloning study that metastatic potential could not be directly correlated with a decreased pericellular FN expression, but they also found that tissue from metastatic lesions, showing a pattern of poor differentiation and cultured cells from the same tumour contained no cell-associated FN. This might be due to degradation by different enzymes secreted by the tumour cells (Jones and DeClerck 1980; Weber et al. 1975) a hypothesis which also would explain the absence of pericellular FN in almost all tumours of this study. The fact that the compressed cytoplasm of the signet ring cells stained considerably more strongly than the cytoplasm of the other invasive carcinoma cells of lobular origin gives the impression that FN is synthesized by many if not all breast carcinoma cells. From the pericellular staining observed in this and other in vivo studies FN is probably also synthesized by normal breast epithelium (Birembaut et al. 1981; Labat-Robert et al. 1980; Stampfer et al. 1981).

Ultrastructural studies will be necessary to determine whether the demonstrable cytoplasmic FN within most tumours of the present study is due to increased synthesis, cricumstantial accumulation or passive/active intake of exogenous FN. Increased synthesis seems likely, as some embryonic cells, to which the tumour cells have certain behavioural and morphological similarities, have been demonstrated to produce the glycoprotein in increased amounts (Linder et al. 1975; Wartiovaara and Vaheri 1980).

The results of this study confirm what several in vitro investigations

have indicated, that FN synthesis occurs in some human malignant breast carcinomas. It further demonstrates that tumour cells with a high dissociation potential contain large amounts of FN and that cytoplasmic FN is increased in poorly differentiated non-solidly growing invasive ductal carcinomas. This is in contrast to other breast cancer functional markers such as Epithelial Membrane Antigen (EMA) and Carcinoembryonic Antigen (CEA). EMA gives a weaker and more unpredictable cytoplasmic staining reaction in undifferentiated breast carcinomas (Sloane and Ormerod 1981), while CEA gives an inconsistant cytoplasmic staining of all breast carcinomas independent of type or degree of differentiation (Waalkes et al. 1984).

FN is by no means a tissue specific antigen, but it may be a marker of tissue differentiation in some human breast carcinomas. More extensive studies, however, will be necessary in order to support this statement.

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