Angiogenin Expression and Prognosis in Primary Breast Carcinoma

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ABSTRACT
Angiogenin is a protein originally isolated as an inducer of new blood vessel growth, and it has been reported to be an effective substrate for tumor cell adhesion. To understand the role of angiogenin in cancer progression, we evaluated the expression of angiogenin in 459 cases with primary breast carcinoma and in 40 benign breast specimens using an immunoassay. Higher angiogenin concentrations were observed in carcinomas in comparison with fibrocystic disease (mean, 17.3 versus 10.9 ng/mg; P = 0.008), but not with fibroadenomas. We selected 5 ng/mg cytostol protein of angiogenin as the normal cutoff for primary breast carcinoma. Eighty-eight percent of carcinomas expressed elevated angiogenin levels and 12% had low levels. We observed an association between elevated levels of angiogenin and low/moderate histological grade (P = 0.001) and small tumor size (P = 0.026), but not with age, menopausal status, lymph node status, stage of disease, or hormonal receptor status. With a median follow-up of 31 months, breast cancer patients with elevated angiogenin levels had significantly longer disease-free survival (DFS) than patients with low angiogenin (log-rank, P = 0.003). This effect was equally observed in node-negative and node-positive cases. In a multivariate analysis of DFS, only angiogenin, tumor size, and histological grade showed statistical significance. A multivariate analysis of overall survival showed that angiogenin and tumor size were the only significant variables. Serum samples from the breast cancer patients at the time of surgery were available in 194 cases. We evaluated the levels of circulating angiogenin using the same immunoassay as in tumor tissue. Serum angiogenin levels were higher in cancer patients than in 40 healthy controls (mean, 401.2 versus 206.0 ng/ml; P < 0.0001). In breast cancer patients, we observed no correlation between the serum concentrations and the tissue levels of angiogenin (r = 0.115; P = 0.110). In addition, serum levels of angiogenin did not have a prognostic impact on the DFS of breast cancer patients (log-rank, P = 0.581). Our results indicate that elevated levels of tissue angiogenin, but not of circulating angiogenin, are a favorable prognostic factor in primary breast carcinoma, which is consistent with a role of angiogenin as a cancer cell substrate.

INTRODUCTION
The development of metastasis is one of the greatest obstacles for cancer cure. An assembly of coordinated cellular processes is responsible for metastasis, and angiogenesis is essential for both the expansion of the primary tumor and the growth of the metastasis. Angiogenesis is induced by a variety of proteins including the family of FGFs, vascular endothelial cell growth factor, and angiogenin. Human angiogenin is a single chain, 123 amino acid residue polypeptide with a molecular mass of 14.2 kDa (1). The gene for human angiogenin exists as a single copy and has been localized to chromosome 14 in the region q11-q13 (2). Although originally isolated from the serum-free conditioned medium of the HT-29 human colon adenocarcinoma cell line (3), angiogenin is not a tumor-specific product. Its mRNA is expressed by normal cells (4), and the protein is present in normal plasma and milk (5, 6). Angiogenin was detected, purified, and named on the basis of its ability to induce new blood vessel growth, and it is known that this protein is a member of the RNase superfamily. Human angiogenin exhibits approximately 35% similarity to pancreatic RNase A (1, 2, 7), although its RNase activity is somewhat limited relative to RNases, with specificity directed toward ribosomal and tRNA systems rather than standard-RNase substrates (8–10). Its ribonucleolytic activity seems to be necessary but not sufficient for angiogenic activity (11). Angiogenin seems to interact with endothelial cells via a specific receptor(s), because it has been shown to activate their phospholipase pathways (12, 13) and bind specifically to their surface. There is evidence to suggest that the binding of angiogenin to endothelial cell surface is mediated by actin (14, 15). In vitro studies demonstrate that this binding is the first step in an internalization-nuclear translocation-nucleolus accumulation process leading to angiogenesis (16). In addition, angiogenin binds to the extracellular matrix (17), and it acts as an adhesion molecule for endothelial cells, fibroblasts (18), and tumor cells (19). Therefore, angiogenin may be capable of participating in different related processes such as angiogenesis, adhesion, and invasion through specific interactions with receptors on endothelial and tumor cells. In this regard, it is not clear whether actin is the functional receptor responsible for the production of all of the observed effects of angiogenin. Recently, a Mr 170,000 putative receptor for angiogenin has been detected on the surface of endothelial cells (20). Also, an angiogenin-binding cell-surface proteoglycan from HT-29 adenocarcinoma cells has been iden-

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2 The abbreviations used are: FGF, fibroblast growth factor; DFS, disease-free survival; ER, estrogen receptor; PR, progesterone receptor.
tified, suggesting that tumor cell adhesion to angiogenin could be mediated by this receptor (19).

Although the physico-chemical properties of angiogenin and the mechanisms of in vitro signal transduction and receptor binding have been described, little is known about these functions in normal or pathological cellular processes. Furthermore, the protein expression of angiogenin in human malignant tumors has not been reported. Information on the expression of angiogenin in tissues and serum will facilitate an understanding of the role of this protein in cancer progression. We here demonstrate that angiogenin is detected at elevated levels in a majority of breast carcinomas and that the expression of tumor angiogenin, but not of circulating angiogenin, is an independent prognostic indicator of favorable outcome in patients with operable breast cancer.

MATERIALS AND METHODS

Patients. We studied 499 unselected patients who underwent breast surgery from January 1992 to December 1994. The patients had nonmalignant breast lesions (n = 40) or stage I-III breast carcinoma (n = 459). Cancer cases did not have distant metastasis, which were excluded by chest radiograph, bone scan, and liver ultrasonography. Tumor stage was defined according to the International Union against Cancer classification, and the number of involved axillary lymph nodes and histological grade were determined by pathological examination. Chemotherapy was administered to all patients with axillary node involvement, in premenopausal patients with tumor size >1 cm, and in postmenopausal patients with negative ER. Tamoxifen was given to all patients with positive ER. Radiation therapy was administered in cases treated with conservative surgery and in patients with four or more axillary nodes. All patients with primary breast cancer were followed postoperatively. Clinical examinations were performed every 3–6 months, according to the institution guidelines. Relapse was defined as the first documented evidence of new disease manifestation. DFS was calculated from the time of surgery to the date of first recurrence. Data of patients who did not have a relapse were censored at the last follow-up visit. Although not an end point of this study, overall survival was also recorded. The follow-up was closed in September 1996.

Tissue Sample Processing. Tumor tissues removed at surgery were immediately frozen in liquid nitrogen and stored at −80°C until used. Tissue samples were processed following the procedures for steroid receptors status analysis. Briefly, tumor specimens were minced and homogenized in ice-cold buffer consisting of 10 mM Tris-HCl (pH 7.4), 1.5 mM ethylenediamine tetracetic, 10 mM monothioglycerol, and 10 mM sodium molybdate, using several intermittent bursts of a Polytron. After centrifugation for 10 min at 800 × g, the supernatant was centrifuged at 105,000 × g for 1 h. Aliquots of cytosol (supernatant) were stored at −80°C until required for the angiogenin assays. Protein concentration was determined by the method of Lowry et al. (21).

Angiogenin ELISA. The assay system uses a highly specific monoclonal antibody for human angiogenin bound to the wells of a microtiter plate (R&D Systems, Inc., Minneapolis, MN). The standards (200 μl) with known amounts of angiogenin and diluted samples (1/10) were pipetted into the wells and incubated for 1 h. The samples were assayed in duplicate. After three washings, polyclonal anti-human angiogenin antibody conjugated with horseradish peroxidase was added and incubated for 1 h. After three additional washings, a substrate solution (tetramethyl-benzidine-H2O2 mixture) was added to the wells, and color was developed in proportion to the amount of angiogenin bound into the initial step. The color development was stopped with sulfuric acid, and the intensity of the color was measured using a spectrophotometer set to 450 nm. The angiogenin values are expressed in ng/mg of cytosolic protein.

Angiogenin Immunoblotting. Cytosolic protein (10 μg) was separated by SDS-PAGE on 18% polyacrylamide gel and transferred to nitrocellulose membrane. Nitrocellulose was then saturated in 3% BSA in TBST (10 mM Tris-HCl pH 8, 150 mM NaCl, 0.1% Tween 20) overnight at 4°C, incubated in the same buffer containing a polyclonal antiangiogenin antibody (10 μg/ml) for 3 h at room temperature, washed 3 × 5 min in TBST (10 mM Tris-HCl pH 8, 150 mM NaCl, 0.1% Tween 20), incubated 45 min at room temperature with IgG alkaline phosphatase conjugate (0.2 μg/ml), washed as before, incubated until color development with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyolphosphate mixture in AP buffer (100 mM Tris pH 9, 100 mM NaCl, 5 mM MgCl2), washed to stop the color reaction, and air-dried to room temperature. Antiangiogenin antibody for Western blotting experiments was supplied by Dr. H. Tschesche (University of Bielefeld, Germany).

Serum Angiogenin Determination. Before surgery, blood samples were obtained from breast cancer patients. We also collected blood samples from healthy donors. These samples were centrifuged, and aliquots of the sera were frozen until angiogenin levels were determined. The ELISA system described previously (R&D Systems, Inc.) was used for the analysis of serum samples that were diluted to 1/200. The results are expressed in ng/ml.

Statistical Analysis. To identify the angiogenin normal cutoff value, we used the corrected minimum P method suggested by Altman et al. (22). In brief, we performed log-rank analysis on breast cancer patients’ DFS of multiple arbitrary cutoff points. The Ps were corrected using the formula \[ P_{corr} = \frac{1}{1 + 2.35 \log_e{P_{min}}} \], selecting the cutoff showing the more significant \( P_{corr} \). Differences in mean values were assessed with the Kruskal-Wallis test. The association of angiogenin levels with qualitative clinicopathological parameters was assessed with the \( \chi^2 \) test, using the Mantel-Haenszel test to assess for linear association. The Kaplan-Meier estimate was used to calculate DFS, and the log-rank test was used to make comparisons. For multivariate analysis, the Cox proportional hazards regression model was used. Correlations were calculated by the Spearman test.

RESULTS

We studied 459 patients with primary breast carcinomas (stage I-III). The median age of the patients at diagnosis was 57 years (range, 26–86 years). One hundred seventy-four of the 459 patients were premenopausal (38%); the remaining 285 were postmenopausal (62%). Most of the breast carcinomas (90%) were invasive ductal carcinomas; 8% were invasive lob-
patients with breast carcinoma, 124 had stage I, 260 had stage II, and 35% were well or moderate differentiated and 68 had stage III disease. Forty percent of the patients had T1 tumors, 44% had T2 tumors, and 16% had T3–T4 tumors. The number of axillary lymph nodes involved at diagnosis ranged from 0–31 (mean, 3). Forty-nine percent of the patients were N0; 29% had 1 to 3 involved axillary lymph nodes; 13% had 4 to 9, and 9% had 10 or more. Sixty-nine per cent of the patients had positive ER and 56% had positive PRs.

The postsurgical adjuvant therapy was hormone therapy alone in 178 patients (39%), chemotherapy plus hormone therapy in 145 patients (32%), chemotherapy alone in 98 patients (21%), radiotherapy alone in 22 patients (5%), and no adjuvant treatment in 15 patients (3%).

A second group was formed by 40 benign breast diseases, which included 8 fibroadenomas and 32 fibrocystic diseases. The median age of the patients was 49 years (range, 20–85 years).

Angiogenin Detection and Cutoff Point Determination. In an initial series of 128 primary breast carcinomas, we found that elevated expression of angiogenin had a favorable impact on the DFS. To select the normal cutoff for angiogenin, we tested different cutoff points between 3 and 25 ng/mg, using the corrected Ps of the corresponding log-rank tests. The best dichotomy between patients with good and adverse prognosis was obtained with the cutoff values of 5 and 8 ng/mg (Pcor = 0.026 and Pcor = 0.031, respectively; Fig. 1). To validate the results in an independent series, we selected a consecutive cohort of 167 patients with primary breast cancer. Using this cohort, we chose the cutoff of 5 ng angiogenin/mg of cytosol protein (log-rank, P = 0.04).

In the 459 cases with primary breast carcinoma, the values of angiogenin ranged from 0–147.9 ng/mg protein, with a median of 13.3 and a mean ± SD value of 17.3 ± 15.0. Elevated values (≥5 ng/mg) were observed in 88% of carcinomas.

To verify the results that we obtained with the angiogenin ELISA, we selected cases with low, intermediate, or high values in which to detect angiogenin by immunoblotting analysis, using an antibody against human angiogenin. The comparison of the methods showed a marked agreement between the staining intensity in the immunoblot and the corresponding ELISA values (Fig. 2).

In the 40 cases with nonmalignant breast lesions, the values of angiogenin ranged from 0–38 ng/mg protein, with a median of 11 and a mean ± SD value of 12.3 ± 9.6. Elevated values were observed in 77% of benign breast specimens. The mean levels of angiogenin in patients with malignant breast tumors were not significantly different from those of patients with fibroadenoma (Table 1). However, we observed differences in angiogenin protein between breast carcinomas and fibrocystic disease (17.3 ± 15.0 versus 10.9 ± 8.7; P = 0.008).

Angiogenin Association with Other Prognostic Factors. Angiogenin levels correlated with histological grade and with tumor size. Cases with well/moderate differentiated tumors showed elevated levels of angiogenin more frequently than those with poorly differentiated tumors (92% versus 81%; P = 0.001). Similarly, elevated angiogenin levels were associated with smaller tumors: 92% of T1 carcinomas, 86% of T2 carcinomas, and 82% of T3–T4 carcinomas had elevated angiogenin (P = 0.026). In contrast, we observed no significant association between angiogenin and age, menopausal status, axillary lymph node involvement, stage, ER status, or PR status (Table 2).

Survival Analysis. With a median follow-up of 31 months (range, 8–58), relapse was observed in 69 cases. Relapsing patients had elevated angiogenin levels less frequently than nonrelapsing patients (13% versus 27%; P = 0.006).

Univariate analysis of DFS indicated that smaller tumor size (P < 0.0001), less extensive lymph node involvement (P < 0.0007), smaller stage (P < 0.0001), lower histological grade
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In the patients with breast carcinoma (Table 3), circulating angiogenin in these selected matched cases. The same cancer patients in which cytosol angiogenin had been studied were available in 194 cases. We determined the levels of circulating angiogenin in these selected matched cases. The mean levels of serum angiogenin in cancer patients were significantly associated with longer DFS (Table 4). Age and histological grade had a significant association with longer DFS (Table 4). During the follow-up, 32 patients have died. In an univariate analysis, all of the variables considered showed an association with DFS. The Kaplan-Meier estimates of DFS, according to angiogenin levels, are shown in Fig. 3.

Because the prognostic value of angiogenin might be different in the patients with or without axillary lymph node involvement, we evaluated separately the DFS analysis in node-negative and node-positive patients. We observed that angiogenin maintained its prognostic value in the two population subsets (P = 0.026 and P = 0.026, respectively).

To perform a multivariate analysis of DFS, we selected the variables showing significance in the univariate model: tumor size (P < 0.0001), ER positive status (P < 0.0001), PR positive status (P < 0.0001), and elevated angiogenin levels (P = 0.003) had a significant association with longer DFS (Table 4). Age and menopausal status did not show correlation with DFS. The Kaplan-Meier estimates of DFS, according to angiogenin levels, are shown in Fig. 3.

Although not an end point in our study, we evaluated overall survival in the patients with breast carcinoma (Table 3). During the follow-up, 32 patients have died. In an univariate analysis, all of the variables considered showed an association with overall survival: tumor size (P < 0.0001), angiogenin (P < 0.0002), histological grade (P = 0.0036), PR status (P = 0.0126), ER status (P = 0.028), and lymph node involvement (P = 0.0449). In a multivariate analysis, however, tumor size and angiogenin were the only variables retaining statistical significance (P < 0.0001 and P = 0.0095, respectively).

**Circulating Angiogenin Levels.** Serum samples of the same cancer patients in which cytosol angiogenin had been studied were available in 194 cases. We determined the levels of circulating angiogenin in these selected matched cases. The mean levels of serum angiogenin in cancer patients were 401.2 ± 167.2 ng/ml (range, 17.7–1844.8). Sera were also collected from 40 healthy donors. The mean levels of angiogenin in normal human serum were 206.0 ± 131.5 ng/ml (range, 110–785). We observed significant differences in circulating angiogenin levels between cancer patients and healthy controls (P < 0.0001). Using 400 ng/ml as a cutoff, 111 breast cancer patients showed low values and 84 elevated values. The correlation of tissue and serum angiogenin levels of breast cancer patients is shown in Fig. 4. Serum angiogenin did not show any statistically significant association with tissue angiogenin levels (r = 0.115; P = 0.110). We performed an analysis of DFS in...
Fig. 3 DFS curves in patients with breast carcinoma. A Kaplan-Meier plot shows that patients with elevated angiogenin (≥5 ng/mg) had an improved prognosis compared with patients with low angiogenin (<5 ng/mg). The difference was significant using the log-rank test (P = 0.003). ANG, angiogenin.

Table 3 Univariate and multivariate analysis of survival

<table>
<thead>
<tr>
<th>Variable</th>
<th>DFS Overall survival</th>
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<tr>
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<td>Univariate b</td>
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<tr>
<td>Angiogenin</td>
<td>0.003</td>
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<tr>
<td>Tumor size</td>
<td>&lt;0.0001</td>
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<tr>
<td>Histologic grade</td>
<td>&lt;0.0001</td>
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<tr>
<td>Lymph node involvement</td>
<td>&lt;0.0007</td>
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<tr>
<td>ER status</td>
<td>&lt;0.0001</td>
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a Stratification of variables as in Table 2.

b P, log-rank test.

c P, cox proportional hazards regression model.

relation with the serum levels of angiogenin, and we did not observe an association between circulating angiogenin and DFS (log-rank, P = 0.581).

DISCUSSION

It has been postulated that angiogenin, an angiogenesis-related protein and a substrate for tumor cell adhesion, participates in the growth of malignant tumors or their metastasis (3, 4, 9, 14, 15, 17–20). The expression of angiogenin mRNA has been originally reported in gastrointestinal malignancies (23, 24). The angiogenin protein expression, however, has not been reported in human breast carcinomas, and an evaluation of the clinical significance of angiogenin in a large series of cancer patients has not been performed. We here demonstrate that angiogenin is expressed at elevated levels in 88% of breast carcinomas, and that angiogenin expression is associated with a favorable prognosis.

We analyzed differences in angiogenin protein levels in benign and malignant breast tissues. The levels of angiogenin in patients with malignant breast tumors were not different from those of patients with fibroadenoma but were significantly different from those of patients with other benign breast lesions (fibrocystic disease). Other authors found the increased protease production in breast tumors may increase the extractability of another angiogenic protein (bFGF) from stroma (25). The reason why fibrocystic disease expresses lower levels of angiogenin may be also related to protease production.

To define the normal cutoff for tissue angiogenin, we used a double methodological approach, following the recommendations for the selection of normal cutpoints for biological markers that have been proposed recently (22). First, using the minimum P method, we tested different cutoff points, and selected two tentative values. Second, we studied an independent series of breast carcinomas to validate the angiogenin cutoff, and found that 5 ng/mg provided the best discriminative value in a DFS analysis.

When we correlated angiogenin with clinicopathological factors, we found that elevated angiogenin was associated with smaller tumors and with carcinomas showing low or moderate histological grade, implying an association between the expres-
observance of angiogenin and less aggressive tumors. We did not observe a correlation between angiogenin and lymph node involvement. These findings would argue against a direct role for angiogenin in the process of early tumor spread.

We evaluated the impact of several variables on patient prognosis. In our series, tumor size, lymph node involvement, grade, and hormone receptor status had prognostic significance. Small tumor size (26, 27), absence of lymph node involvement (26, 27), and low histological grade (28) have been consistently shown to be favorable prognostic factors, as have been positive ER and PR status (29). Therefore, it would seem that our series is comparable with other reported series of operable carcinoma. Our study has shown that elevated expression of angiogenin is an additional favorable prognostic factor in breast carcinoma. This occurred both in node-negative and node-positive patients, and the prognostic value of angiogenin was maintained in a multivariate analysis of DFS. This is the first report demonstrating that expression of tumor angiogenin is a significant independent prognostic factor for DFS in operable breast carcinoma.

Because elevated tumor angiogenin was a favorable prognostic factor in breast cancer patients, we evaluated the levels of circulating angiogenin and compared them with the tumor levels of the protein in 194 matched samples. We found that circulating angiogenin levels were significantly higher in breast cancer patients than in healthy controls. Angiogenin levels in controls were similar to those reported by Shapiro et al. (5). In breast cancer cases, we did not observe a correlation between tumor and serum angiogenin levels. Moreover, serum angiogenin did not have any impact on DFS of breast cancer patients. These data, together with the fact that the levels of serum angiogenin observed were reasonably high, are consistent with the idea that a source for serum angiogenin different from the breast carcinoma cells exists in the human body. The liver may be a major source for serum angiogenin because Weiner et al. (30), when examining the tissue distribution of angiogenin mRNA in the rat, demonstrated that angiogenin is predominantly detected in the adult liver.

The favorable prognosis of the elevated levels of angiogenin in breast carcinoma tissue supports the proposed role of angiogenin as a substrate for tumor cell adhesion. The loss of angiogenin expression in breast carcinomas may facilitate the migration of tumor cells and the subsequent metastatic spread, because the loss of cell adhesion is essential for the dissemination and growth of solid tumors. To test this hypothesis, we have investigated whether angiogenin can serve as a substrate for breast cancer cells. Our in vitro results demonstrate that angiogenin acts as an adhesion molecule for MCF-7 and MDA-MB-231 breast adenocarcinoma cells (data not shown). In this regard, other authors have found that angiogenin supports endothelial and fibroblast cell adhesion (18), and HT-29 adenocarcinoma cell line adhesion (19) from which angiogenin was first obtained (3). The primary function of angiogenin in vivo may be in processes other than the regulation of vascular growth, because the pattern of angiogenin gene expression is not temporally related to vascular development in the rat (30). Moenner et al. (31) reported that the widespread expression of angiogenin by different human cells in culture suggests a physiological role not necessarily limited to angiogenesis. In ovarian cancer, Barton et al. (32) did not find a correlation between the serum expression of angiogenin and tumor vascularity. The different known functions described for angiogenin, including promotion of cell adhesion (18, 19) and invasion (33), as well as distinct RNase activity (8–10), stimulation of second messengers (12, 13), and nuclear translocation and nucleolar accumulation (16), suggest that it can serve multiple roles in cancer progression.

The prognostic evaluation of new angiogenic molecules in breast carcinoma has shown that these proteins may not always indicate a poor clinical course. Although vascular endothelial growth factor expression has been associated with an adverse prognosis of breast carcinomas (34), the expression of bFGF has
not. We and others (35, 36) have found that the expression of bFGF is associated with a favorable prognosis in human breast cancer, possibly related to the preferential expression of bFGF in myoepithelial cells, a cellular type usually seen in the less advanced carcinomas. Originally, it was believed that angiogenin was a tumor-specific protein: tumors and transformed cell lines associated with angiogenin expression include colonic, gastric, hepatocellular, pancreatic, ovarian carcinomas, and epidermoid, lung, colon, and cervix carcinoma cell lines (3, 23, 24, 31, 32). However, now it is known that angiogenin is also expressed by a variety of nonmalignant cell types including endothelial, vascular smooth muscle, fibroblast, peripheral blood lymphocytes, macrophages, and normal epithelial cells (5, 31, 37, 38). Although the source of angiogenin in breast carcinomas has not been addressed in our study, the in situ detection of angiogenin mRNA in pancreatic carcinoma showed expression in cancer cells and also in the fibroblasts surrounding the cancer cells (24). Additional immunohistochemical study will help in quantitating the relative weight of tumor or stromal cell sources.

Our study has clearly demonstrated that the expression of angiogenin in breast carcinoma extracts is associated with a favorable patient prognosis, and we suggest that this may be related with its function as a substrate for tumor cell adhesion. Further research on the role of angiogenin as a molecule responsible for the adhesion of tumor cells will help in providing a clearer understanding of the metastatic process.

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