Pregnancy-Associated Plasma Protein A

A Clinically Significant Predictor of Early Recurrence in Stage I Breast Carcinoma Is Independent of Estrogen Receptor Status

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Pregnancy-associated plasma protein-A (PAPP-A) immunopositivity and extensive tumor necrosis have been shown to correlate significantly with early (<24 months) recurrence in patients with Stage I breast carcinoma negative for estrogen receptor (ER). In this study we have extended our analysis of Stage I disease to include 30 ER-positive cases. Twenty-five traditonal clinical and pathologic features were reviewed in addition to the immunoperoxidase staining characteristics of PAPP-A for examination of the independent relationship of immunopositivity both to early recurrence and to ER and progesteron receptor (PR) status. Clinical recurrence developed in 6 of 30 (20%) patients, 4 of the 30 (13%) patients experiencing early recurrence. Pearson chi-square tests revealed significant correlations between early recurrence and PAPP-A tumor positivity ($P < 0.002$), younger age ($P < 0.009$), and premenopausal status ($P < 0.03$). Stepwise regression analysis showed that PAPP-A-positive staining correlated independently with early recurrence. Of the 10 PAPP-A-positive tumors, 4 (40%) recurred within 2 years, compared with no early recurrences in the 20 PAPP-A-negative cases. With a comparison of frequency of PAPP-A positivity of ER-positive (30%) tumors and ER-negative tumors (40%), there was no correlation with ER or PR status. PAPP-A tumor positivity is independent of ER status and is a clinically significant independent predictor of early recurrence in both ER-positive and ER-negative Stage I patients. (Am J Pathol 1985, 121:342–348)

ECTOPIC production of various pregnancy-associated and placental proteins have been recently discovered in human breast carcinoma. A number of studies have shown evidence that production of these substances may be an indicator of prognosis.[1-5] In two previous studies, we demonstrated immunoreactivity for pregnancy-associated plasma protein A (PAPP-A), one of the many recently described pregnancy-associated proteins, in human breast carcinoma. In addition, we have shown that tumor immunopositivity for PAPP-A correlated significantly with early (<24 months) recurrence in patients with both Stage II and Stage I breast carcinoma negative for estrogen receptor (ER).[4,5] We have now extended our study of Stage I disease to include ER-positive patients. Both PAPP-A immunopositivity and clinicopathologic features known to correlate with the biologic behavior of breast carcinoma were assessed for their ability to predict early recurrence. In addition, combining the data from the previous study of Stage I ER-negative patients with the ER-positive group permitted study of the relationship between ER and progesterone receptor (PR) status and PAPP-A positivity.

Materials and Methods

All patients of the Johns Hopkins Hospital treated surgically for carcinoma of the breast from 1977 to 1981 who had no pathologic or clinical evidence of metastases and whose tumors had ER binding of >10 fmol/mg of cytoplasmic protein by the dextran-coated charcoal method[6] were initially evaluated for this study. Progesterone receptor (PR) level was not a criterion for inclusion in the study. Any PR value greater than zero was considered positive. Patients for whom there were

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inadequate clinical records or pathologic material, who had received pre- or post-operative irradiation or chemotherapy or who on review were found to have either lymph node metastases or only noninvasive carcinoma were excluded from the study. This resulted in a population of 30 patients.

Tumor Immunostaining

Representative sections of the primary tumor were stained for pregnancy-associated plasma protein A (PAPP-A) with the use of the unlabeled antibody immunoperoxidase method.[7] Rabbit anti-human PAPP-A (1:125) (Dako Laboratories, Santa Barbara, Ca) diluted in TRIS-HCl buffer, pH 7.6, was used for antigen localization. Control studies included substitution of nonimmune rabbit serum for the rabbit anti-human PAPP-A and staining of normal human placenta as a positive control for PAPP-A. Absorption studies with formalin-fixed tissue have been previously reported.[4] Additional testing of the sensitivity and specificity of the DAKO antibody have been performed on serum from normal pregnant patients with the use of an ELISA technique.[8]

Because tumor immunostaining was seldom uniform, staining was evaluated and graded with the use of a system similar to that derived by Gleason[9] for grading prostatic carcinoma, in which major and minor morphologic components were included in the evaluation. In our study the components were determined by staining pattern, rather than histologic pattern. For each case, the staining was graded on a 0–3+ scale for both the major and minor staining patterns of the primary tumor. The results were then expressed as the sum of the major and minor staining components (for example, if most of the tumor showed 3+ staining, and the minor component was negative, the tumor was graded as 3+0=3). According to this system, therefore, the most strongly positive tumors (5+ to 6+) were tumors with uniformly strong positive staining, whereas tumors with weaker focal positivity ranged from 2+ to 4+. In this manner, both the distribution and intensity of staining were taken into account. In order for a tumor to be graded as positive, unequivocal staining needed to be present at least focally. Cases with focal ambiguous staining were not regarded as positive.

Other Histologic Features

The traditional pathologic features evaluated were similar to those used by Kuhajda et al[5] and Fisher et al[10] and are summarized in Table 1. The clinical features are self-explanatory; selected pathologic features were analyzed as follows. The major histologic types of breast carcinoma were those used by Fisher et al.[10] Representative slides of tumor, uninvolved breast tissue, skin, nipple, and axillary lymph nodes from each case were examined, and the pathologic features were evaluated semiquantitatively.

Table 1—Clinical and Pathologic Features Evaluated in Addition to Immunostaining

Clinical features
Age of patient
Location of tumor (side and quadrant)
Size of tumor
Menopausal status
Symptoms (bleeding, discharge, pain, ulceration)
Family history of cancer
Parity
Radiographic identification (mammography, isotopic scan, CT scan)
Type of mastectomy (radical, modified radical)
Short term recurrence (within 2 years)
History of biopsy preceding mastectomy
Pathologic features of the tumor
Histologic type of tumor
Estrogen receptor (fmol/mg)
Progesterone receptor (fmol/mg)
Histologic grade
Nuclear atypia
Necrosis (absent, focal, extensive)
Tumor border (circumscribed versus infiltrating)
Inflammation (lymphocytes, plasma cells, neutrophils)
Mitoses (none, few, many)
Lymphatic invasion
Axillary invasion
Perineural invasion
Muscle invasion
Paget's disease
Skin involvement
Microcalcification
Mucin (absent, mild, moderate, marked)
Squamous metaplasia
Pathologic features of uninvolved breast tissue
Stromal reaction (absent, fibrous, sclerotic)
Fibrocystic (proliferative, nonproliferative)
Sinus histiocytosis (mild, moderate, marked)
Germinal center hyperplasia (mild, moderate, marked)
Diffuse cortical hyperplasia (mild, moderate, marked)

Tumors were divided into one of three histologic grades based primarily upon the degree of differentiation or tubule formation, nuclear to cytoplasmic ratio, and degree of hyperchromatism. The number of mitoses and extent of necrosis were graded independently, but these features also contributed to the assessment of the overall histologic grade. Grade I tumors had extensive tubule formation with minimal nuclear atypia. Grade II tumors showed moderate cytologic atypia with varying degrees of differentiation, and grade III tumors demonstrated pleomorphic cells, a high nuclear to cytoplasmic ratio, and a solid growth pattern.

In keeping with the convention used in other tissues for histologic grading, we chose to grade the nuclei in the following order: the most pleomorphic anaplastic nuclei were Grade III, small uniform nuclei with relatively inconspicuous nucleoli were Grade I, and Grade

II nuclei were intermediate. As noted by others, breast carcinomas expand as circumscribed masses with a pushing border or as irregular, stellate masses with infiltrating margins.[11] Tumors were classified microscopically as either infiltrating or circumscribed. The degree and type of inflammation was also evaluated for each case. Necrosis in the infiltrating tumor was graded as absent, focal, or extensive. Extensive necrosis is defined as necrotic tumor visualized on a scanning (4×) objective. Intraductal necrosis and comedocarcinoma were not included in the overall assessment of necrosis. The presence or absence of lymphatic and vascular invasion, Paget's disease, and microcalcification was noted. Additionally, histologic features of nonneoplastic breast tissue similar to those utilized by Fisher et al[10] were assessed, including stromal reaction in both proliferative and nonproliferative fibrocystic disease. Axillary lymph nodes were evaluated for the presence of germinal center hyperplasia, sinus histiocytosis, or diffuse cortical hyperplasia with criteria similar to those used by Hunter et al.[12]

Statistical Analysis

The data were analyzed with the use of the BMDP statistical software package[13] in the IBM 370/3031 computer of the information systems department of the Johns Hopkins Medical Institutions. The means, standard deviation, Pearson chi square, Yates corrected chi square, and Student *t* test were obtained for selected variables. Stepwise discriminant analysis was performed for determination of which variables correlated independently with recurrence. Discriminant analysis seeks to provide classification functions (linear combinations) or the variable that best separates the cases into the recurrent and nonrecurrent groups. In stepwise discriminant analysis, variables are removed from the classification function one at a time until group separation ceases to improve significantly. In addition, life-table analysis was also performed with the use of both the generalized Wilcoxon (Breslow) and generalized Savage (Mantel-Cox) test statistics.[14,15]

Results

Clinical Features and Early Recurrence

The 30 patients in this study ranged in age from 31 to 89 years, with a mean of 66 years. All were women. Follow-up ranged from 10 to 78 months, with a mean of 39 months. Clinical recurrence developed in 6 of 30 (20%), 4 of 30 (13%) patients with early (<2 years) recurrence. Quantification of the ER status showed a range of 11–120 fmol/mg, with a mean of 42 fmol/mg; PR ranged from 0 to 187 fmol/mg, with a mean of 38 fmol/mg. Eleven cases had PR values <10 fmol/mg. All of the clinical features listed in Table 1 were evaluated for a possible association with early recurrence. Pearson chi-square tests revealed significant correla-

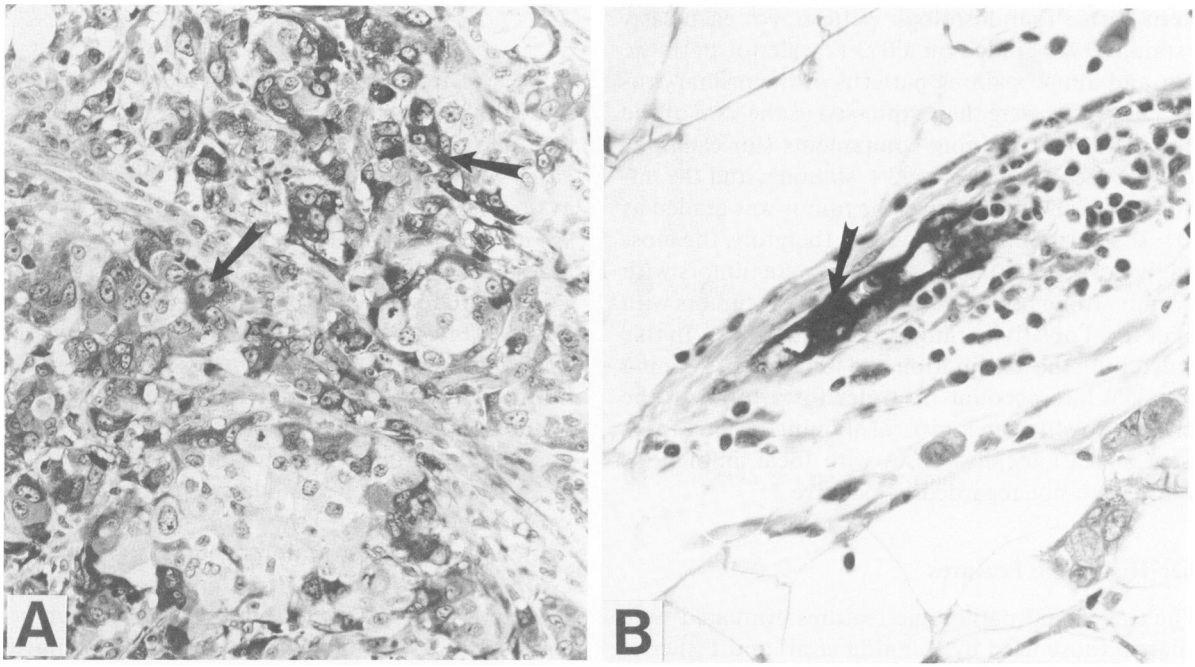

Figure 1—PAPP-A staining in infiltrating carcinoma of breast. Note the heterogeneity of staining with adjacent strongly positive (*arrows*) and negative tumor cells. **B**—PAPP-A staining commonly occurs in endothelial cells (*arrow*). There is adjacent negatively staining infiltrating breast carcinoma. (Hematoxylin counterstain, **A**, ×270; **B**, ×350)

tions between early recurrences and younger age ($P < 0.009$) and premenopausal status ($P < 0.03$). None of the other clinical features correlated significantly with early recurrence.

Histopathologic Features of PAPP-A Immunostaining and Correlation with Early Recurrence

PAPP-A is normally found in the syncytiotrophoblastic cells of the human placenta.[16] In sections of normal term placenta used as positive controls, PAPP-A gave variable staining in the syncytiotrophoblastic cells, the most intense staining being in the cytotrophoblastic shell cells. This focal staining for PAPP-A in the syncytiotrophoblast of term placentas has been previously noted.[16] Because PAPP-A is a secretory glycoprotein, cellular positivity was strictly intracytoplasmic in both the positive control and breast carcinomas (Figure 1A). PAPP-A also intensely stained endothelial cells lining the vessels both within the tumors and in the sections of normal breast (Figure 1B). Focal staining was the pattern seen exclusively in all of the immunoreactive breast carcinomas. None of the tumors showed diffuse positive staining with equal intensity throughout. Instead, there were zones of positivity with staining of both individual cells and groups of cells with varying intensity (Figure 1A). The occasional staining of "normall" breast structures and fibrocystic disease with PAPP-A had been previously reported.[5]

The relationship of positive PAPP-A tumor immunostaining and early recurrence is highlighted in Table 2. Of the 10 PAPP-A positive tumors, 4 (40%) recurred within 2 years, compared with no early recurrences in the 20 PAPP-A-negative cases ($P < 0.002$). There was no correlation between staining intensity or staining pattern with prognosis in this or the previous studies. Unequivocal positivity, even if focally present, was a significant prognostic indicator.

Comparison of Early Recurrence in PAPP-A Positivity in Stage I Estrogen-Receptor-Negative and -Positive Patients

In the previous study with Stage I ER-negative patients, there was a strong association between PAPP-A positivity and early recurrence. Of the 16 patients who had early recurrence, 9 (56%) were PAPP-A-positive, compared with 3 of 24 (11%) of the nonrecurrent group ($P < 0.001$).[5] Combining data from both Stage I ER-positive and -negative patients led to a significant correlation (generalized Wilcoxon [Breslow], $P < 0.002$) with recurrence as shown by life-table analysis (Figure 2). There was no significant difference in the frequency of PAPP-A positivity between ER-positive (30%) and ER-negative (40%) tumors. In addition, there was no significant difference between the frequency of recurrence in ER-positive (13.3%) and that in ER-negative (20%) patients. PAPP-A positivity showed no correlation with PR status.

Table 2—Positive PAPP-A Immunostaining and Early Recurrence in ER-Positive Stage I Breast Cancer

	PAPP-A$^+$	PAPP-A$^-$
Recurrent (n = 4)	4/4 (100%)	0/4 (0%)
Nonrecurrent (n = 26)	6/26 (23%)	20/26 (77%)
Total (n = 30)	10/30 (33%)	20/30 (66%)

$P < 0.002$ (Pearson chi-square).

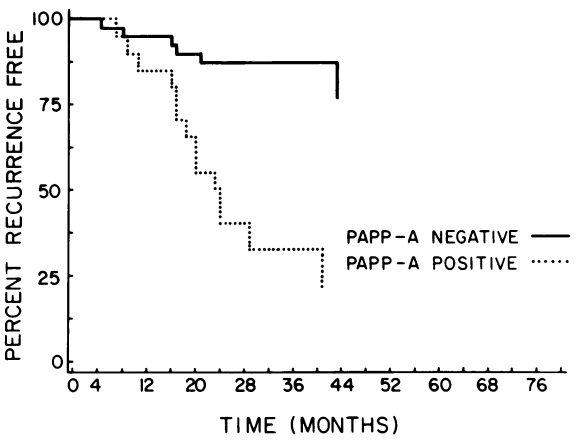

Figure 2—Life-table analysis substituting time of tumor recurrence for survival time with both ER-positive and -negative Stage I breast cancer patients. Approximately 50% of the patients with PAPP-A$^+$ tumors developed recurrent disease within 24 months, compared with 13% in the PAPP-A$^-$ group.

Other Histopathologic Features

There were 5 Grade I, 21 Grade II, and 4 Grade III carcinomas. Tumor grade, nuclear atypia, and Mitotic index did not correlate with recurrence. One tumor had focal necrosis, but none showed extensive necrosis. Review of the axillary lymph nodes showed 2 cases with sinus histiocytosis and one each with germinal center hyperplasia and diffuse hyperplasia. None of the other histopathologic features correlated with recurrence.

Discussion

Despite recent advances in the detection of early breast cancer, 25–35% of patients with Stage I disease die of metastatic breast carcinoma.[17,18] Many investigators have attempted to predict the biologic behavior of breast carcinoma through the assessment of various histopathologic criteria. Numerous features have been found to correlate with tumor virulence, such as histo-

logic grade,[10] nuclear grade,[19] necrosis,[20] lymph node sinus histiocytosis,[12] lymphocytic infiltration of tumor,[19] invasion of blood vessels, and pattern of tumor borders.[19] Although these studies have advanced our understanding of the biologic behavior of breast carcinoma, none of these features have correlated well enough with clinical disease to be useful in planning therapy for individual patients.[10] Thus, stage of disease, histologic type of breast cancer, size of the tumor, ER status, and number of axillary lymph node metastases remain the most important criteria in the assessment of the biologic behavior of breast carcinoma.[21]

With the advent of the immunoperoxidase technique and various other immunochemical methods, a variety of ectopically produced substances have been detected in human breast carcinoma. These substances range from oncofetal antigens such as carcinoembryonic antigen (CEA),[7] small polypeptide hormones such as human chorionic gonadotropin (hCG),[5] and secretory glycoproteins with hormonelike activity, the so-called pregnancy-associated proteins.[1-5] It is this last group, the pregnancy-associated proteins, which show promise in the study of the biology of breast carcinoma.

Pregnancy-associated proteins are roughly divided into 2 groups: those with hormonal or enzymatic activity such as hCG, which acts upon sites distant from the placenta, and those with local effects upon the immune or coagulation system, such as PAPP-A and pregnancy-specific-β_1-glycoprotein (SP-1). Most of these substances have been described in the serum in tumors with patients with breast carcinoma, each with its own unique clinical and biologic implications.

In two previous studies, we have demonstrated that PAPP-A may be ectopically produced by breast carcinoma and, when present, strongly correlates with early recurrence.[4,5] In our study of Stage II breast carcinoma patients, 82% of the patients with PAPP-A-positive tumors had early recurrence of disease, whereas 84% of the patients with PAPP-A-negative tumors remained disease-free after 2 years ($P < 0.0005$).[4] None of the other traditional histopathologic or clinical features evaluated correlated with early recurrence. Concerning early breast carcinoma, in our study of Stage I ER-negative breast carcinoma, there was significant, independent correlation of both PAPP-A positivity and extensive tumor necrosis with early recurrence.[5] Of those patients who had early recurrence, 9 of 16 (56%) were PAPP-A-positive, compared with 1 of 24 (4%) in the nonrecurrent group ($P < 0.001$). Combining extensive necrosis and PAPP-A positivity, in the 5 cases with both extensive necrosis and PAPP-A positivity, all patients experienced early recurrence of disease. When both features were absent, only 3 of 23 (13%) had recurrence. Those tumors with either extensive necrosis or PAPP-A positivity comprised an intermediate risk group, with 57% and 80% recurrence, respectively. Thus, in Stage II and Stage I ER-negative patients, PAPP-A positivity in the primary breast cancer was an indicator of poor prognosis with a clinically significant correlation with early (less than 2 years) recurrence.

Extending our study of Stage I breast carcinoma to include ER-positive patients, we saw that PAPP-A again showed a significant correlation with early recurrence. In this study of 30 patients, clinical recurrence developed in 6 of 30 (20%), 4 of 30 (13%) of patients experiencing early recurrence. Of the 10 PAPP-A-positive tumors, 4 (40%) had early recurrence, compared with no early recurrences in the 20 PAPP-A-negative cases ($P < 0.002$, Table 2). In addition to positive PAPP-A immunostaining, younger age ($P < 0.009$) and premenopausal status ($P < 0.03$) also correlated with early recurrence. When stepwise regression analysis was performed, however, the correlation of PAPP-A with early recurrence was independent of patient age. Thus, as in our previous studies, PAPP-A identified a subset of patients with virulent disease, as measured by early recurrence. Life-table analysis (Figure 2) in the combined populations of Stage I ER-positive and -negative patients further illustrated the association of PAPP-A positivity and recurrence.

With regard to the relationship of PAPP-A to ER status, frequency of PAPP-A positivity was similar for both ER-positive (33%) and ER-negative (30%) tumors. The independence of PAPP-A staining and ER protein is important biologically, because ER status itself has been shown to be associated with early recurrence and a shorter disease-free interval.[22,23] Thus, PAPP-A and ER status are two biologic parameters which correlate independently with early recurrence. Table 3 illustrates the interrelationship of PAPP-A, ER status, and early recurrence in the combined 70 cases of Stage I ER-positive and -negative cases. Among the patients with both risk factors present, namely, positive PAPP-A and negative ER, 9/16 (56%) experienced early recurrence. When both risk factors were absent, 0/20 (0%) had recurrence. The patients with PAPP-A-positive, ER-positive, and PAPP-A-negative, ER-negative tumors showed a combined frequency of early recurrence of 5/34 (18%), comprising an intermediate risk group. As

Table 3—Relationship of PAPP-A Immunostaining and Estrogen Receptor Status to Early Recurrence in 70 Stage I Breast Cancer Patients

	PAPP-A⁺	PAPP-A⁻
ER-Negative	9/16 (56%)*	1/24 (4%)
ER-Positive	4/10 (40%)	0/20 (0%)

* Percentage of patients who developed early recurrence.

with ER status, PAPP-A staining was also independent of PR status. The limited number of patients in this study did not permit further stratification based on ER and PR status.

The precise mechanism by which PAPP-A and ER protein modify the biologic behavior of breast carcinoma is unknown. Several recent studies, however, raise hypotheses concerning the role of these substances in tumor biology. It has been shown that ER-negative breast carcinomas have higher rates of cell proliferation, as demonstrated by thymidine labeling studies.[24] Histopathologically, ER-negative tumors more frequently show features suggestive of rapid tumor growth, such as higher tumor grade and mitotic index.[25] ER status appears to act as an indirect measure of the rate of tumor cell proliferation, which is reflected clinically as an increased risk of early recurrence in patients with ER-negative tumors.

Details of the relationship of PAPP-A to tumor virulence are not as well understood. PAPP-A is not associated with ER status, tumor grade, or mitotic index and does not appear to be a reflection of the rate of tumor growth. Recent investigations of the normal biologic function of PAPP-A, however, suggest that it locally alters the host's immune and coagulation system, providing a microenvironment which may be advantageous for tumor growth. Purified PAPP-A, either alone or in the presence of human serum, significantly inhibits lymphocyte responsiveness to phytohemagglutinin stimulation.[26,27] It has been postulated that PAPP-A modulates lymphocyte function at the level of lymphokine production.[26] Because PAPP-A-like material has been found both in human seminal plasma and ovarian follicular fluid,[28,29] it has been suggested that PAPP-A serves to modulate the maternal immune response initially to sperm antigens and, following fertilization, to the fertilized ovum. Similarly, production of PAPP-A by breast cancer may serve to suppress locally the host's immune response to the tumor. In addition to the interaction of PAPP-A with the immune system, PAPP-A has also been shown to interact with both the blood coagulation and fibrinolytic system. Purified PAPP-A has been found to inhibit the thrombin-induced coagulation of plasma, probably by activation of antithrombin III.[30] It is thought that the high local concentration of PAPP-A in the intervillous space of the placenta, where blood flow is sluggish, serves to prevent activation of the coagulation system and blood clot formation. These studies suggest two possible mechanisms which might explain the correlation of PAPP-A positivity and early recurrence. Production of PAPP-A by tumor cells may serve both to suppress locally the host's immune response to the tumor and to alter locally the coagulation system, enabling malignant cells to both gain access to and travel within vascular spaces to produce distant metastases.

Clinically, immunohistochemical localization of PAPP-A in breast carcinoma correlates significantly with early recurrence of disease and thus may provide a more accurate means of predicting the clinical behavior of early breast carcinoma. Pending further study with larger patient populations, PAPP-A immunostaining may better enable the selection of patients who might benefit from earlier and more intensive antitumor therapy. In addition, PAPP-A tumor positivity is independent of ER status and other histopathologic measures of tumor proliferation. Further basic studies are necessary for examination of the molecular biology of PAPP-A and its role in the mechanisms of tumor virulence.

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