Polyadenylate Polymerase Enzymatic Activity in Mammary Tumor Cytosols: A New Independent Prognostic Marker in Primary Breast Cancer

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ABSTRACT

Polyadenylate polymerase (PAP) is one of the enzymes involved in the formation of the polyadenylate tail of the 3\' end of mRNA. High levels of PAP activity were associated with rapidly proliferating cells. Here we evaluate the prognostic value of PAP activity in breast cancer patients. PAP specific activity values were measured by a highly sensitive assay in the tumor cytosols of 228 women with primary breast cancer. The median follow-up period was 58 months. PAP specific activity values ranged from 2.1–39.4 units/mg protein in the breast tumor cytosols, and the activity was correlated with the level of expression of the antigen. An optimal cutoff value of 5.5 units/mg extracted protein was first defined by statistical analysis. PAP status was then compared with other established prognostic factors in terms of relapse-free survival (RFS) and overall survival (OS). PAP activity levels had a tendency to increase with tumor-node-metastasis (TNM) stage and were higher in node-positive patients. Evaluation of the prognostic value of PAP was performed using univariate and multivariate analyses. Univariate analysis showed that PAP-positive patients had a less favorable prognosis for both RFS (relative risk (RR) = 2.35; P < 0.001) and OS (RR = 3.15; P < 0.001). PAP significantly added to the prognostic power for RFS (RR = 2.51; P = 0.0012) and OS (RR = 4.21; P < 0.001) in multivariate analysis, whereas patient age, tumor size, and nodal and ER status remained independent factors for predicting survival. When only node-negative patients were examined, PAP was found to be an independent factor for predicting RFS (RR = 3.68; P = 0.0032) and OS (RR = 4.81; P < 0.001). PAP did not appear to have a prognostic significance for node-positive patients. PAP is a new prognostic factor for early recurrence and death in breast cancer patients. Our results suggest that PAP may be used as an independent unfavorable prognostic factor in node-negative breast cancer patients because there were no significant associations between PAP and the other prognostic indicators evaluated in this group of patients.

INTRODUCTION

Breast cancer is the most common malignancy among women and is responsible for an estimated 24% of all cancers and 18% of all cancer deaths (1). Ultimately, about 1 of 13–14 women will develop breast cancer during their lifetime, and at least half of these patients will die as a consequence of metastatic disease. One-third of node-negative breast cancer patients are expected to have a distant recurrence within 10 years because occult disseminated disease, which is difficult to detect, may have been present at the time of diagnosis. Furthermore, the course of the disease and response to treatment vary greatly (1).

In view of the heterogeneity of breast carcinomas, in terms of both their biological profile and their clinical outcome, the need for reliable prognostic parameters is obvious. Classical prognostic factors in primary breast carcinoma are tumor size, nodal status, and distant metastases (TNM status) and age. Histopathology and nuclear grading are also standard prognostic variables (2). The presence of steroid receptors serves predominantly as an indicator of hormone responsiveness (3). Ploidy [DNA content (4, 5)] and proliferative capacity [S-phase fraction (6)] are the most well-characterized prognostic factors (7). These measurements have been shown to predict disease-free survival and OS in node-negative and node-positive breast cancer. Additional prognostic indicators such as oncogenes, growth factors, and secretory proteins have been investigated and appear to characterize the behavior of the tumor with respect to differentiation extent, growth rate, and metastatic pattern. The expression of the protease cathepsin D has been reported to be an unfavorable prognostic parameter (8) and has been found to promote invasive growth and metastasis of tumor cells (9). Overall, there is a need to identify as many cellular parameters as possible that will help define the biological profile of the breast tumor cell.

PAP, whose biological function is the polyadenylation of mRNAs, appears to be regulated within the cell cycle and has been shown to vary greatly in terms of enzymatic activity among normal and neoplastic tissues (10, 11). The poly(A) tail found in almost all eukaryotic mRNA is important in enhancing translation initiation and determining mRNA stability (12). Control of the poly(A) tail synthesis has the potential to be a key regulatory step in gene expression. PAP has been reported to reflect the proliferative activity of the cell, and it is also associated with less differentiated cells (11, 12). Most studies report that rapidly proliferating and actively metabolizing lymphocytes have higher levels of PAP activity (13, 14). Furthermore, it has been suggested to be a unfavorable prognostic factor in chronic leukemias (15–17).

PAP activity has been studied initially in the cytosol of breast tumors from 62 untreated patients, and significant association was observed between high PAP activity values and the TNM stage of the disease, node invasiveness, and c-erbB-2 overexpression (18).

The objective of the present study was to assess the significance of PAP activity in breast cancer prognosis. We measured PAP activity in the tumor cytosols of 228 breast cancer patients and analyzed the relation of PAP to other clinicopathological variables for the RFS and OS using univariate and multivariate analyses.

MATERIALS AND METHODS

Population Studied. Tumor specimens from 228 patients who underwent surgery for primary breast cancer between 1989 and 1993 at the Oncologic Hospital of Athens St Savvas as well as 15 nonmalignant breast tissues for negative control were evaluated in this study. Tumor specimens were drawn from a pool of frozen specimens originally submitted to the Laboratory of Hormone Receptors for steroid receptor analysis. A computerized database...
containing updated information concerning each patient, together with receptor status, nodal status, size of the primary tumor, number of positive nodes, age and menopausal status of the patients, and/or differentiation grade of the tumor, was available for statistical analysis.

The age of patients ranged from 24–96 years; the median age was 62 years. All patients had a histologically confirmed diagnosis of primary breast cancer and received no treatment before surgery. Modified mastectomy (98 patients) and breast-conserving lumpectomy (121 patients) with axillary lymph node dissection were performed on 96% of the patients. For patients who had axillary node dissection, the positivity rate for cancer involvement of lymph nodes was 61.8%. The sizes of the tumors resected during surgery ranged from 0.6–7.2 cm, and the mean and median sizes were 2.9 and 2.8 cm, respectively.

Clinical staging was performed according to the Postoperative International Union against Cancer TNM classification system (19). Histological grade of the tumors was determined according to criteria reported by Bloom and Richardson (20) and was known for 224 patients. Postoperative treatment was known for 217 of 228 patients, and postoperative locoregional radiotherapy was given to 158 patients. Disease relapse was defined as the first documented evidence of local or regional axillary recurrence or distant metastasis.

Follow-up information was available for 223 patients and included survival status (alive or deceased) and disease status (relapse free or recurrence/metastasis) along with the dates of the events and cause of death, if applicable. The median follow-up was 58 months. The distribution of follow-up times for patients still alive at the time of analysis ranged from 24–74 months with a median of 62 months; only six and two patients had been followed for less than 48 and 36 months, respectively. Follow-up times for the entire cohort, however, ranged from 10–75 months with a median of 58 months. The RFS in each case was the time interval between the date of surgical removal of the primary cancer and the date of the first documented evidence of relapse. The OS was the time interval between the date of surgery and the date of death or the date of last follow-up for those who were alive at the end of the study.

Preparation of Cytosolic Extracts. Tumor tissues (n = 228) were stored at –80°C until pulverization in liquid nitrogen and cytosolic extraction. The extraction procedure consisted of treatment of the tissue powders (10–50 mg) with a cell lysis buffer (500 μl) containing 50 mM Tris (pH 8.0), 150 mM NaCl, 5 mM EDTA, 1% NP40, and 1 mM phenylmethylsulfonyl fluoride for 30 min on ice and subsequent separation of cell debris from the cytosols by centrifugation at 15,000 × g for 30 min at 4°C. Supernatants were assayed for total protein concentration immediately after centrifugation by the Lowry method.

PAP Activity Assay. The assay measures the incorporation of [5′-3H]ATP into acid-insoluble material using poly(A) as initiator, as described previously (14, 18). One unit of enzyme activity is defined as 1 nmol of radioactive radioucleotide incorporated per hour. Specific activity is expressed as units of activity per milligram of protein.

Western Blot Analysis. Cell lysates were run on 8% SDS-PAGE gels and transferred electrophoretically to Immobilon-P (Millipore) membranes. Rabbit polyclonal antiserum raised against recombinant bovine PAP expressed in Escherichia coli (courtesy of Dr. E. Wahl and A. Jenny) was used at a dilution of 1:2500. Western blots were visualized with the enhanced chemiluminescence system by Amersham according to the manufacturer’s instructions. The films were scanned using the UMAX Scanner (Vista-S6) and quantitated using the Image 1.44 program.

Steroid Hormone Receptor Analyses. Steroid hormone receptors were quantified as described elsewhere (21). The results of the dual ligand binding assay, in which dextran-coated charcoal was used to separate bound ligand from free ligand, were interpreted by Scatchard analysis (22). Tumors with ER and PR concentrations of ≤10 fmol/mg protein were considered receptor negative, those with ER and PR concentrations of 10–300 fmol/mg were characterized as positive, and those with receptor concentrations above such values were considered strong positive, as described previously (23, 24).

Statistical Analysis. For analysis of the data, patients were subdivided into groups on the basis of different clinical or pathological parameters. Because the distribution of PAP activity levels was not Gaussian, the analysis of differences in PAP values between two groups was performed with the nonparametric Mann-Whitney U test. Similarly, relationships between more than two groups were determined by the Kruskal-Wallis test. In this analysis, PAP was used as a continuous variable. PAP values were also classified into two categories (PAP-positive and PAP-negative groups), and associations between PAP status and other qualitative variables were analyzed using the χ² and Fisher’s exact tests, where appropriate. An optimal cutoff point equal to 5.5 units/mg protein was found by χ² analysis. ER and PR values were categorized into strong positive, positive, and negative status as described above. Tumor size was classified into three categories: (a) <2 cm; (b) 2–5 cm; and (c) >5 cm. Lymph node status was either positive (any positive number of nodes) or negative. Age was categorized into three groups: (a) <45 years; (b) 45–55 years; and (c) >55 years. Survival analyses were performed by constructing Kaplan-Meier RFS and OS curves (25), where differences between the curves were evaluated by the log-rank test and by estimating the RRs for relapse and death using the Cox proportional hazards regression model (26). Cox regression analyses using SAS statistical software (SAS Institute, Cary, NC) were used to calculate the RR and 95% CI. Only patients for whom the status of all variables was known were included in the multivariate models.

RESULTS

Distribution of PAP Activity and Relationship to PAP Antigen Levels

The PAP activity of the 228 cytosolic samples varied widely from 2.1–39.4 units/mg protein; the median was 6.3 units/mg protein, and the mean was 9.9 units/mg protein. Fig. 1 shows the distribution of the activity, which was slightly positively skewed. The range of PAP activity in the cytosols derived from 15 nonmalignant samples was 1.5–4.8 units/mg protein; the mean and median values were 3.5 and 2.8 units/mg protein, respectively. To investigate the variation in PAP activity values recorded in the breast tumor cytosols, Western blot analysis was performed to determine the level of PAP antigen in the cytosols. One major protein band was present in all tumor cytosols examined, whereas in extracts from the MCF-7 breast cancer cell line, additional forms of PAP were apparent (Fig. 2). Furthermore, the data indicated that PAP activity values correlated with the level of expression (intensity) of the M₅₀ 80,000–90,000 protein. Thus, we are inclined to conclude that the differences in PAP enzymatic activity in breast tumor cytosols were due to different levels of expression of an M₅₀ 80,000–90,000 protein rather than differences in posttranslational modification.

Relationship of PAP Activity to Other Prognostic Variables

An optimal cutoff value was defined by χ² analysis, based on the ability of PAP values to predict the RFS and OS for the population studied. As shown in Fig. 3, the specific activity of 5.5 units/mg protein was shown to be the optimal cutoff value (χ² = 12.7 and
risk of relapse and a 3-fold higher risk of death in patients with PAP-positive tumors compared with those whose tumors were PAP negative. The Kaplan-Meier survival curves (Fig. 6) also showed that PAP-negative patients had more favorable RFS and OS rates than PAP-positive patients. The difference in survival rates between the two groups was greater for OS than for RFS. In the multivariate analysis of PAP, the Cox regression models were adjusted for age, nodal status, tumor size, grade, and cathepsin D, ER, and PR status, which were used as categorical variables (except for tumor size, which was used as a continuous variable) as described above. Patient age, tumor size, and ER and nodal status were thus shown to be independent factors for predicting both RFS and OS. PAP significantly added to the prognostic power in the multivariate model of RFS (RR = 2.51; \( P = 0.0012 \)) and OS (RR = 4.21; \( P < 0.001 \)) analyses.

**Univariate and Multivariate Analysis in Patients Classified by Nodal Status.** Because node-positive patients differ substantially from node-negative patients in terms of prognosis and postoperative treatment, separate univariate and multivariate Cox regression models were developed to evaluate the effect of PAP on RFS and OS for each of the two groups of patients. The results are shown in Table 2 and Fig. 7. Node-negative patients with PAP-positive tumors tended to have an approximately 5-fold increase in risk for relapse or death. PAP activity proved to be an independent factor for predicting RFS (RR = 3.68; \( P = 0.0032 \)) and OS (RR = 4.81; \( P < 0.001 \)) in node-negative patients (Fig. 7, A and B). Cathepsin D and ER significantly added to the prognostic power in the multivariate model of analysis for RFS and OS, respectively. In node-negative patients, no statistically significant difference was observed in RFS and OS between PAP-positive and -negative tumors (Fig. 7, B and D).

**DISCUSSION**

PAP activity has been reported in the past to have prognostic value, at least in chronic leukemias (16, 17). Our preliminary data show that PAP activity is related with other parameters linked to poor prognosis (18). PAP values had a tendency to increase with tumor grade and were higher in node-positive patients. An association of PAP activity with c-erbB-2 overexpression has also been observed (18). Overexpression of c-erbB-2 is considered to be an unfavorable prognostic indicator for both node-negative and node-positive patients (27, 28).

In this study, PAP activity was measured in the cytosols of 228
women, and these values were analyzed comparatively with the follow-up data of 223 of the patients. On dividing the patients into PAP-positive and PAP-negative groups using a cutoff value of 5.5 units/mg protein, a relation emerged between PAP activity and other unfavorable prognostic parameters. A higher percentage of tumors positive for PAP activity was recorded within the node-positive group in comparison with other histological types. These data confirmed previous observations linking high PAP activity levels with unfavorable prognostic parameters. A higher percentage of tumors positive for PAP activity was recorded within the node-positive group.

This is the first study of the prognostic value of PAP activity in breast cancer. Follow-up information for 223 patients indicated that patients with PAP-positive tumors had a 2-fold increase in risk of relapse and a 3-fold increase in risk of death, as compared with those with PAP-negative tumors. PAP activity significantly added to the prognostic power of parameters such as age, nodal status, tumor size, grade, and cathepsin D and steroid receptor status in the multivariate models of analysis for both RFS and OS. The prognostic significance of PAP activity was more prominent within the group of node-negative patients. A 5-fold increase in the risk for relapse or death was calculated for node-negative patients whose tumors showed high PAP activity. Our data indicate that PAP activity may be an independent factor of poor prognosis.

Because very little is known about the physiological role of PAP in breast tissue, a hypothesis explaining the mechanism by which PAP expression may confer poor prognosis in breast cancer, especially in node-negative patients, is at present difficult to formulate; it is also difficult to correlate PAP measurements with global or individual mRNA polyadenylation, a highly regulated multicomponent process. A strong positive association between high ER content (>300 fmol/mg) and PAP activity was observed. The presence of ER in tumor cells is a well-established predictor of response to endocrine therapy but is a weak prognostic indicator. Moreover, a very high ER concentration may be a negative prog-

| Variable | Total (%) | PAP negative (%) | PAP positive (%) | P
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<td>Age (yrs)</td>
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| <45 | 44 (19.3) | 20 (45.5) | 24 (54.5) | 0.47<sup>a</sup>
| 45–55 | 53 (23.2) | 29 (54.7) | 24 (45.3) | 0.001<sup>a</sup>
| >55 | 131 (57.5) | 59 (45.0) | 72 (55.0) | 0.001<sup>a</sup>
| Tumor size (cm) | | | |
| ≤2 | 56 (24.7) | 24 (42.9) | 32 (57.1) | 0.42<sup>a</sup>
| 2–5 | 152 (66.9) | 76 (50.0) | 76 (50.0) | 0.041<sup>a</sup>
| >5 | 19 (8.4) | 7 (36.8) | 12 (63.2) | 0.001<sup>a</sup>
| Nodal status | | | |
| Negative | 84 (38.2) | 48 (57.1) | 36 (42.9) | 0.001<sup>a</sup>
| Positive | 136 (61.8) | 60 (44.1) | 76 (55.9) | 0.001<sup>a</sup>
| Unknown | 4 | | | 0.001<sup>a</sup>
| Grade<sup>c</sup> | | | |
| I | 8 (3.6) | 5 (50.0) | 4 (50.0) | 0.63<sup>a</sup>
| II | 172 (76.8) | 80 (46.5) | 92 (53.5) | 0.63<sup>a</sup>
| III | 44 (19.6) | 24 (54.5) | 20 (45.5) | 0.63<sup>a</sup>
| Unknown | 4 | | | 0.63<sup>a</sup>
| Histology | | | |
| Ductal | 169 (73.7) | 64 (37.9) | 105 (62.1) | 0.001<sup>a</sup>
| Lobular | 20 (8.8) | 0 (0.0) | 20 (100.0) | 0.001<sup>a</sup>
| Other | 12 (6.0) | 0 (0.0) | 12 (100.0) | 0.001<sup>a</sup>
| Unknown | 27 | | | 0.001<sup>a</sup>
| Stage<sup>d</sup> | | | |
| I | 28 (12.8) | 20 (71.4) | 8 (28.6) | 0.001<sup>a</sup>
| II | 164 (74.9) | 76 (46.3) | 88 (53.7) | 0.033<sup>a</sup>
| III | 27 (12.3) | 11 (40.7) | 16 (59.3) | 0.033<sup>a</sup>
| Unknown | 9 | | | 0.033<sup>a</sup>
| ER status<sup>e</sup> | | | |
| Negative | 28 (12.3) | 12 (42.9) | 16 (57.1) | 0.001<sup>a</sup>
| Positive | 156 (68.4) | 92 (59.0) | 64 (41.0) | 0.12<sup>a</sup>
| Strong positive | 44 (19.3) | 4 (9.1) | 40 (90.9) | 0.12<sup>a</sup>
| PR status<sup>e</sup> | | | |
| Negative | 40 (17.6) | 24 (60.0) | 16 (40.0) | 0.013<sup>a</sup>
| Positive | 136 (59.6) | 68 (50.0) | 68 (50.0) | 0.013<sup>a</sup>
| Strong positive | 52 (22.8) | 16 (30.8) | 36 (69.2) | 0.013<sup>a</sup>
| Cathepsin D status<sup>e</sup> | | | |
| Negative | 104 (47.1) | 44 (42.3) | 60 (57.7) | 0.12<sup>a</sup>
| Positive | 117 (52.9) | 60 (51.3) | 57 (48.7) | 0.12<sup>a</sup>
| Unknown | 7 | | | 0.12<sup>a</sup>
| Adjuvant treatment | | | |
| None | 33 (15.2) | 20 (60.6) | 13 (39.4) | 0.091<sup>a</sup>
| Tamoxifen | 116 (53.5) | 48 (41.4) | 68 (58.6) | 0.091<sup>a</sup>
| Chemotherapy ± tamoxifen | 68 (31.3) | 36 (52.9) | 32 (47.1) | 0.091<sup>a</sup>
| Unknown | 11 | | | 0.091<sup>a</sup>

<sup>a</sup> Fisher’s exact test.
<sup>b</sup> Bloom-Richardson grading system (20).
<sup>c</sup> TNM system.
<sup>d</sup> ER negative: <10 fmol/mg; positive, 10–300 fmol/mg; strong positive, >300 fmol/mg.
<sup>e</sup> Cutoff point, 60 pmol/mg.
nostic indicator. Patients with very high ER content have been reported to have a poor prognosis compared with patients with a low concentration of ER (29, 30). It was recently reported that whereas the presence of ER and expression of the proliferation-related marker Ki-67 are mutually exclusive in normal proliferating epithelium, in breast cancer, they are often coexpressed (31). If PAP is increased in proliferating cells, it is not surprising that its expression correlates with ER content.

The poly(A) tail is present at the 3’ end of virtually all mRNAs and affects both cytoplasmic mRNA stability and translatability (12). Formation of this structure involves endonucleolytic cleavage of the mRNA precursor coupled with poly(A) synthesis, a reaction that requires several protein factors. Two multisubunit factors are required for specification of the poly(A) site and formation of a stable protein-RNA complex. Subsequently, two additional proteins, as well as PAP, join the complex and are required for cleavage of the pre-mRNA and synthesis of the poly(A) tail (32). Regulation of individual components of the polyadenylation process has been established, for example, during the cell cycle and cellular differentiation (13, 33, 34). Furthermore, inappropriate RNA processing is prevented by the BRCA1-associated RING domain protein (BARD1) that interacts with the polyadenylation factor CstF-50 and inhibits polyadenylation in vitro (35).

In mammalian cells, high levels of PAP activity have been reported in rapidly proliferating cells and in neoplastic cells (11, 14–17, 36). Earlier reports attribute increased levels of PAP activity in neoplasia to phosphorylation (37). However, recent data indicate that among the several species of PAP characterized by varying degrees of phosphorylation, the hyperphosphorylated species represents a form with reduced enzymatic activity. It has been shown that PAP can be phosphorylated in vivo and in vitro by p34(cdc2)/cyclin B (maturation/mitosis-promoting factor) at four nearby nonconsensus cyclin-dependent kinase sites (38). PAP becomes hyperphosphorylated both during meiotic maturation of Xenopus laevis oocytes and in HeLa cells arrested at M phase, times in the cell cycle at which maturation/mitosis-promoting factor is known to be active (13, 39). Reduced PAP activity probably contributes to the well-established reduction in polyadenylated mRNA and/or protein synthesis known to occur in M-phase cells (13, 38). In the breast cytosols studied, only one species of PAP was detected by the polyclonal antibody used, and the intensity of the band corresponded to the enzymatic activity measured in each sample. The same antibody recognized additional

![Fig. 6. RFS (A) and OS (B) curves in patients with PAP-positive and PAP-negative breast tumor cytosols, followed for a median of 58 months. The cutoff value for PAP-positive status was 5.5 units/mg protein.](image-url)
species in MCF-7 extracts and multiple forms of PAP in other cell lines (40). The possibility that additional forms of the enzyme exist in the intact breast tumor cells cannot be excluded. However, the above-mentioned data confirm that the high activity measured in certain samples reflects overexpression of the enzyme.

In breast cancer, the degree of lymph node invasion is one of the major prognostic factors for predicting relapse and death (3, 41, 42). On the other hand, about 30% of node-negative tumors relapse, and it has been proposed to apply systematic adjuvant chemotherapy to all patients, including those who are node-negative (43). However, this adjuvant treatment may be detrimental to patients who do not require it. In our study, PAP-positive status in node-negative patients is not only an adverse prognostic factor, but PAP-positive patients in this group have substantially worse prognosis than node-positive patients, regardless of PAP status. This may suggest the potential utilization of PAP to predict which of the node-negative patients should be treated.

The level of PAP activity in the cytosols of breast tumors may be a parameter related to proliferation and/or transcriptional activity of the tumor cell. Alternatively, high levels of PAP activity may reflect deregulated expression of this protein, which in turn may contribute to the malignant phenotype of the cell. Both possibilities merit further examination. Nevertheless, our data indicate that high levels of PAP activity characterize a more aggressive tumor type, and PAP has been proven to be a new independent prognostic indicator, particularly in node-negative patients.

ACKNOWLEDGMENTS

We thank Dr. W. Keller’s laboratory (Biozentrum, Basel University, Switzerland) for the generous gift of PAP polyclonal antiserum.
PAP ACTIVITY IN BREAST CANCER


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