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RESEARCH ARTICLE

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Relationships of protein biomarkers of the urokinase plasminogen activator system with expression of their cognate genes in primary breast carcinomas

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Abstract

Background: uPA, its receptor uPAR, and inhibitors PAI-1 and PAI-2 play key roles in membrane remodeling/invasion and in predicting response to chemotherapy. We identified novel relationships of these biomarkers with ER/PR that indicate clinical utility for assessing breast carcinoma outcomes.

Methods: Retrospective studies were performed with de-identified results of (a) uPA, uPAR, and PAI-1; (b) estrogen (ER) and progestin receptor (PR); and (c) clinical outcomes. Relative expression of 22 000 genes from microarray of RNA from LCM-procured breast cancer cells was used with R Studio version 3.4.1.

Results: Primary ER/PR status was related to uPA, uPAR, or PAI-1 levels. ER− or PR− cancers expressed elevated *uPA*, *uPAR*, and *PAI2* mRNA compared to ER+ or PR+ cells. Inverse relationships between ER/PR protein and expression of *uPA*, *uPAR*, and *PAI-2* were observed, whereas HER2 status was unrelated. qPCR analyses showed *RERG* and *NQO-1* expressions were elevated in uPA− lesions, while *CD34* and *EDG-1* were elevated in uPAR− cancers. *ERBB4* was overexpressed in PAI-1+ carcinomas. Cox regression analyses revealed relationships of ER/PR status and uPA system members with regard to clinical outcomes of breast cancer.

Conclusions: *uPA*, *uPAR*, *PAI1*, or *PAI2* expression was increased in either ER− or PR− cancers similar to that of protein content in ER−/PR− carcinomas, suggesting sex hormones regulate the uPA system in breast cancer. Results revealed protein content of uPA system members was related to ER/PR status of primary lesions. Use of LCM-procured carcinoma cells uncovered relationships between expression of known cancer-associated genes and protein content of uPA system members. Collectively, results indicate evaluation of ER and PR protein of primary breast cancers combined with analyses of uPA, uPAR, and PAI-1 protein content improves assessment of clinical outcomes.

KEYWORDS

breast carcinoma, estrogen receptor, gene expression, laser capture microdissection (LCM), PAI-1, PAI-2, progestin receptor, uPA, uPAR

1 | INTRODUCTION

1.1 | Conventional biomarkers used in the management of breast cancer

Standard of care for management of breast cancer includes assessments of levels of ER, PR, and HER2 protein in the tissue biopsies.[1-3] For instance, patients with breast cancers exhibiting a biomarker subtype of ER+/PR+/HER2− typically have a better prognosis than those with triple-negative breast cancer. Patients with a biomarker subtype of ER+/PR+/HER2+ are candidates for hormonal treatments (eg, tamoxifen, letrozole) as well as treatment with herceptin or other humanized antibody-based therapies. However, patients with triple-negative breast cancers have poor survival and few treatment options other than chemotherapy, radiation, and surgical excision of cancers.[2,4-7]

Clinically relevant cutoff values for assays, which quantified biomarkers, were established previously for each receptor that indicated potential for response to drug therapy regimens (eg, tamoxifen, herceptin). Within the past three decades, the principal assays for the three analytes have been semi-quantitative, immunohistochemical-based procedures. Due to the lack of assay standardization and related problems, the College of American Pathologists (CAP) and the Society for Clinical Oncology (ASCO) established guidelines for improving uniformity of measurements and reporting of IHC results.[2,7,8]

Although conventional biomarkers (eg, ER, PR, and HER2 proteins) have demonstrated utility in management protocols of breast cancer, improvements in personalized treatment plans are at the forefront of cancer research. Genomic screens (eg, gene expression profiles) have been developed, which assess expression levels for genes whose actions and protein products are associated with breast cancer behavior. For instance, an FDA-approved microarray test known as MammaPrint™ uses expression of 70 genes to assess risk of breast cancer development and risk of recurrence[9-11] (website: https://www.agendia.com/our-tests/mammaprint/).

1.2 | Review of plasminogen activator system and its role in cancer growth

A ubiquitous network known as the urokinase plasminogen activator (uPA) system is a proteolytic enzyme system consisting of four gene members, which collectively remodel tissue basement membranes.[12,13] The system consists of uPA, which is a serine protease, its respective uPA receptor (uPAR), which is associated with the extracellular components of a target cell, and two inhibitors of uPA association with uPAR (PAI-1 and PAI-2). One of the principal functions of the uPA system is to convert plasminogen to plasmin to aid in the dissolution and breakdown of fibrin at clot sites in blood vessels. In addition to the dissolution of clots, the uPA system also has reported roles in a range of biochemical processes such as control of inflammation, angiogenesis, embryogenesis, wound healing, and cellular apoptosis.[14] From a clinical standpoint, the uPA system is widely documented in processes of tumor migration, angiogenesis,[15,16] modulation of tumor microenvironments, and metastasis in a number of cancers such as lung,[17] breast,[6] ovarian,[18,19] endometrial,[19] and pancreatic.[20] However, it is critical to consider that reports of the efficacy of uPA system members as prognostic markers in other cancers (ie, other than in breast carcinomas) remain contradictory.[21]

1.3 | Clinical application of the uPA system

The uPA system has been studied regarding cancer outcomes for nearly three decades, with one of the first suggestions for use as a prognostic marker by Duffy (cf. [12]). Following the original report of Duffy[22] multiple studies[21,23,24] demonstrated use of uPA/PAI-1 expression levels as predictors of cancer outcomes in node-negative breast cancer. At the turn of the millennium, large investigations confirmed the use of quantified uPA and PAI-1 from breast cancer patients as a measure for assessment of treatment response, progression, and outcome.[25-27] Furthermore, breast cancers exhibiting high uPA/PAI-1 levels are associated with patients with poor prognosis, yet are most responsive to adjuvant chemotherapy.[24,28] Additionally, patients having high uPA/PAI-1 levels in node-negative breast cancers that are ER+ may benefit from anti-HER2 therapy in HER2+ breast cancer patients. Currently, the American Society of Clinical Oncology (ASCO) encourages medical oncologists to utilize quantified uPA/PAI-1 levels in patients to direct decisions regarding adjuvant chemotherapy in early-stage and node-negative breast cancers.[29]

A major question remaining was related to the relationships of either uPA, PAI-1, or uPAR protein content in the context of the estrogen receptor (ER) or progestin receptor (PR) status of the primary breast carcinoma. Using extensive data mining approaches of existing databases, relationships of protein content of the biomarkers, uPA, uPAR, and PAI-1 in primary breast cancer biopsies with other clinical features of the carcinomas (eg, nodal status) and of the patients (eg, menopausal status) were studied to determine alternative biomarker combinations (eg, with either ER or PR status) that better predict clinical outcomes of breast cancer.

2 | MATERIALS AND METHODS

2.1 | Remark

Summaries of patient and specimen characteristics are displayed by REMARK tables[30] for the two distinct populations analyzed in this study. The first patient population summary provided in Table 1 represents those patients whose tissue biopsy measurements of uPA, uPAR, and PAI-1 were used in the investigations outlined in the Results section. Protein biomarker measurements were performed by earlier investigators in the laboratory according to the protocols described for determination of uPA system members.[18,19,31] A summary of patient characteristics and tissue-based measurements for the population used in the microarray studies is given in Table 2.

TABLE 1 Clinicopathological properties of study population of primary breast carcinomas and patients used in analysis of biomarker protein status

Continuous variables	Mean (SD)
Age (y)	55.0 (14.5)
Tumor size (mm)	29.8 (14.6)
	Median (IQR)
Progression-free survival (mo)	52 (2-77)
Overall survival (mo)	62.5 (33-78)
Discrete variables	**n (%)**
Menopausal Status (n = 749)	
Premenopausal	359 (47.9)
Postmenopausal	386 (51.5)
Unknown	4 (0.6)
Biomarker measurements (protein)	
uPA	745
uPAR	616
PAI-1	749
PFS events	11 (10.0)
PFS censored	99 (90.0)
OS events	27 (24.5)
OS censored	83 (75.5)
Pathology of primary	
IDC	80 (76.2)
ILC	7 (6.7)
IDC + ILC	1 (1.0)
Other histologic types	17 (16.2)
Nodal status (n = 105)	
N 0	48 (45.7)
N 1-3	27 (25.7)
N > 3	17 (16.2)
Unknown	13 (12.4)
Tumor grade (n = 105)	
1	10 (9.5)
2	32 (30.5)
3	38 (36.2)
4	2 (1.9)
Unknown	23 (21.9)
Tumor stage (n = 105)	
0	4 (3.8)
1	26 (24.8)
2	50 (47.6)
3	9 (8.6)
4	9 (8.6)
Unknown	7 (6.7)
Steroid receptor status (n = 498)[a]	
ER+	263 (52.8)
ER–	235 (47.2)
PR+	271 (54.4)
PR–	227 (45.6)
HER2 status (n = 425)[b]	
HER2+	254 (59.8)
HER2–	171(40.2)

[a]Cutoff values for ER and PR are described in the methods.
[b]Cutoff value for HER2 protein levels for the NEN/DuPont ELISA was 1.7 hnu/μg protein, while that used for the TRITON Diagnostics EIA was 129.9 hnu/mg protein.

Patients were treated with standard of care of time of tissue biopsy collection, and tissue measurements were performed according to the protocols presented earlier.[3,31,32]

2.2 | Determination of plasminogen activator system biomarkers

ELISA analyses previously quantified uPA, uPAR, and PAI-1 levels on extracts of freshly collected and frozen tissue biopsies, stored at −86°C, using IMUBIND™ kits (formerly American Diagnostica Inc; currently BioPacific Diagnostics, Inc) applying cutoff values previously reported.[23] Cutoff values of 3.0, 2.9, and 2.2 ng/mg protein were utilized for protein measurements of biomarkers uPA, uPAR, and PAI-1, respectively. Biomarker cutoff values for uPA and PAI-1 were determined in accordance with clinically defined parameters,[23] whereas the cutoff value that we employed for uPAR protein was the median of 614 measurements from the patient population. Kits for assessing PAI-2 protein were unavailable at the time of these analyses.

Biochemical analyses of uPA, uPAR, and PAI-1 were performed on extracts as described previously using freshly collected and frozen tissue biopsies before being assayed[18,19,31,33] using a sandwich ELISA procedure (IMUBIND™, American Diagnostica Inc; currently BioPacific Diagnostics, Inc). The uPA assay recognizes pro-uPA, high-molecular-weight uPA, receptor-bound uPA, and uPA complexed with PAI-1 and PAI-2. With the uPAR assay, both soluble and native (membrane-associated) uPAR as well as complexes of either uPAR/uPA or uPAR/uPA/PAI-1 are all recognized. The PAI-1 assay determined both active and inactive forms of free PAI-1 and PAI-1 complexes according to the manufacturer.[31,33] The final concentration of each analyte was expressed in ng of uPA, uPAR, or PAI-1 per mg extract protein.

2.3 | Determination of estrogen receptor and progestin receptor protein levels

Estrogen receptor and PR protein levels were determined previously using either the Abbott Laboratories enzyme immunoassay

TABLE 2 Clinicopathological properties of microarray study population of primary breast carcinomas and patients

Continuous variables	Mean (SD)
Age (yr)	58.4 (14.9)
Tumor size (mm)	29.8 (16.3)
	Median (IQR)
Progression-free survival (mo)	57 (26.0-82.5)
Overall survival (mo)	65 (41.0-89.5)
Discrete variables	**n (%)**
Menopausal status	
Premenopausal	58 (23.5)
Postmenopausal	135 (54.6)
Unknown	54 (21.9)
Race	
White	211 (85.4)
Black	34 (13.8)
Other	2 (0.8)
PFS events	96 (38.9)
PFS censored	151 (61.1)
OS events	75 (30.4)
OS censored	172 (69.6)
Pathology of primary	
IDC	201 (81.4)
ILC	15 (6.1)
IDC + ILC	2 (0.8)
Other histologic types	29 (11.7)
Nodal status	
N 0	126 (51.0)
N 1-3	55 (22.3)
N > 3	46 (18.6)
Unknown	20 (8.1)
Tumor grade	
1	14 (5.7)
2	69 (27.9)
3	94 (38.1)
4	1 (0.4)
Unknown	69 (27.9)
Tumor stage	
0	3 (1.2)
1	60 (24.3)
2	140 (56.7)
3	35 (14.2)
4	4 (1.6)
Unknown	5 (2.0)
Steroid receptor status[a]	
ER+	146 (59.1)
ER–	101 (40.9)
PR+	151 (61.1)
PR–	96 (38.9)
HER2 status (n = 45)[b]	
HER2+	28 (62.2)
HER2–	17 (37.8)

(Continues)

TABLE 1 (Continued)

[a]Cutoff values for ER and PR are described in the methods.
[b]Cutoff value for HER2 protein levels for the NEN/DuPont ELISA was 1.7 hnu/μg protein (7+ of 14 biopsies), while that used for the TRITON Diagnostics EIA was 129.9 hnu/mg protein (21+ of 31 biopsies).

(EIA kit) or the radio-labeled ligand binding assay (NEN/DuPont kit) on freshly prepared cytosols as described previously.[1,3,32,34] Using the latter FDA-approved kits, which employ either [^{3}H]estradiol-17β or [^{3}H]R5020 depending upon the receptor type being determined, specific ligand binding capacity, reflecting both receptor level, expressed as fmol/mg cytosol protein, and activity expressed as the apparent dissociation constant (K_d value as M), were determined previously by Scatchard analysis. Determination of ER and PR levels by EIA employed an FDA-approved kit formerly distributed by Abbott Laboratories.[3,32] The distribution according to ER and PR status of the primary breast carcinomas used in these investigations is given in Tables 1 and 2.

2.4 | Detection of HER2/neu in primary breast cancers

HER2/neu oncoprotein status was measured in primary breast carcinomas using either one of the two experimental antibody-based assays as described previously.[3,31,35,36] Cutoff value measured in HER2–neu units (hnu) utilized for NEN/DuPont ELISA (which became Oncogene Science Diagnostics) was 1.7 hnu/μg protein (7+ of 14 biopsies), while that used for the TRITON Diagnostics EIA was 129.9 hnu/mg protein (21+ of 31 biopsies).

2.5 | Assessment of gene expression in LCM-procured cells using microarray analyses

Results described in these investigations were collected previously from de-identified primary breast cancer carcinomas obtained from 1988 to 1996, in IRB-approved studies and stored in de-identified databases of the Hormone Receptor Laboratory, which holds CLIA and Commonwealth of Kentucky licenses.[37-41] Selection and examination of the patient population were performed using REMARK criteria[1] as described previously.[37,40-44] Patients were treated with standard of care at time of diagnosis. Patient-related characteristics and tissue-based properties (Table 1), stored as de-identified parameters in our unique comprehensive databases, were explored to determine relationships between relative gene expression and clinical parameters.

Briefly, tissue sections of frozen de-identified tissue biopsies were processed previously for laser capture microdissection (LCM) with a PixCell IIe™ Instrument (Arcturus/Thermo Fisher), as described previously.[34,37-41] As reported earlier,[38,45,46] total RNA was extracted from LCM-procured cells and amplified before microarray as described earlier. Relative expression levels of each of 20 000 genes obtained from microarray uniquely represented only those mRNA species of breast carcinoma cells.[34,38-40,45,46]

2.6 | Assessment of gene expression in breast carcinoma tissue sections using qPCR analyses

Using qPCR results determined in our laboratory by other investigators,[37,39-41,47] gene expression levels for almost 100 genes, known to be associated with various cancers, were used in these studies to explore relationships with members of the uPA system.

2.7 | Univariable Cox regressions and survival analyses

Statistical computations, violin plots, and Kaplan-Meier plots were performed using R version 3.2.5. Utilizing commands from R package *survival*,[48] univariable Cox regressions of expression levels of each gene candidate estimated *P*-values of hazard ratios (HRs) to determine genes suggesting clinical significance. *P*-values were adjusted for multiple comparisons using the Benjamini and Hochberg (BH) method with <0.30 selected as the "discovery" cutoff as applied earlier.[43,49-51]

Univariable Cox regression was performed on each gene candidate using relative expression levels to discern relationships with progression-free survival (PFS) and overall survival (OS). This allows the use of relative gene expression values as a single covariate to investigate extent to which expression levels of a single gene in the cohort predicted PFS or OS of breast cancer patients. Hazard ratios were derived from univariable Cox regression models and calculated for each of the candidate genes. Summaries of Cox regression analyses, which utilized gene expression from LCM-procured breast carcinoma cells, are provided in Tables 3 and 4.

3 | RESULTS

3.1 | Interrelationships of biomarker protein status of the primary breast carcinoma as a function of patient age

Scatter plots were constructed in order to ascertain the relationship between patient age and quantified biomarker protein in the primary breast cancer (Figure 1). Linear regression analyses were performed, and a *P*-value of <0.05 was considered significant. Our results indicated that age of the patient was not significantly associated with the level of either uPA, uPAR, or PAI-1 biomarker protein in the primary breast cancer.

TABLE 3 Univariable Cox regression analyses of relative gene expression according to ER status of LCM-procured breast carcinoma cells

Gene symbol	β	HR	95% CI (HR)	*P* value	Adjusted *P*-value
PFS/ER+					
uPA	0.17	1.185	(0.93,1.51)	0.165	0.375
uPAR	0.14	1.153	(0.39,1.19)	0.421	0.561
PAI-1	0.07	1.068	(0.82,1.63)	0.660	0.660
PAI-2	−0.39	0.680	(0.8,1.43)	0.177	0.354
OS/ER+					
uPA	0.21	1.231	(0.21,0.92)	0.168	0.335
uPAR	0.13	1.136	(0.92,1.66)	0.516	0.588
PAI-1	0.13	1.135	(0.77,1.67)	0.466	0.516
PAI-2	−0.83	0.437	(0.81,1.59)	**0.030**[a]	0.122
PFS/ER-					
uPA	−0.08	0.923	(0.14,0.76)	0.535	0.753
uPAR	−0.39	0.677	(0.85,1.61)	**0.013**[a]	0.093
PAI-1	0.19	1.213	(0.59,1.53)	0.238	0.630
PAI-2	0.06	1.061	(0.66,1.39)	0.454	0.614
OS/ER-					
uPA	0.10	1.105	(0.05,0.44)	0.488	0.554
uPAR	−0.14	0.870	(0.86,1.86)	0.342	0.459
PAI-1	0.39	1.471	(0.51,1.45)	**0.039**[a]	0.275
PAI-2	0.09	1.097	(0.74,1.66)	0.271	0.382

Note: Relative expression of each gene was utilized for ER+ patients (n = 146); relative expression of each gene was utilized for ER− patients (n = 101).

[a]Bold values indicate genes as those with an adjusted *p* value below 0.3.

TABLE 4 Univariable Cox regression analyses of relative gene expression according to PR status of LCM-procured breast carcinoma cells

Gene symbol	β	HR	95% CI (HR)	*P* value	Adjusted *P*-value
PFS/PR+					
uPA	0.25	1.286	(0.99,1.66)	**0.055**[a]	0.221
uPAR	0.16	1.177	(0.87,1.58)	0.282	0.342
PAI-1	0.12	1.128	(0.83,1.54)	0.443	0.443
PAI-1	−0.16	0.853	(0.6,1.22)	0.384	0.404
OS/PR+					
uPA	0.39	1.481	(1.07,2.05)	**0.018**[a]	0.072
uPAR	0.21	1.232	(0.89,1.71)	0.213	0.426
PAI-1	0.10	1.102	(0.77,1.58)	0.600	0.657
PAI-2	−0.15	0.864	(0.57,1.3)	0.486	0.600
PFS/PR-					
uPA	−0.12	0.885	(0.55,1)	0.310	0.413
uPAR	−0.30	0.737	(0.97,1.35)	**0.048**[a]	0.191
PAI-1	0.05	1.054	(0.7,1.12)	0.725	0.725
PAI-2	0.13	1.143	(0.79,1.41)	0.109	0.218
OS/PR-					
uPA	−0.02	0.982	(0.93,1.74)	0.894	0.894
uPAR	−0.10	0.908	(0.98,1.4)	0.522	0.696
PAI-1	0.24	1.269	(0.67,1.22)	**0.137**[a]	0.274
PAI-2	0.16	1.170	(0.76,1.28)	**0.084**[a]	0.212

Note: Relative expression of each gene was utilized for PR+ patients (n = 151); relative expression of each gene was utilized for PR− patients (n = 96).

[a]Bold values indicate genes as those with an adjusted *p* value below 0.3.

3.2 | Determinations of biomarker protein status as a function of either ER or PR protein status of the primary breast carcinoma

Violin plots were computed of biomarker protein content (ng biomarker/mg total protein) of each uPA system member according to either ER or PR status of the primary carcinoma. Using highly quantified assays for steroid receptor status that have received FDA approval[34] (DuPont 1988 PR Assay Kit, Abbott 1988 ER−EIA monoclonal assay; DuPont 1989 ER Assay Kit, Abbott 1998 PGR-EIA monoclonal assay), cutoff values were utilized as reported earlier.[3,32,34] An adjusted *P*-value of <0.30 was employed as the discovery cutoff for significance as described previously.[43]

Results of analyses are displayed in which either ER or PR protein was measured either by radio-ligand binding or by EIA (Figure 2). When either ER or PR levels were measured by radio-ligand binding, it was observed that protein content of both uPA and uPAR was elevated in receptor-positive carcinomas compared to those of receptor-negative carcinomas (Figure 2A,B and D,E). In contrast, it was observed that protein content of uPAR was significantly elevated in ER− cancer biopsies compared to those ER+ specimens as measured by EIA (Figure 2H). Furthermore, protein levels of PAI-1 were significantly elevated in either ER− or PR−negative cancers measured by EIA compared to receptor-positive cancers (Figure 2I,L).

For each relationship, violin plots juxtaposed distributions of uPA, uPAR, or PAI-1 expression values of either ER+ vs ER− or PR+ vs

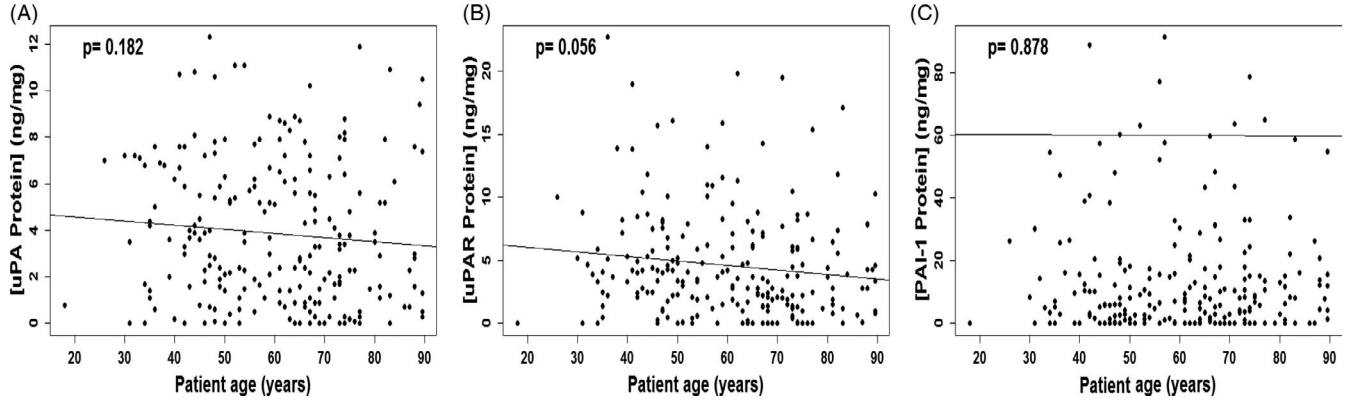


FIGURE 1 Scatter plot analyses of patient age versus protein biomarker content of members of the urokinase plasminogen activator system. A, Relationship of age versus [uPA], n = 217; B, age versus [uPAR], n = 218; C, age versus [PAI-1], n = 218

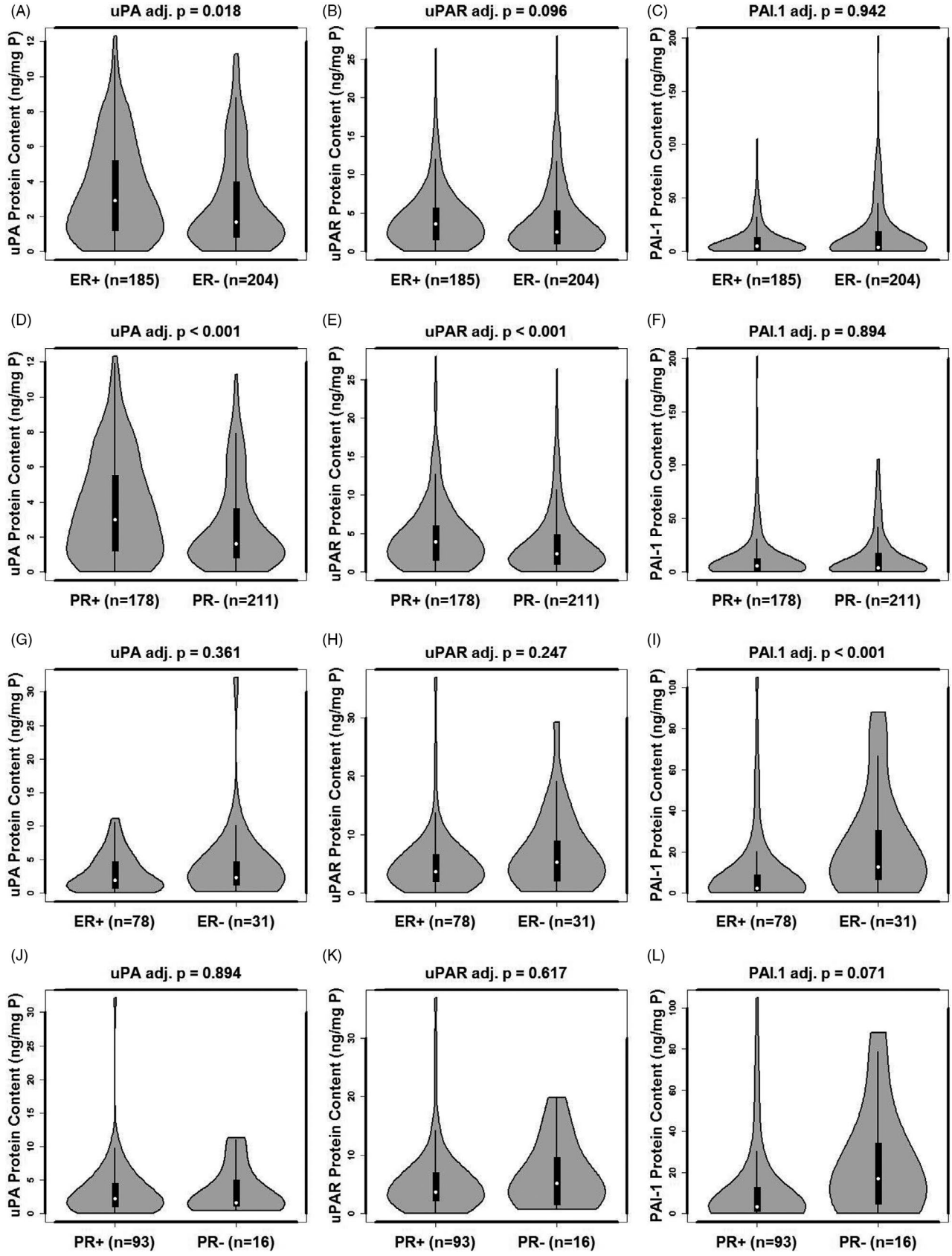


FIGURE 2 Relationship of biomarker protein status of the uPA system with ER or PR status of the primary carcinoma. Steroid receptor protein content (ER or PR) in plots A-F was determined by radio-ligand binding, and content in plots G-L was assessed by EIA. Adjusted *P*-values of each comparison are shown above each pair

PR− primary breast cancers to examine potential regulation by these sex steroid hormones. Distributions were compared using an unpaired independent two-sample Mann-Whitney-Wilcoxon test. Plots display the kernel of the distribution of log uPA, uPAR, or PAI-1 expression for either ER or PR subtypes from breast carcinomas. Median of log relative expression is indicated by a white circle, and the interquartile range is denoted with a black bar.

3.3 | Assessment of biomarker protein status as a function of HER2 protein status of the primary breast carcinoma

To assess the relationship between HER2 protein status of a primary breast carcinoma and uPA system biomarker, violin plots were constructed (Figure 3). An adjusted *P*-value of <0.30 was employed as the cutoff value for significance. Our analyses indicated that HER2 status of the primary breast cancer was not related to protein content of either uPA, uPAR, or PAI-1.

3.4 | Analysis of biomarker gene expression of the uPA pathway members as a function of ER or PR protein status of the primary breast carcinoma

Since ER is used routinely as a biomarker for prediction of breast cancer recurrence and therapy selection such as tamoxifen administration,[2] relative expression of either *uPA*, *uPAR*, *PAI-1*, or *PAI-2* genes was examined through construction of violin plots according to ER+ or ER− status of the primary lesion (Figure 4). An adjusted *P*-value of <0.30 was employed as a discovery cutoff for significance.[43] Note that relative gene expression was estimated using LCM-procured breast carcinoma cells as described in Section 2.1. These analyses revealed that expression of the *uPA* gene was significantly elevated in ER− cells compared to ER+ cells (Figure 4A). Analyses also indicated that expression of either *uPAR* or *PAI-2* genes was significantly elevated in ER− breast cancer cells compared to ER+ cells at an adjusted *P*-value of <0.001 (Figure 4B,4). Expression of *PAI-1* gene was not significantly expressed in regard to ER status of the primary lesion.

In addition, PR status of the primary lesion was evaluated in relation to expression of each candidate gene in LCM-procured cells. Therefore, violin plots of PR+ and PR− lesions were constructed in relation to relative gene expression of each gene of interest (Figure 4). Analyses indicated that expression of *uPA* and its respective receptor, *uPAR*, were significantly elevated in PR− lesions (Figure 4 e-f). In comparison with PR+ cells, elevated expression of both *PAI-1* and *PAI-2* genes was detected in PR− breast cancer cells (Figure 4G,H).

To determine whether there is a relationship between either ER or PR protein levels in a primary lesion and relative expression of each gene of interest, scatter plots were constructed. Total ER or PR protein content (fmol/mg P) of each primary breast carcinoma was plotted as a function of relative expression for each gene of interest (Figure 5). Note that since microarray analyses also provided results of *PAI-2* gene expression in LCM-procured cells, determination of these relationships was possible with each of the four members of the plasminogen activator system.

Representative analyses of statistically significant analyses are shown in Figure 5. These data indicated a negative relationship between ER protein content and relative expression of *uPA*, *uPAR*, and *PAI-2* genes that was statistically significant (Figure 5A-C). When the relationship between PR protein content of the cancer biopsies was examined in relation to expression of each candidate biomarker, expression of *uPAR* and *PAI-2* genes was significant (Figure 5D,E).

3.5 | Interrelationships of relative gene expression of *uPA*, *uPAR*, *PAI-1*, *and PAI-2* as a function of HER2 protein status of the primary breast carcinoma

The HER2/neu protein status of a primary breast cancer is considered with ER and PR as a biomarker for prediction of breast cancer outcome and selection of treatment regimen.[2,7,52] Relative expression of each gene of members of the uPA system determined by microarray was evaluated in relationship to HER2 protein status (Figure 6). Analyses

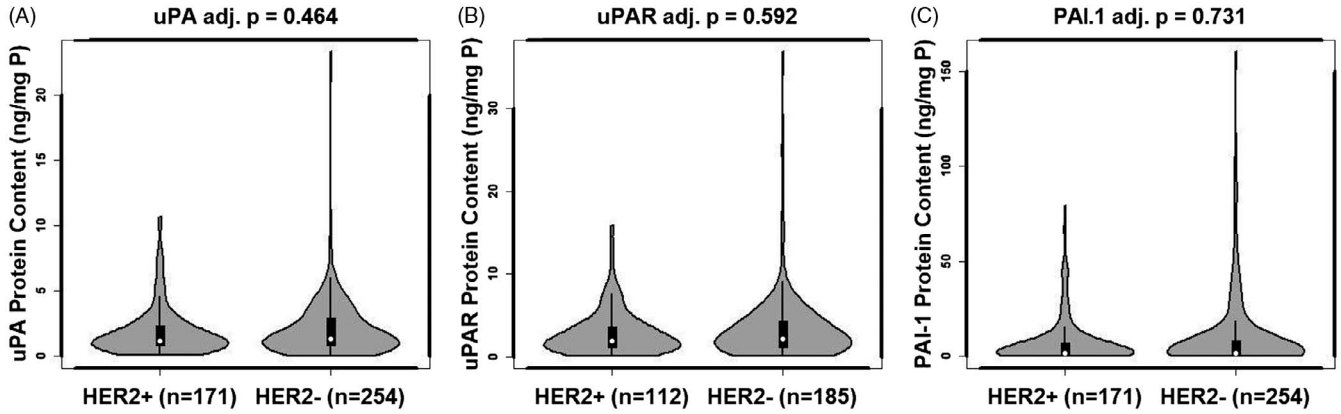


FIGURE 3 Violin plots of protein biomarker content of urokinase plasminogen activator system members comparing distributions by HER2 status. Breast carcinomas were classified as either HER2+ or HER2− according to cutoff values for measurements obtained from either NEN/DuPont or TRITON assays as described in Section 2.1

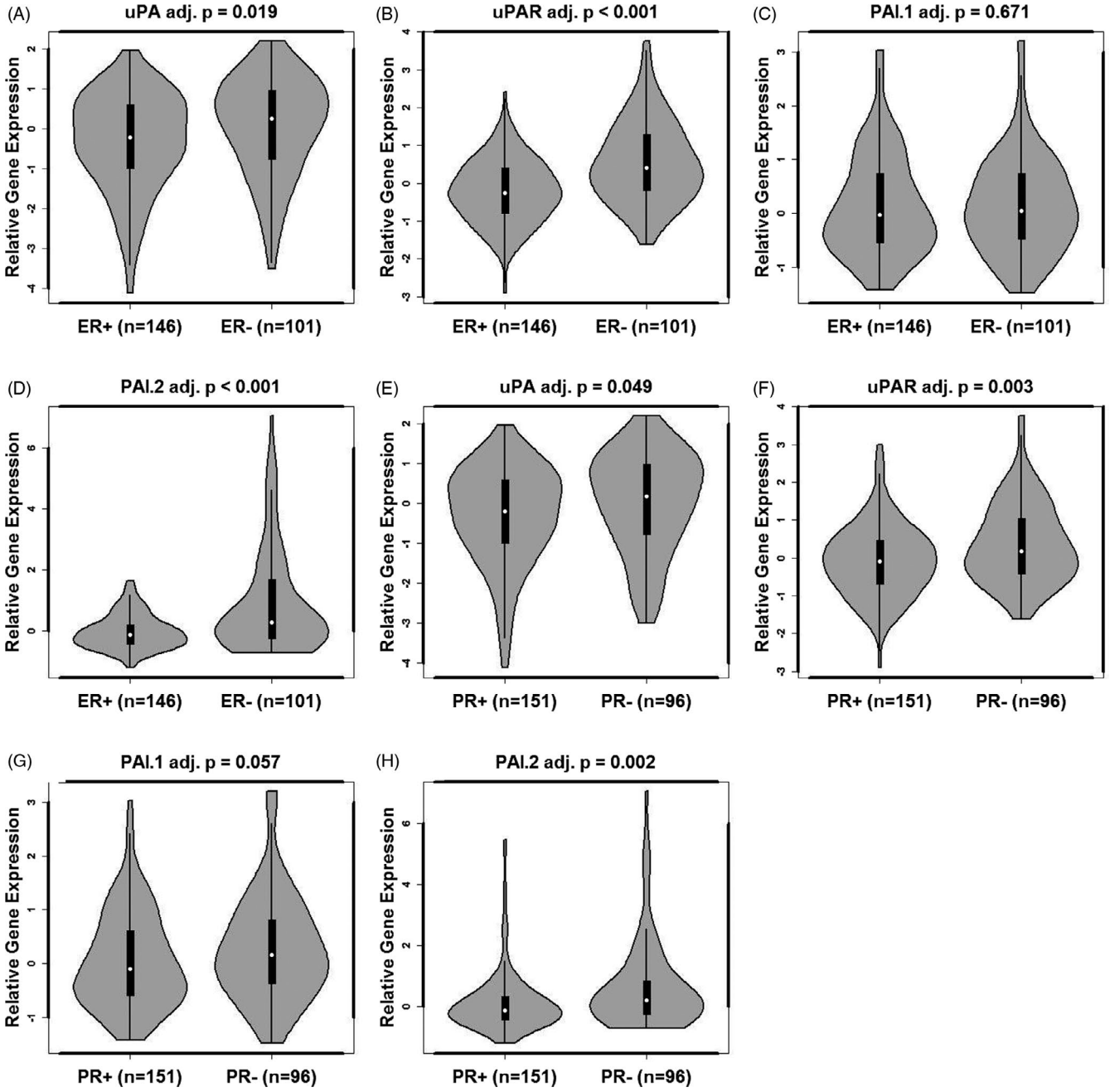


FIGURE 4 Violin plots utilizing relative expression of genes of the uPA system estimated by microarray compared to either ER or PR status of the breast primary lesion for 247 patients. Breast carcinomas were classified as either ER+ or ER– and either PR+ or PR– utilizing measurements obtained by either EIA or radio-ligand binding

indicated that HER2 status of the primary breast cancer was not significantly related to expression of any of the four candidate genes analyzed.

3.6 | Biomarker gene expression of the LCM-procured carcinoma cells as a function of menopausal status of the patient

Since the menopausal status of breast cancer patients is a clinically useful management parameter, violin plots were created to examine the relative expression of each candidate gene in LCM-procured carcinoma cells and menopausal status of patients (Figure 7). Violin plots were constructed for each gene of interest according to premenopausal or postmenopausal status, and an adjusted *P*-value of <0.30 was employed as the discovery cutoff. Patients who were 54 years of age or younger at the time of biopsy were classified as premenopausal. Notably, expression of *uPAR* gene was significantly elevated in premenopausal patients at an adjusted *P*-value of <0.30 (Figure 7B).

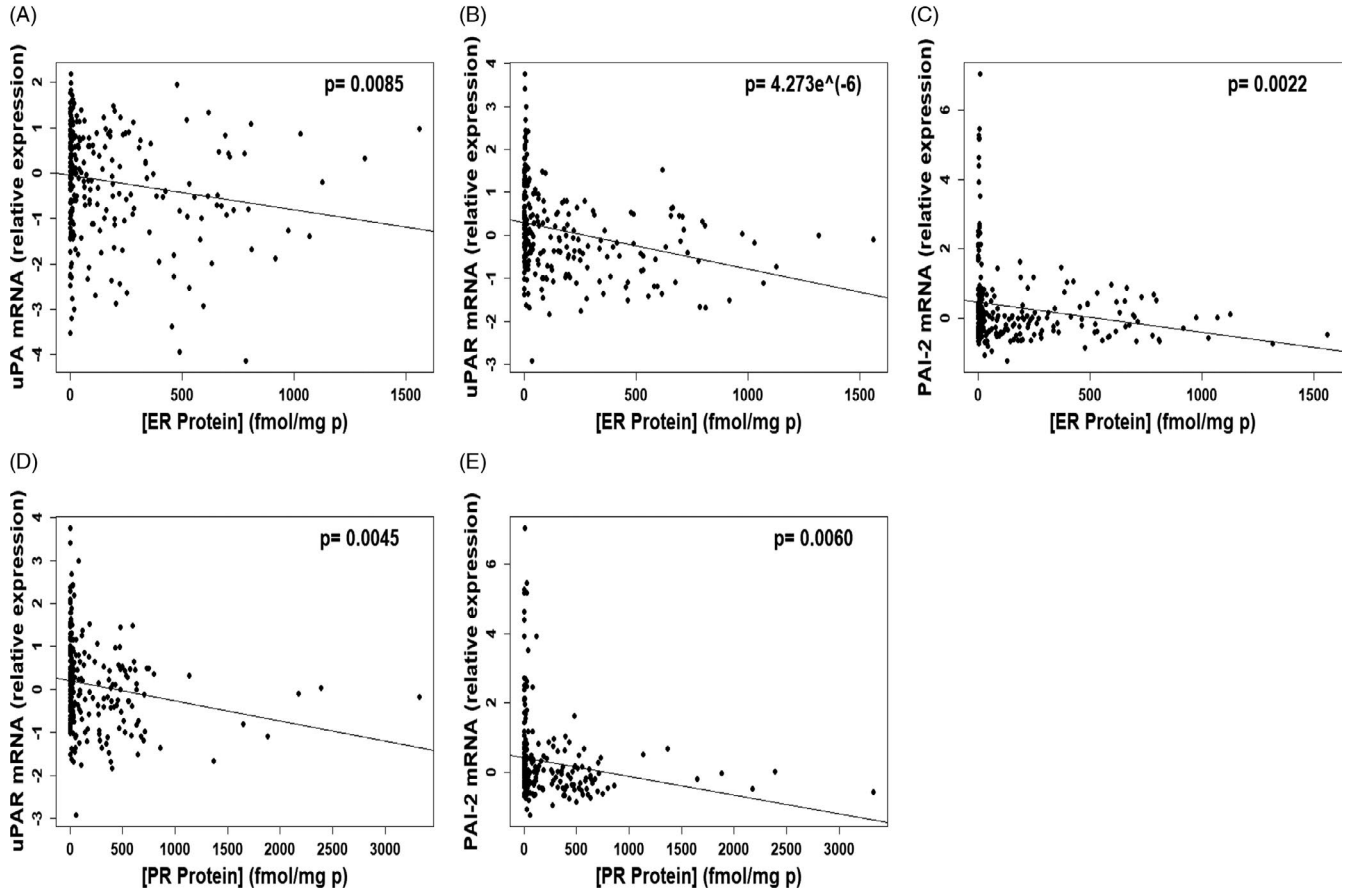


FIGURE 5 A-C, Relationship of ER protein versus candidate gene expression and D,E, PR protein content in relation to relative expression of candidate genes. Of the 8 possible relationships of gene expression of steroid receptor status, only 5 exhibited statistical significance. Representative relationships of ER protein versus candidate gene expression (A-C) and PR protein content in relation to relative expression of candidate genes (D,E) are shown

3.7 | Relative expression of each biomarker gene in the uPA pathway as a function of nodal status of the patient

Nodal status of a patient presenting with breast carcinoma is of critical importance to clinical management[2,23] although not all node-positive breast cancers exhibit early recurrence. Relative expression of each gene of the uPA system was estimated in LCM-procured carcinoma cells by microarray and plotted in relationship to either positive (n = 122) or negative (n = 125) nodal status for 247 patients. For this initial set of violin plot analyses, patients with 1 or more nodes exhibiting breast carcinoma were considered node-positive (Figure 8).

Since nodal status of patients is also considered in different categories based upon different patterns of breast carcinoma progression, recurrence, and other clinical outcomes,[1,2,23] analyses were performed. Violin plots utilized relative expression of either *uPA*, *uPAR*, *PAI-1*, or *PAI-2* genes estimated by microarray of RNA extracted from LCM-procured carcinoma cells plotted in relationship to nodal status, either negative (n = 125), 1-3 positive nodes (n = 69), or >3 positive nodes (n = 53) as shown in Figure 9.

Analyses indicated that the nodal status of a patient with breast carcinoma was not related to relative expression of either *uPA*, *uPAR*, *PAI-1*, or *PAI-2* genes estimated by microarray of RNA extracted from LCM-procured carcinoma cells (Figure 9).

3.8 | Interrelationships of biomarker status of the primary breast carcinoma with expression of candidate genes known to be associated with cancer

From our previous studies of gene expression in primary breast carcinomas,[10,11,34,39] almost 100 genes of interest have been identified whose expression we have been identified using expression levels validated by qPCR using intact tissue sections from fresh frozen biopsies. Relationships between expression of candidate cancer-related genes and protein biomarker content were analyzed through construction of violin plots (Figure 10). Relative gene expression estimated by qPCR, an adjusted *P*-value of <0.30 employed for significance, and biomarker cutoff values described previously were employed in these analyses. It was observed that *MSX1* expression was significantly elevated in *uPA*+ cells (Figure 9A), whereas expression of *RERG* was elevated in uPA− carcinomas (Figure 9B).

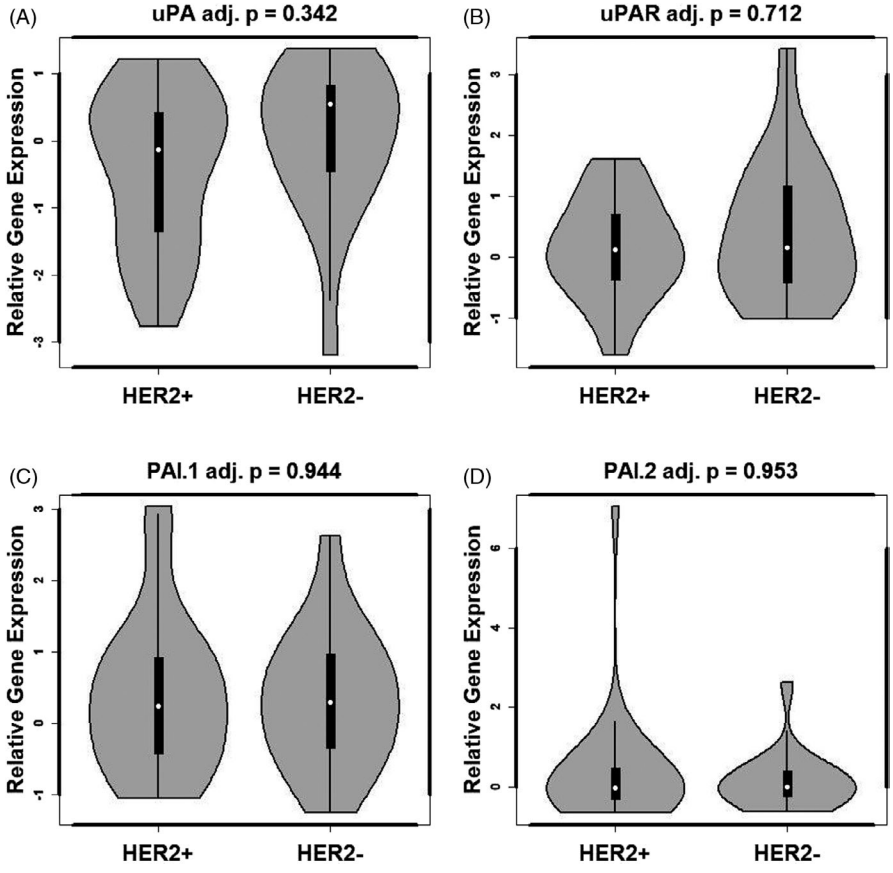


FIGURE 6 Representative violin plots utilizing microarray expression of genes of the uPA system comparing HER2 protein status for 45 patients. Breast carcinomas were classified as either HER2+ (n = 28) or HER2− (n = 17) with measurements obtained from either NEN/DuPont or TRITON assays as described in Section 21

In contrast, results indicated that *LRBA* expression was significantly elevated in *uPAR*− carcinomas (Figure 9C). Subsequent analyses revealed that *SIAT8A* expression was significantly elevated in *uPAR*+ breast cancer (Figure 9D). When qPCR gene expression was examined in relation to PAI-1 status, it was observed that elevated expression of *COMT* and *TGFB1* was observed in *PAI-1*+ cells (Figure 9E,F).

Although the sample size was small (n = 29), the expression of a number of genes previously reported to be associated with breast carcinomas was examined as a function of the relative expression of *uPA*, *uPAR*, *PAI-1*, and *PAI-2* genes. Scatter plots of the genes identified from analyses shown in Figure 10 were constructed (Figure 11). None of the genes examined indicated their relative expression was associated with that of either *uPA*, *uPAR*, or *PAI-1* genes when a *P*-value of <0.05 was employed for significance.

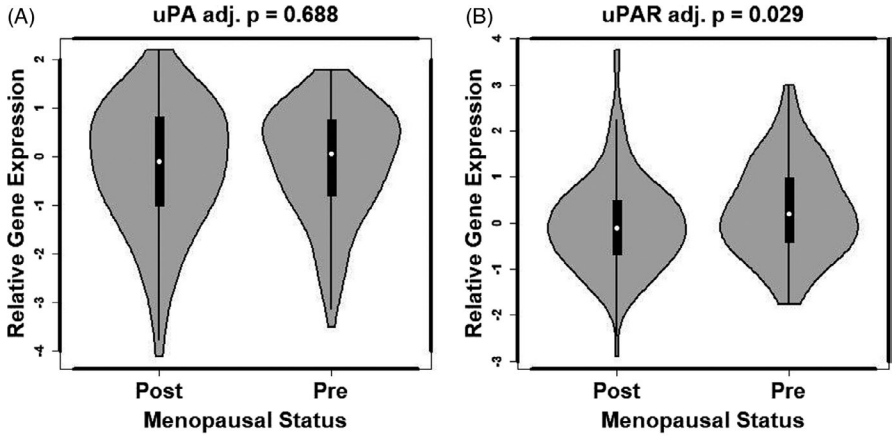
3.9 | Univariable Cox regression analyses of relative gene expression according to ER and PR status of LCM-procured breast carcinoma cells

To determine the relationship between expression of each gene from microarray of the uPA system and steroid receptor status, Cox regression analyses of genes were performed with regard to ER or PR status of the primary breast cancer. An adjusted *P*-value of <0.30 was selected for discovery (Tables 3 and 4). Cox regressions revealed that expression of *PAI-2* in carcinoma cells was significantly related to OS in ER+ breast cancers. In ER− breast cancers, *uPAR* expression was related to prediction of PFS, whereas *PAI-1* expression predicted OS. Furthermore, when carcinomas were classified according to PR status, *uPA* expression was significantly related to PFS and OS of patients with PR+ primary carcinomas. In PR− cancers, expression of either *uPAR* or *PAI-2* was related to prediction of PFS. Similarly, expression of either *PAI-1* or *PAI-2* was related to prediction of OS in PR− breast cancer patients from LCM-procured cells of breast carcinomas according to ER protein status.

4 | DISCUSSION

Investigations reported focused on relationships of the biomarkers, uPA, uPAR, and PAI-1, in primary breast cancer biopsies with other clinical features of the carcinomas (eg, nodal status) and of the patients (eg, menopausal status) to determine alternative protein biomarker combinations (eg, with either ER or PR status) that predict clinical outcomes (PFS and/or OS) of breast carcinoma patients in an improved manner. In addition, gene expression results obtained by microarray of RNA extracted from LCM-procured breast carcinoma cells[34,37,39-41,43,44,53] were compared with clinical outcomes and carcinoma-based parameters for the study populations of breast cancer patients.

Results revealed that an increase of uPA, uPAR, or PAI-1 protein content was not correlated with patient age. Relationships between other biomarkers for breast cancer (eg, ER or PR protein)



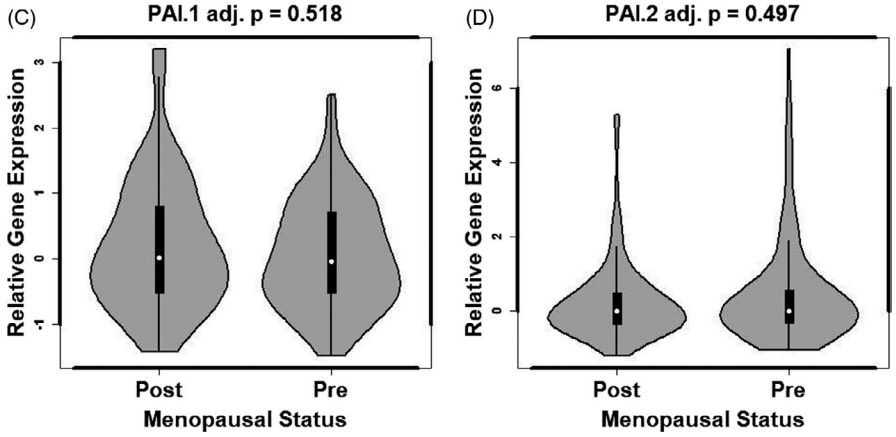
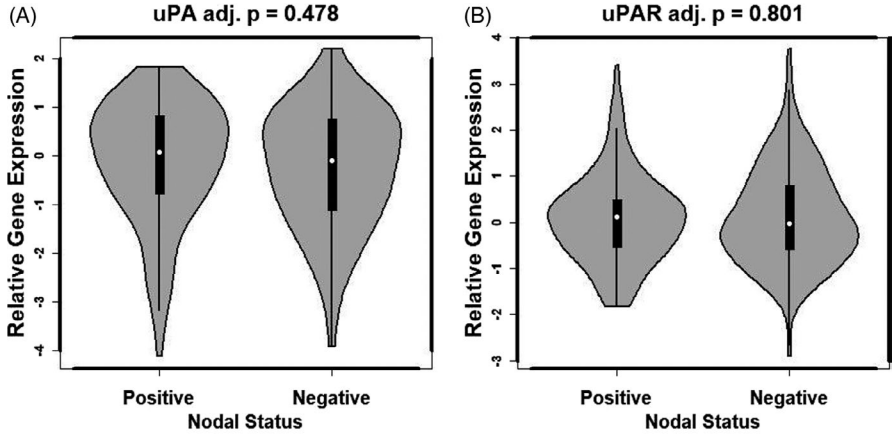


FIGURE 7 Violin plots comparing microarray expression levels of each gene of interest in LCM-procured breast carcinomas of either premenopausal (n = 102) or postmenopausal (n = 145) breast cancer patients



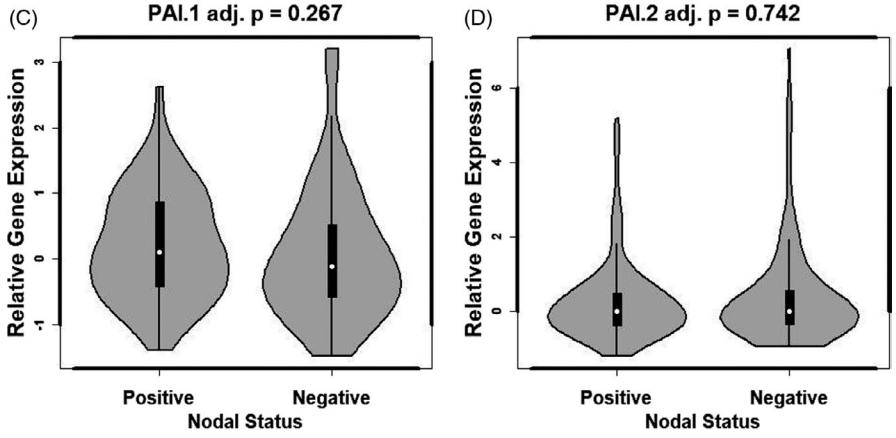


FIGURE 8 Violin plots utilizing relative expression of genes of the uPA system estimated by microarray in relationship to either positive or negative nodal status for 247 patients. Patients with 1 or more nodes exhibiting breast carcinoma were considered node-positive

in intact tissue biopsies and patient age have exhibited associations for breast cancer patients, though the literature is conflicting.[54,55] Similarly, our findings established relationships between biomarker protein content of the uPA system and either ER or PR status of the primary carcinoma. Violin plots indicated either uPA or uPAR content was elevated in carcinomas when they also exhibited elevated levels of either ER or PR protein content as measured by radio-ligand binding. However, when ER or PR content was determined by EIA, receptor-negative carcinomas exhibited elevated levels of uPA content.

Differences in relationships of protein content and status may be due to the type of assay employed despite utilization of standard cutoff values for both the radio-ligand binding and the EIA.[32] Literature reports of evidence of a weak association of negative ER status and elevated uPA content in carcinomas were similar to our findings when ER was assessed by EIA.[56,57] To extend our investigation of interrelationships between gene expression and proteins status, HER2 status was examined in relation to biomarker protein content. No relationships were detected between HER2 status and protein content of either uPA, uPAR, or PAI-1 for our patient population.

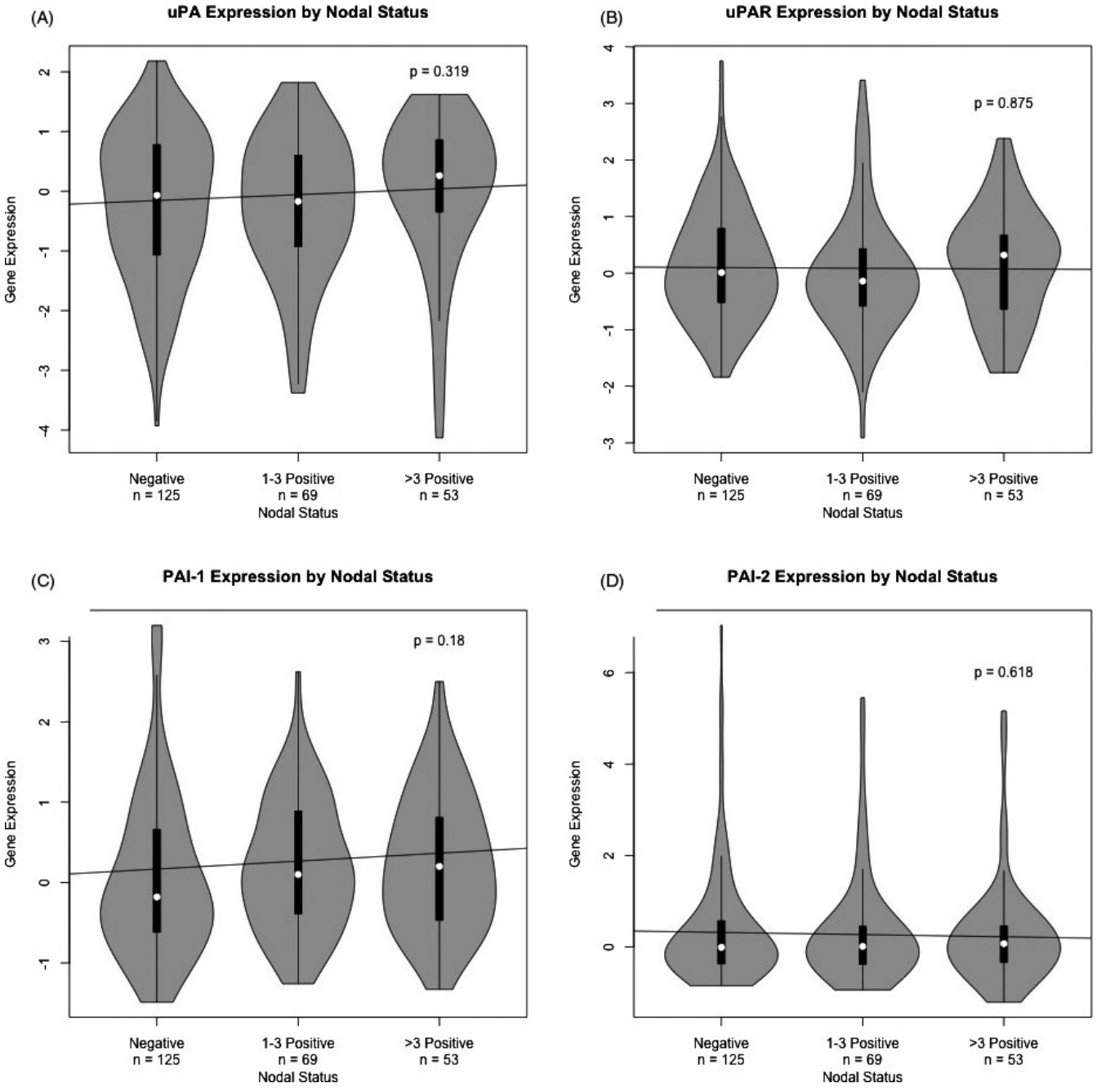


FIGURE 9 Violin plots utilizing relative expression of genes of the uPA system estimated by microarray in relation to either negative (n = 125), 1-3 positive (n = 69), or >3 positive (n = 53) nodal status for 247 primary breast carcinoma patients

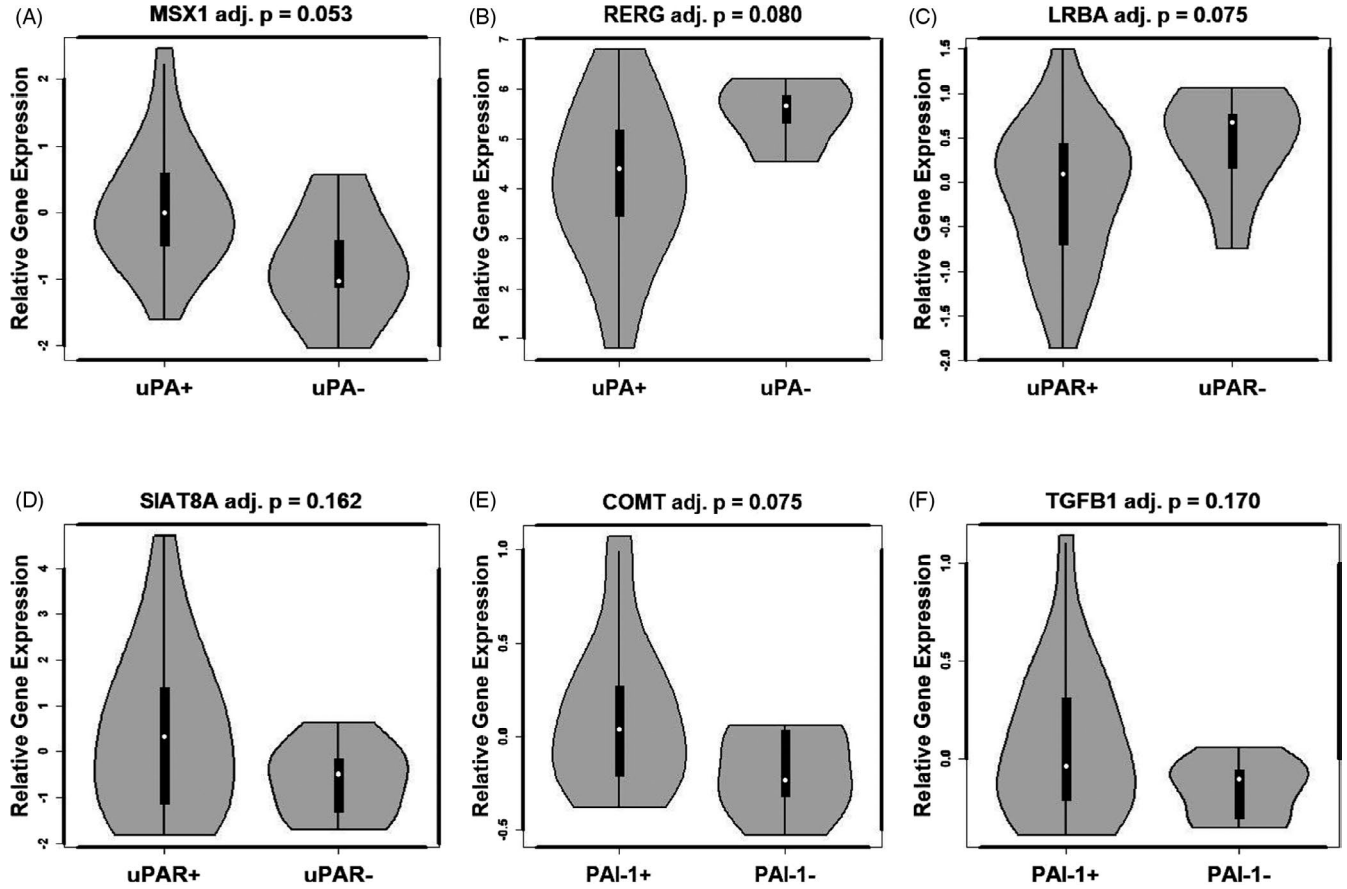


FIGURE 10 Violin plots of the qPCR expression levels of representative genes known to be associated with breast cancer as a function of plasminogen activator system protein status (n = 29 patients)

Interrelationship analyses utilized expression from microarray of LCM-procured breast carcinoma cells collected under stringent conditions. In addition, IMUBIND™ kits were employed to quantify uPA, uPAR, and PAI-1 content using cellular extracts from heterogeneous tissue samples of breast cancer.[18,19,31,33] Thus, results presented may reflect a heterogeneous tumor microenvironment, which is composed of stromal elements.[40,56] Largely, results indicated that biomarker protein content may be related to patient age and ER/PR status, but no relationship with HER2 status was observed among our patient population.

Established relationships have been reported between uPA, uPAR, and PAI-1 content of a primary carcinoma and clinical features such as ER/PR status and outcomes.[18,19,33,58] However, few studies examined relationships for both protein content and gene expression of uPA system members within a unique patient population, such as that used in these investigations. Our exploratory and complementary analyses investigated gene expression results, collected from LCM-procured breast carcinoma cells, according to either ER, PR, or HER2 status of the primary. Additionally, expression of plasminogen activator inhibitor 2 (*PAI-2*) gene was incorporated due to limited research regarding its expression in breast cancer.

Expression of either *uPA*, *uPAR*, *PAI-1*, or *PAI-2* genes was significantly elevated in either ER− or PR− carcinomas similar to the observed elevation of protein content in receptor-negative carcinomas measured by EIA. Scatter plots revealed that expression of either *uPA*, *uPAR*, or *PAI-2* appears to be negatively correlated with both ER and PR protein of the primary. Collectively, results largely indicate that increased protein content or expression of uPA system members is significantly associated with receptor-negative carcinomas (ie, ER− or PR−). In contrast, either uPA or uPAR protein content was elevated in ER+ cells determined by a radio-ligand binding assay.

Nodal status of patients with breast cancer was examined according to gene expression of each uPA system member and each patient was classified in two different categories. Differential outcomes according to the manner in which nodal status is assigned have been documented.[1,28] Therefore, nodal status was designated as positive if one or more nodes exhibited breast cancer, in the manner of previous studies. In contrast, nodal status was assigned according to the number of nodes, which exhibited breast cancer according to certain studies. Collectively, *PAI-1* was elevated in node-positive breast carcinomas, whereas the latter classification did not provide statistically significant differences. In the context of a clinical setting, it may be useful to assign positive nodal status as one or more nodes exhibiting breast cancer for assessment of *PAI-1*. However, classification of status according to the number of nodes that are positive holds strong clinical utility for the prediction of early recurrence and clinical outcome.[23,59]

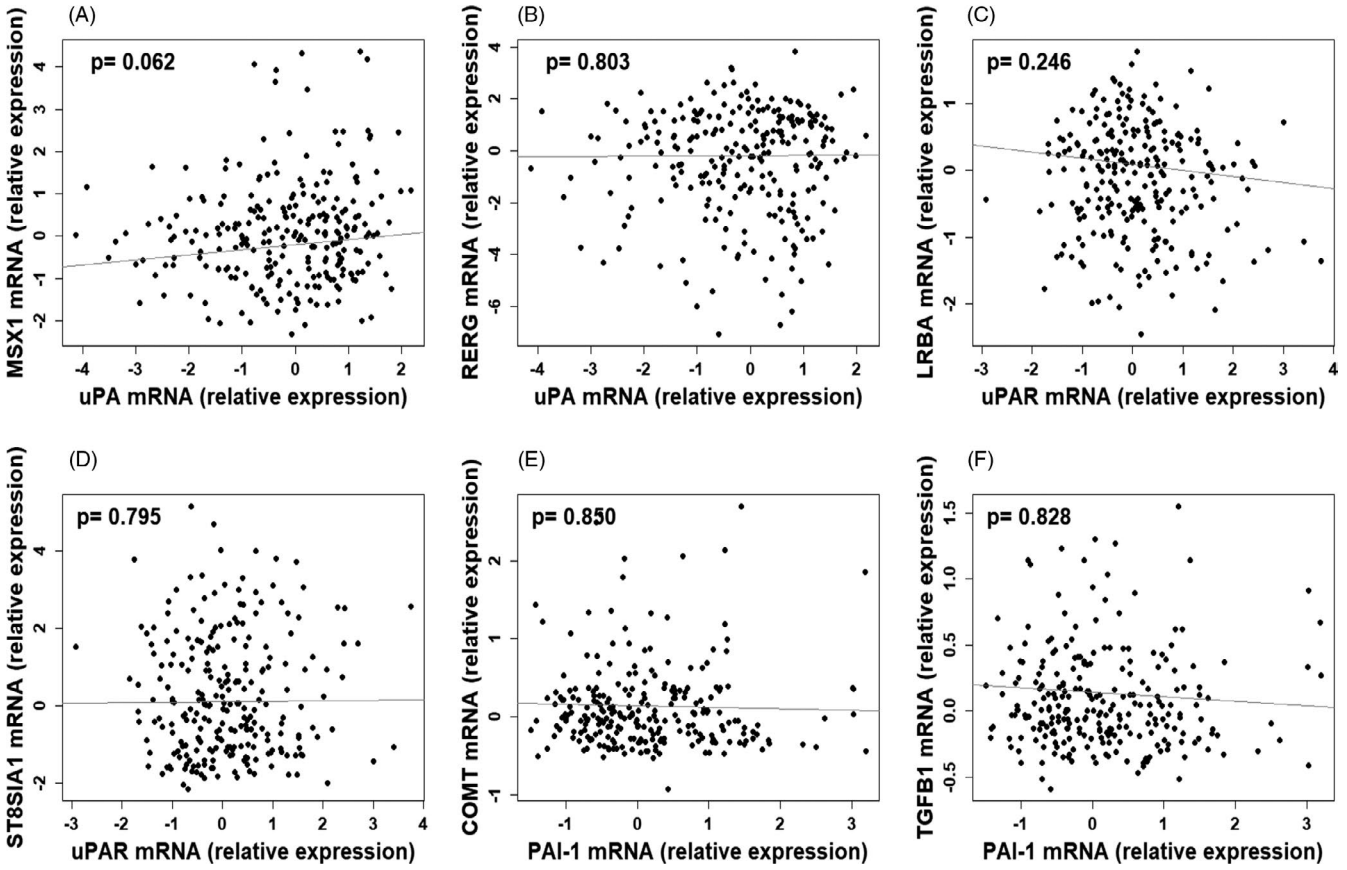


FIGURE 11 Scatter plots of relative expression of candidate cancer-associated genes identified in Figure 9 as a function of relative gene expression of members of the urokinase plasminogen activator system. Results shown represent microarray expression levels for each of the genes shown

Interrelationships of qPCR expression of a number of genes reported to be associated with breast cancer outcomes were studied as a function of biomarker status. In addition, the uPA system has reported roles in processes of inflammation, angiogenesis, embryogenesis, wound healing, cellular apoptosis tumor migration, and angiogenesis.[14-16] Expression of genes such as *TGFB1* or *LRBA* was significantly associated with either PAI-1 or uPAR status. Similarly, significant differences in expression of genes *MSX1*, *RERG*, *SIAT8A*, and *COMT* according to biomarker status were observed.

The status of either uPA, uPAR, or PAI-1 protein content was assigned according to standard cutoff values provided by IMUBIND™ kits, whereas expression of each gene was estimated via qPCR on intact carcinoma tissue sections.[37]However, analyses employing expression levels obtained from microarray analysis of LCM-procured breast carcinoma cells showed no significant relationships. Thus, the clinical utility of our preliminary findings warrants additional investigation in the context of the microenvironment of breast carcinomas. Differences in expression patterns for the genes of interest could be a reflection of the genomic profile of an intact breast cancer specimen containing many cell types (e.g., stromal cells, invading lymphocytes, inflammatory cells) (ie, collection of qPCR expression) compared to that of isolated carcinoma cells collected non-distructively by LCM.[40]

Expression of *PAI-2* gene was significantly related to OS of patients with ER+ breast cancers. In ER− carcinomas, expression of either *uPAR* or *PAI-1* was related to PFS or OS, respectively. In contrast, literature reports focused on protein content and indicate PAI-2 level is related to increased prognosis for breast cancer patients, whereas other studies have reported no significance for the use of PAI-2 level as a determinant of clinical outcome.[60,61] Additionally, Gelder et al[62] analyzed PAI-2 level of cytosols prepared from ER+ primary breast cancers and discovered a significant relationship between high PAI-2 level and length of response to tamoxifen (ie, first-line therapy for recurrent breast cancer). Thus, our findings establishing the relationship between *uPAR*, *PAI-1*, and *PAI-2* genes with clinical outcomes of patients with breast cancers exhibiting various ER status support the concept that estrogen levels are involved in regulation of these biomarkers.

Kotzsch et al[63] examined *uPAR* gene expression obtained from microarray in breast tumor specimens for invasive ductal carcinomas. It was reported that elevated *uPAR* gene expression levels in tumor cells, and not from stromal cells, were predictive of PFS. Our findings support the work of Kotzsch et al[63] and identify the prognostic value of *uPAR* gene expression in ER− carcinomas. In breast cancer patients, it is widely known that elevated PAI-1 level is associated with poor OS, whereas few studies have evaluated *PAI-1* gene expression. Our analyses identified a relationship between *PAI-1* gene expression and OS for ER− patients. Since ER− patients are not

candidates for traditional treatments (ie, SERM, raloxifene, tamoxifen), further examination of *PAI-1* gene expression may serve as a strategy for breast cancer management as evidenced by our findings. In PR+ patients, *uPA* gene expression obtained from microarray of LCM-procured breast carcinoma cells was significantly related to prediction of either PFS or OS. In PR− patients, *PAI-2* expression collected from LCM-procured breast carcinoma cells was predictive of PFS and OS.

Current guidelines for management of primary breast carcinomas instruct clinicians to utilize analyses of ER, PR, and HER2 protein levels collectively to assess risk of recurrence and therapy selection.[7,52] The results of these investigations strongly suggest that combinations of results from ER or PR protein content with measurements of either uPA, uPAR, or PAI-1 protein levels of a primary breast cancer biopsy provide improved assessment of a patient's clinical outcome. Furthermore, analyses of expression of genes for the *uPA*, *uPAR*, *PAI-1*, and *PAI-2* as well as those for *ESR1* (gene expressing ER mRNA and protein) and *PGR* (gene for expressing PR mRNA and protein) using LCM-procured breast carcinoma cells indicated relationships between these biomarkers and disease-free progression and OS of breast cancer patients.

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