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Prostate-Derived Ets Transcription Factor Overexpression is Associated with Nodal Metastasis and Hormone Receptor Positivity in Invasive Breast Cancer[1]

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Abstract

Prostate-derived Ets transcription factor (PDEF) has recently been associated with invasive breast cancer, but no expression profile has been defined in clinical specimens. We undertook a comprehensive PDEF transcriptional expression study of 86 breast cancer clinical specimens, several cell lines, and normal tissues. PDEF expression profile was analyzed according to standard clinicopathologic parameters and compared with hormonal receptor and HER-2/*neu* status and to the expression of the new tumor biomarker Dikkopf-1 (DKK1). Wide ranging PDEF overexpression was observed in 74% of tested tumors, at higher levels than the average expression found in normal breasts. High PDEF expression was associated with hormone receptor positivity (*P* < .001), moderate to good differentiation (less than grade III, *P* = .01), and dissemination to axillary lymph nodes (*P* = .002). PDEF was an independent risk factor for nodal involvement (multivariate analysis, odds ratio 1.250, *P* = .002). It was expressed in a different subgroup compared to DKK1-expressing tumors (*P* < .001). Our data imply that PDEF mRNA expression could be useful in breast cancer molecular staging. Further insights into PDEF functions at the protein level, and possible links with hormone receptors biology, bear great potential for new therapeutic avenues.

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Keywords: Prostate-derived Ets transcription factor (PDEF), Dikkopf-1 (DKK1), breast cancer, expression profile, tumor biomarkers.

Introduction

Genome-wide expression profiles have provided a genotypic ground that supports the main invasive breast cancer phenotypes, namely, the estrogen receptor (ER)-positive luminal epithelial type, the ER-negative basal epithelial type, and breast carcinoma overexpressing the HER-2/*neu* receptor [1]. ER-positive tumors form the largest group of breast cancers, but encompass heterogeneous tumors of variable aggressiveness [2–4]. ER-positive tumor-specific genes, which promote or prevent early dissemination or resistance to antiestrogenic therapies, remain to be identified, included as molecular staging tools, and used as new therapeutic targets to personalize breast cancer treatment and improve outcomes.

Epithelial-specific Ets transcription factors could potentially be exploited in this regard [5,6]. Several Ets have been linked mainly to ER-negativity and HER-2/*neu* breast cancer [7–10]. Prostate-derived Ets transcription factor (PDEF), one of the last Ets identified, was the first to be characterized in hormone-sensitive prostate cancer as a promoter of the protease prostate-specific antigen, in cooperation with androgen receptor and other transcription factors [11,12]. Bioinformatic tools and various gene expression quantification methods subsequently documented PDEF mRNA overexpression in invasive breast cancer [13], in atypical ductal hyperplasias, and in carcinomas *in situ* [14], when compared to normal breasts. PDEF mRNA has also been detected in micrometastatic axillary lymph nodes [15]. Despite high mRNA expression, immunohistochemical data suggest that PDEF protein expression could be lost in prostate and breast carcinomas [16,17]. PDEF silencing and overexpression assays in breast and prostate metastatic cancer cell lines resulted in antimetastatic effects [18–20], but prometastatic effects has also been documented in other metastatic- and benign disease–derived breast cell lines [14].

Up to now, however, the PDEF expression profile has not been described, at the mRNA level, in clinical breast cancer specimens. In order to orient future work at the protein level,

Abbreviations: DKK1, Dikkopf-1; ER, estrogen receptor; PDEF, prostate-derived Ets transcription factor; ROC, receiver–operator curve; RT-PCR, reverse transcription–polymerase chain reaction

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we report a comprehensive PDEF transcriptional expression study of 86 breast cancer clinical specimens, several cell lines, and normal tissues. PDEF expression profile was analyzed according to standard clinicopathologic parameters, compared with hormonal receptor and HER-2/*neu* status, and to the expression of the new tumor biomarker Dikkopf-1 (DKK1). We observed that PDEF expression is strongly associated with the ER-positive breast cancer phenotype and that PDEF mRNA overexpression in primary tumors could also be an independent risk factor for cancer dissemination to lymph nodes.

Materials and Methods

Patient Specimens and Cell Lines

Breast cancer tissues of consecutive patients who had provided written consent to contribute to the CHUM-FRSQ Tumor Bank (Montreal, QC, Canada) between September 2003 and February 2006 were selected by the pathologist after surgical resection of tumors 1.5 cm or greater in diameter. The recruitment protocol and management of clinical specimens and information were previously approved by institutional authorities. Fresh tissues were stored at 4°C in RNAlater (Sigma, St. Louis, MO) for RNA stabilization. All patients underwent sentinel node dissection and, when positive for nodal metastasis, complete axillary lymph node dissection. Cell lines used for the first detection of PDEF at the mRNA and protein levels (breast cancer MCF7, MDA-MB-231, BT-20, HCC-1428, HCC-2218, renal embryonic 293T, and melanoma SK23) were obtained from the American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 (Wisent, St.-Bruno, QC, Canada) supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin/streptomycin (both from Wisent), 2 mM L-glutamine, and 10 μg/ml gentamicin (both from Invitrogen, Grand Island, NY). HCC breast cancer lines also required 10 mM Hepes solution plus 1 mM sodium pyruvate (both from Invitrogen). Mononuclear cells were obtained by patient blood centrifugation on a lymphocyte separation medium (Cellgro, Herndon, VA) and culture in complete AIM-V medium (Invitrogen), as described previously [21].

RNA Extraction and Reverse Transcription–Polymerase Chain Reaction (RT-PCR)

Cancer specimens were homogenized with Medimachine (Dako Cytomation, Glostrup, Denmark) according to the manufacturer's instructions. Total RNA was extracted with a reagent (Qiazol; QIAGEN GmbH, Hilden, Germany), followed by a cleanup and concentration procedure, using the RNeasy Mini or Micro Kit (QIAGEN) and stored at −80°C. Five of 91 clinical specimens were rejected because of poor quality of the extracted mRNA (OD_{260}/OD_{280} absorption ratio below 1.6) or a β-actin expression level detected beyond the last dilution of the standard curve when tested in real-time PCR (see below). A panel of pooled mRNA from 19 normal tissues and 6 peritumoral normal breast mRNA were also analyzed (both from Clontech, Mountain View, CA).

We synthesized cDNA from 1 μg of mRNA with the Omniscript Reverse Transcriptase (RT) Kit (QIAGEN), using oligo-dT (Invitrogen) at 42°C for 1 hour. Intron-spanning PCR primer pairs were designed for PDEF (5′ primer GACATCGAGACGGCCTGCAAGCTG; 3′ primer ACATGGCGCACAGCTCCTTG; amplicon 150 bp) and for β-actin, exploited as a housekeeping gene (5′ primer GGAAGGCTGGAAGAGTGCC; 3′ primer GTGATGGTGGGC ATGGGT C; amplicon 300 bp) (Invitrogen). PCR was performed with the Quantitect SYBR Green PCR kit (QIAGEN). Optimal annealing temperatures for both PDEF and β-actin were determined by a gradient (51–65°C). Real-time quantitative RT-PCR was performed with 0.4 μM of each PDEF primer or 0.8 μM of each β-actin primer, 6.25 μl of 2× SYBR Green mix (providing 2.5 mM $MgCl_2$), 2.5 μl of cDNA (1:25 dilution), and water in a thermal cycler (Rotorgene 3000; Corbett Life Science, Sydney, Australia). The optimized cycling conditions were 10 minutes at 95°C for the initial polymerase activation, then 32 cycles for 40 seconds at 94°C, 40 seconds at 56°C, 50 seconds at 72°C, and a final melting curve from 72 to 95°C. Fluorescence was measured at the end of each extension step. The gain was adjusted automatically on the first tube at the end of the first cycle (channel FAM/SYBR, source 470 nm, detector 510 nm, gain adjusted between 2 and 5 fluorescence). The absence of primer dimers and the specificity of the PCR products were documented by melting curve analysis and electrophoresis migration in 2% agarose gel stained with ethidium bromide.

PDEF Quantification

The relative PDEF expression ratio over β-actin was reported in relation to MCF7 cell line expression established at a value of 1 [22]. The equation takes into account the PCR efficiencies (E) of both genes and the difference (Δ) between the moment at which the fluorescence of a given sample *versus* MCF7 crosses the threshold (C_t). The equation is as follows:

$$\text{Ratio PDEF}/\beta-\text{actin} = (E_{\text{PDEF}})^{\Delta C_t(\text{MCF7}-\text{sample}) \text{ for PDEF}} / (E_{\beta-\text{actin}})^{\Delta C_t(\text{MCF7}-\text{sample}) \text{ for } \beta-\text{actin}}$$

Standard curves were generated every two runs with serial dilutions of a pool of cDNA taken from the above-mentioned breast cancer cell lines. The curves allowed the software from Corbett to calculate the lowest thresholds of the log-linear amplification phase above the fluorescence background and the efficiencies of PCRs derived from the high linearity slopes (Pearson correlation coefficient $r > 0.99$). Mean thresholds and efficiencies were used to compare the expression of all samples. C_t obtained at or after the last detectable point of the dilution curves (1:3125 for PDEF and 1:15,625 for β-actin) was considered negative. All samples were tested in duplicate in at least two independent runs, whereas MCF7 and the controls (MCF7 without RT, water, or lymphocytes) were systematically included in every run. Intra- and interassay C_t variations, calculated with the Relative Expression Software Tool, are represented by error bars [23].

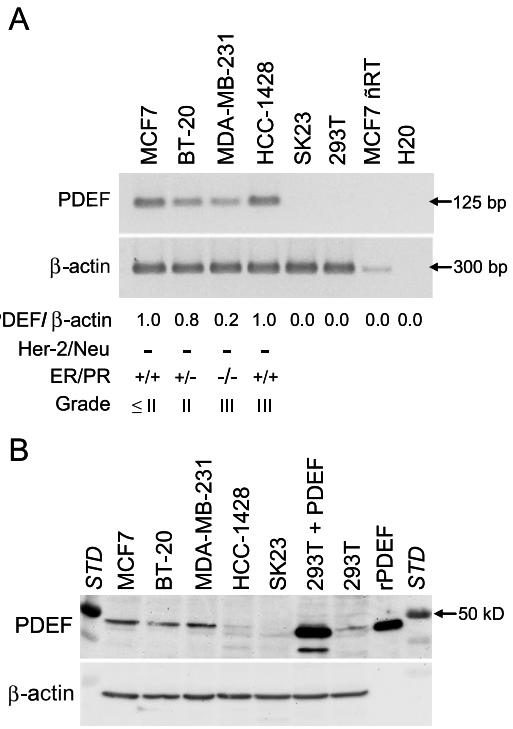

Figure 1. *PDEF mRNA and protein expression in cancer cell lines. (A) Migration of PDEF amplicon obtained in cancer cell lines by real-time RT-PCR, with relative quantification (PDEF/β-actin, if MCF7 = 1.0). Four cell lines derived from metastatic breast cancer (MCF7, BT-20, MDA-MB-231, and HCC-1428) figure with SK23 melanoma, and 293T renal embryonic cells used as negative controls. (B) PDEF expression at the protein level by Western blot analysis. All results are representative of at least three independent experiments. ER, estrogen receptor; PR, progesterone receptor; −RT, without reverse transcription; rPDEF, recombinant PDEF; 293T + PDEF, 293T cells transfected with PDEF.*

DKK1 Expression Measurements and Quantification

Real-time RT-PCR with intron-spanning DKK1 primers was performed in a thermal cycler (LightCycler; Roche, Mannheim, Germany) and revealed with SYBR Green (QIAGEN) as described previously [24]. cDNA was synthesized as described for PDEF, from the same RNA extracts, tested at the same time period, and the amplified material was submitted to the same specificity validation as PDEF.

Measurement of PDEF Protein Expression

For Western blot analysis, protein extracts were prepared from the above-mentioned cell lines for 20 minutes in lysis buffer (20 mM Tris−HCl, pH 8, 137 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM Na_3VO_4, and 2 mM EDTA) containing protease inhibitors (1 mM PMSF, 2 μM pepstatin A, and 2 μM leupeptin, all from Sigma). Protein concentration was measured by Lowry's assay with a DC Protein Assay kit (Bio-Rad, Hercules, CA). For recombinant PDEF, the coding sequence was cloned in pQE-30 (QIAGEN) and the recombinant protein was produced in *Escherichia coli* DH5-α. Cell extracts (10 μg/well), resolved by 12% sodium dodecyl sulfate−polyacrylamide gel electrophoresis, were transferred to polyvinylidene fluoride membranes (Immun-Blot, Bio-Rad). The membranes were subjected to 1-hour incubation with rabbit affinity-purified polyclonal anti-PDEF antibody (1:400), kindly provided by Dr. Dennis K. Watson (Hollings Cancer Center, Charleston, SC) [16], or with mouse actin-specific antibody (1:4000; Chemicon, Temecula, CA). The membranes were then washed and proteins revealed after a 1-hour incubation with secondary peroxydase−conjugated antibodies (1:5000 goat anti-rabbit from Santa Cruz Biotechnology, Inc., Santa Cruz, CA; 1:40,000 goat anti-mouse antibody from Chemicon) were detected with a reagent (ECL Plus; Amersham Biosciences, Picastaway, NJ). Chemifluorescence was quantified with an imaging system (Omega 12ic; Ultralum, Clarement, CA).

Statistical Analysis

Associations between PDEF continuous expression and categorical clinicopathologic parameters were evaluated by the independent-sample *t* test or by the one-way analysis of variance (if more than two categories, with previous Levine test to ensure the homogeneity of variance). PDEF *versus* DKK1 proportions clustered by clinicopathologic factors were compared by the Pearson chi-square test or Fisher's exact test for small samples. These tests were done using a software for Windows (SPSS 13.0; LEAD Technologies, Chicago, IL), with the generation of receiver−operator curves (ROCs) to evaluate the sensitivity of PDEF expression for the prediction of clinicopathologic factors. Univariate and multivariate logistic regressions were used to compare the strength of associations between PDEF continuous expression and metastatic lymph node involvement. Logistic regression analysis was performed with software R, version 2.3.1, with the deviance test for multivariate analysis (The R project for statistical computing [http://www.r-project.org]).

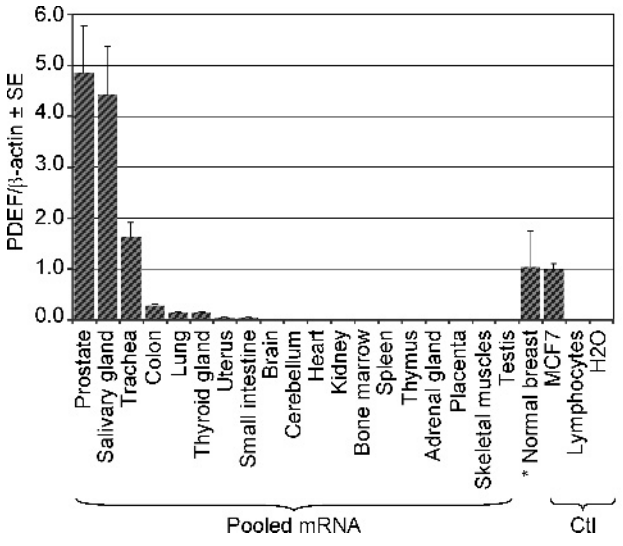

Figure 2. *PDEF expression in normal tissues. PDEF mRNA expression of a panel of 19 mRNA pools of normal tissues. *"Normal breast" corresponds to the average level of six normal peritumoral breast tissues taken from patients operated on for breast cancer. Controls (Ctl) include the MCF7 breast cancer cell line and lymphocytes taken from the blood of breast cancer patients who contributed to the CHUM-FRSQ Tumor Bank. The error bars represent the intra- and interassay Ct variations, calculated with the Relative Expression Software Tool, except for the normal breast, where it represents the standard deviation of the six samples tested.*

All tests were two-sided and *P* values < .05 were regarded as statistically significant.

Results

PDEF mRNA and Protein Expression in Breast Cancer Cell Lines

PDEF expression at the mRNA and protein levels was first evaluated in four breast cancer cell lines (Figure 1). The ER-negative MDA-MB-231 breast cancer cell line expressed the lowest PDEF level, five times lower than other ER-positive lines. As reported previously, protein detection did not always follow mRNA levels [16,17]. For further PDEF mRNA expression analysis of clinical specimens, the MCF7 expression level was chosen as a reference because it corresponded to the median PDEF/β-actin expression level in breast cancer cell lines and is widely available.

PDEF mRNA is Weakly or Not Detected in Vital Organs

PDEF expression was previously reported by different quantification methods in some high epithelial content tissues, namely, the prostate, salivary glands, colon, and normal breast [11,13,16]. We reassessed PDEF expression in normal tissues to compare its magnitude with PDEF expression in breast cancer by a reproducible method (Figure 2). Besides weak expression in the lungs and colon, PDEF was not detected in other vital organs, such as the heart, brain, and

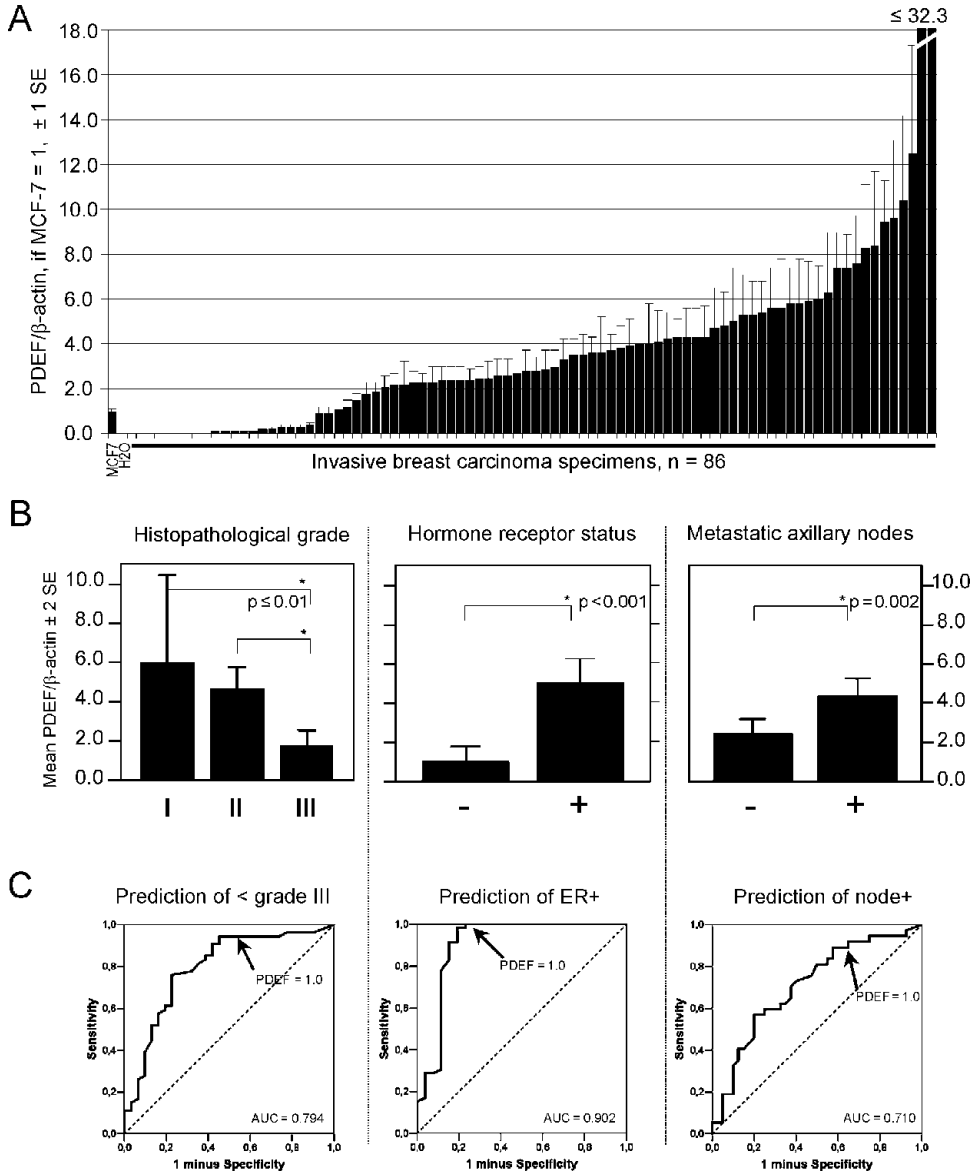

Figure 3. *PDEF expression in invasive breast carcinoma. (A) PDEF expression in 86 tested clinical samples. Sixty-four tumors were found to express PDEF at a level at least equal to the MCF7 breast cancer cell line. (B) Higher PDEF expression was associated with moderate to well-differentiated (grades I and II) tumors, with hormone receptor positivity (estrogen and/or progesterone), and with metastatic nodal involvement at the time of surgery. The same scale was used for the three graphs. (C) Receiver–operator curves (ROCs) allowed the determination of the sensitivity and specificity at which the PDEF expression level was positively associated with the three clinicopathological factors illustrated in (B). The MCF7 expression level (PDEF = 1) is indicated by arrows. AUC, area under the curve; SE, standard error.*

kidneys. Mean PDEF expression in six peritumoral breast tissues was 1.04 ± 0.68, a level similar to MCF7. PDEF was absent from lymphocytes that often infiltrate solid tumors. Altogether, the absence of PDEF expression in most normal tissues and vital organs represents an essential prerequisite for the validation of future tumor biomarker or antigen.

Measurement of PDEF in Breast Cancer Clinical Specimens

PDEF expression in breast cancer cell lines, which are free of normal epithelium, combined with a similar level of expression in peritumoral normal breasts, called for a comprehensive PDEF transcriptional analysis of breast cancer clinical specimens. We were able to extract sufficient RNA from 86 of 91 consecutive tumors. Mean patient age at diagnosis was 64.5 years, and the majority presented with moderately differentiated, sporadic, invasive ductal carcinomas. Among the 86 tumors, 64 (74.4%) were found to express PDEF at a level at least equal to the established MCF7 breast cancer cell line, and higher than the normal breast average expression level (Figure 3*A*). PDEF expression ranged from 0 to 32 times higher than MCF7. Mean PDEF expression in all tumors was 3.76 ± 4.46, with a median of 2.80.

Cluster Analysis of PDEF By Clinicopathologic Parameters

Table 1 summarizes the clinicopathologic parameters of patients and their tumors as well as the significance of the different average PDEF expression cluster levels. Overall,

Table 1. PDEF Expression and Clinicopathological Factors of 86 Invasive Breast Cancers.

Clinicopathological Characteristics	Frequencies*		PDEF Expression and Association with Clinicopathological Factors		
	N	(%)	Mean	95% CI	*P*†
Age					
< 50	15	(17.6)	2.6	1.4–3.7	
50 to 70	36	(42.4)	4.1	2.9–5.3	
> 70	34	(40.0)	3.9	1.8–6.0	.516
Primary or relapse					
Primary	68	(79.1)	3.2	2.5–3.9	
Recurrence	10	(11.6)	7.5	0.2–14.8	
Second primary, contralateral	8	(9.3)	4.0	1.7–6.2	.278
Familial history					
No	60	(69.8)	4.4	3.1–5.7	
Yes	26	(30.2)	2.4	1.4–3.4	.060
Histology					
Ductal	66	(76.7)	3.4	2.6–4.3	
Lobular	13	(15.1)	5.7	0.6–10.7	
Other	7	(8.1)	3.6	0.6–6.7	.267
Histopathological grade‡					
I (good)	13	(15.1)	6.0	1.1–10.9	
II (moderate)	41	(47.7)	4.6	3.5–5.8	
III (poor)	31	(36.0)	1.7	0.9–2.5	.003**
Tumor size					
T1 (≤ 2 cm)	33	(38.4)	3.6	1.7–5.6	
T2 (2.1–5 cm)	46	(53.5)	4.1	3.0–5.2	
T3 (> 5 cm)	7	(8.1)	2.3	0.1–4.6	.614
Metastatic axillary nodes§					
Negative	40	(46.5)	2.4	1.6–3.2	
Positive	37	(43.0)	4.3	3.4–5.3	.002
Combined staging (American Joint Committee on Cancer)					
I	20	(23.3)	2.3	1.5–3.2	
IIA	28	(32.6)	2.7	1.7–3.8	
IIB	16	(18.6)	5.8	4.2–7.3	≤ .003††
IIIA	8	(9.3)	3.1	0.7–5.5	
IIIC	5	(5.8)	3.4	−0.3–7.1	
Estrogene receptor status¶					
Negative	31	(36.0)	2.0	0.6–3.4	
Positive	54	(62.8)	4.9	3.6–6.1	.003
Progesterone receptor status¶					
Negative	37	(43.0)	2.0	1.1–2.9	
Positive	48	(55.8)	5.2	3.8–6.7	.001
HER-2/*neu* overexpression#					
Negative	73	(84.9)	4.0	2.9–5.2	
Positive	5	(5.8)	2.9	−0.5–6.3	.596

PDEF, prostate-derived Ets transcription factor; *CI*, confidence interval.

*Data do not always add up to 86 due to missing values.

†*P* value of Student's *t* test for independent samples or one-way ANOVA when there was more than two categorical variables.

‡Scarff Bloom and Richardson classification, combined grade.

§Nodal status is dichotomized since only 11 tumors were associated with four or more nodes (N2 and N3).

¶Immunohistochemical classification.

#Immunohistochemical classification (TAB 250 and CB11) confirmed by fluorescent *in situ* hybridization when doubtful.

**Average PDEF expression of grade III tumors is significantly lower than grade II or I.

††Average PDEF expression of stage IIB is higher than stage I or IIB tumors, but not significantly different from stage III tumors.

Table 2. Univariate and Multivariate Analyses of Metastatic Nodal Involvement in the Set of Clinical Samples Tested for PDEF Expression.

Clinicopathological Characteristics	Univariate		Multivariate*	
	P	Odds Ratio (95% CI)	*P*	Odds Ratio (95% CI)
PDEF expression (continuous)	.002	1.34 (1.10–1.62)	.002	1.250 (1.004–1.540)
Differentiation, grade III *vs* I and II	.08	0.41 (0.14–1.08)	.42	0.51 (0.14–1.88)
Tumor size, > 2 cm *vs* ≤ 2 cm	.03	3.12 (1.12–8.70)	.10	2.75 (0.89–8.34)
HER-2/*neu*, positive *vs* negative	.93	1.09 (0.14–8.25)	.48	2.38 (0.21–27.00)

CI, confidence interval.
*The multivariate model included 69 tumors, with 8 recurrent tumors for women who had already undergone axillary dissection for the primary tumor, 8 missing values for HER-2/*neu*, and 1 missing tumor grade.

these results provide an association between PDEF expression and sporadic epithelial ER-positive breast cancers that are better differentiated (histopathological grades I and II) than ER-negative tumors (Figure 3*B*). At the time of diagnosis, breast cancer can be regionally disseminated to the axillary lymph nodes. PDEF overexpression in the primary tumor was associated with these more advanced node-positive tumors (Figure 3*B*). No association was found with patient age, tumor size, ductal, or lobular histological type. The limited number of recurrent tumors and tumors overexpressing HER-2/*neu* did not yield statistical significance.

PDEF Expression is Strongly Associated with Hormone Receptor Status

We observed that seven patients who presented an ER-positive primary tumor and who had taken a 5-year course of

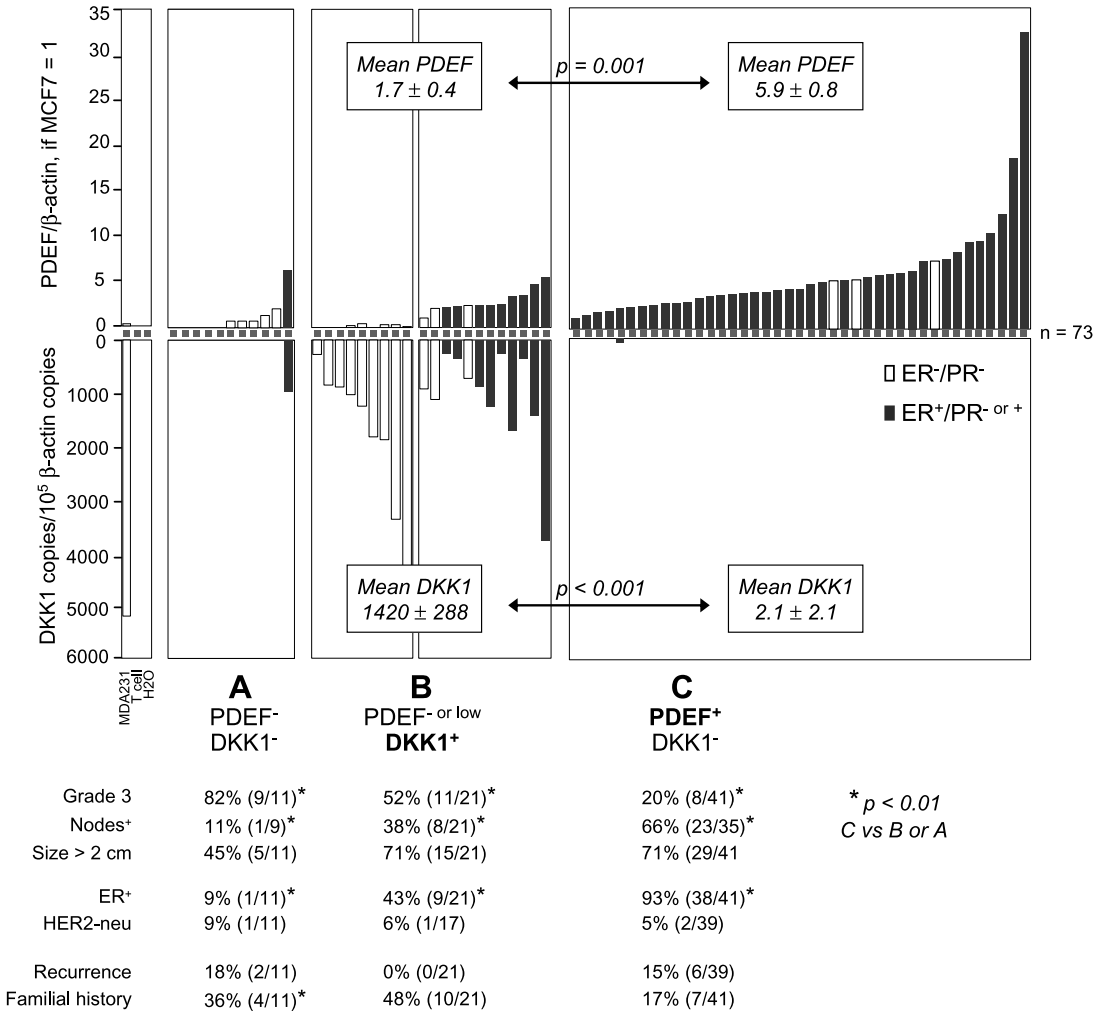

Figure 4. *PDEF and DKK1 differential expression pattern. PDEF (upper charts) and DKK1 (lower charts) transcriptional expression are presented in three groups. Group A comprises less differentiated, hormone receptor–negative tumors. DKK1 is overexpressed in Group B, with concomitant null or weak PDEF expression. Group C corresponds to tumors in which PDEF is overexpressed. Compared with Groups A and B, Group C tumors more frequently involve axillary lymph nodes (Nodes+), although better differentiated (less than grade III) and hormone receptor–positive. ER, estrogen receptor; MDA231, breast cancer cell line (experimental control); PR, progesterone receptor; T cell, T lymphocytes.*

antiestrogen therapy (Tamoxifen) to prevent tumor recurrence nevertheless relapsed with ER-positive tumors coexpressing PDEF. The mean PDEF expression in these tumors was 8.1, but the sample size was insufficient to reach statistical significance (data not shown). Among all tumors expressing PDEF, only 10 did not express ER, further reinforcing a close link between ER and PDEF. We then generated a ROC analysis to appreciate the sensitivity and specificity at which PDEF expression could predict an association with hormone receptor positivity (Figure 3*C*). As expected from the five times higher mean PDEF expression in hormone receptor–positive tumors (Figure 3*B*), the ROC analysis allowed us to predict hormone receptor positivity with 98.3% sensitivity (58/59) and 76.9% specificity (20/26), when PDEF was at or above the MCF7 level. These findings all suggest a strong association between PDEF and the hormone receptor–positive phenotype of breast cancer.

PDEF Overexpression is an Independent Risk Factor for Cancer Dissemination to Axillary Lymph Nodes

The ROC generated for the prediction of nodal involvement was less discriminatory than for hormone receptors (Figure 3*C*). We, however, compared the strength of this association to standard pathological characteristics, because regional dissemination of cancer to lymph nodes is the strongest predictor of recurrence and death from breast cancer. Univariate analysis revealed that PDEF expression was a better predictor of nodal involvement than the degree of differentiation, tumor size and HER-2/*neu* status (Table 2). Continuous PDEF expression remained the only significant independent risk factor in multivariate logistic regression analysis. In this model, sensitivity and specificity were 87.8% (29/33) and 38.8% (14/36), respectively. The associated positive predictive value was 56.9% (29/51), and the negative predictive value, 77.8% (14/18). The odds of presenting nodal metastasis at the time of surgical management were hence increased by 25% for each one-point elevation of PDEF expression (odds ratio 1.250, 95% confidence interval 1.004–1.540, $P = .002$). PDEF expression in the primary tumor thus appeared to be of interest as a molecular staging tool.

PDEF and DKK1 Expressions Delineated Different Subgroups of Aggressive Breast Cancers

The secreted protein DKK1, involved in the Wnt/β-catenin canonic pathway, is emerging as a biomarker of shorter cancer survival [25]. We recently reported that DKK1 was preferentially expressed, at the transcriptional level, in the aggressive subgroup of ER-negative invasive breast cancers [24]. Since it was recently documented that PDEF could modulate the Wnt/β-catenin pathway [19], we wanted to compare the PDEF and DKK1 expression profiles. PDEF expression was effectively lower in DKK1-expressing tumors (1.7 ± 1.4 *vs* 5.9 ± 3.2, $P < .001$) (Figure 4), and DKK1 was not detected among high PDEF-expressing tumors. It was confirmed that PDEF was expressed in a different subgroup of tumors than DKK1. PDEF-expressing tumors were better differentiated (lower than grade III, $P = .01$); a higher proportion expressed ER (93% *vs* 43%, $P = .001$) and were disseminated to axillary lymph nodes (66% *vs* 38%, $P = .01$). In summary, PDEF and DKK1 could delineate distinct aggressive subgroups of invasive breast cancers. The prognostic significance of DKK1 and PDEF in terms of disease-free survival and overall survival remains to be evaluated.

Discussion

This is the first study to assess the expression profile of PDEF, measured as a continuous variable, by a highly reproducible quantification method, in a substantial number of clinical specimens, characterized for classic clinicopathologic parameters. The first main finding was the strong association between PDEF and the hormone receptor–positive breast cancer phenotype. The second was that high PDEF mRNA expression in the primary tumor was associated with a higher risk of regional metastasis to the lymph nodes, which remains the most important prognostic factor for survival at the time of breast cancer diagnosis.

High PDEF mRNA expression needs to be interpreted in relation to low PDEF protein expression found in prostate and breast carcinomas, when compared to normal tissues [16,17,20]. Specifically for breast cancer, immunohistochemical nuclear staining of PDEF was shown to be higher in the peritumoral normal breast epithelium compared to carcinoma cells of all differentiation grades, among 7 and 14 clinical samples [16,20]. These authors noted the near absence of PDEF in the less differentiated grade III tumors. PDEF protein expression was unfortunately not compared with respective mRNA expression or ER status in the clinical samples tested, which limits comparison with our study.

Several observations can, however, be made. We have described significantly low PDEF mRNA expression in grade III tumors when compared to grades I and II (Figure 3*B*), which appears consistent with a PDEF downregulation and low protein detection in grade III tumors. This is reconcilable with a scheme of epithelial to mesenchymal transition to neoplasia, in which PDEF expression could be lost during cancer progression. However, we cannot exclude that the majority of the few clinical specimens stained for PDEF was ER-negative in the studies cited. Low PDEF protein detection could also reflect the expected low PDEF transcription level in this ER-negative tumor phenotype, as we have observed at the mRNA level.

Nevertheless, low PDEF mRNA expression in normal breasts still translates into high protein nuclear detection [16]. Furthermore, low PDEF mRNA expression in breast cancer cell lines can be associated with sizeable protein expression (see MDA-MB-231, Figure 1), and high PDEF mRNA expression, with barely detectable protein (see HCC-1428, Figure 1). If PDEF is not mutated in cancer cells, it suggests the occurrence of posttranscriptional mechanisms, such as protein cleavage, homo- or heterodimerization, as reported for other Ets factors [6], fast degradation due to two sequences rich in proline, glutamic acid, serine, and threonine motifs contained in PDEF [11], or cytoplasmic instead of nuclear pooling [20]. If PDEF would qualify as a therapeutic target, it would

further be important to investigate its protein expression in low PDEF mRNA normal tissues, such as the lungs and colon (Figure 2).

The link between PDEF and cell motility has been the subject of recent publications. In clinical samples, we observed an increased risk of nodal metastasis when PDEF was highly transcript in the primary tumor (Figure 3*B* and Table 2). Other groups have documented PDEF mRNA overexpression in breast cancer micro- and macrometastasis to lymph nodes [15,26]. These data suggest that PDEF can be transcribed in invasive breast cancer and that PDEF could have a role in molecular staging, provided that an impact on patient survival would be further demonstrated prospectively and have an impact on clinical decision making. Considering the heterogeneity of breast cancers presenting lymph node metastasis, it is too speculative to think that the altered expression of one gene alone could accurately predict nodal involvement from a primary tumor sample. We believe that the potential role of PDEF in molecular staging lies in multigene predictive models [3,27].

The association between PDEF mRNA overexpression and nodal metastatis has to be discussed in light of the recent work done on cell migration. Most studies have concluded that PDEF was antimetastatic [16,18–20]. In short, PDEF overexpression in transfected cell lines derived from approximately eight breast or prostate cancer metastases resulted in decreased cell migration, loss of pseudopods, spheroid–morphological changes, and increased proportion of cells in the G_0 phase of the cell cycle. Opposite effects were observed in PDEF knockdown cells (small interfering RNA). Some underlying genetic mechanisms were also proposed to support the decrease in cell invasiveness, conditioned by PDEF, namely, the reduced urokinase plasminogen activator and increased Maspin [16], Survivin downregulation [20], and interaction with the transforming growth factor-β pathway [19]. In contrast, PDEF overexpression yielded prometastatic effects in transfected normal endothelial cells, in normal breast cells, in the MCF10A benign breast fibrocystic disease, in four pleural metastasis–derived breast cancer cell lines, in one melanoma, and in one colon cancer cell line [14]. Interestingly, coexpression of PDEF and activated receptor tyrosine kinase Her-2/*neu*, or colony-stimulating factor receptor, synergistically enhanced MCF10A metastatic attributes. How can the apparent bimodal, anti-, and prometastatic effects of PDEF be explained?

Three aspects may be considered in this regard. First, the loss of PDEF protein expression, combined with high PDEF mRNA expression, could be a stronger marker of prometastatic characteristics in advanced breast cancers. Secondly, PDEF overexpression in lower-grade tumor, as well as in normal breast, may participate in the initiation or progression of early breast cancer. In fact, most antimetastatic results have been documented in MDA-MB-231 mesenchymal-like, ER- and HER-2/*neu*–negative pleural metastasis breast cancer cell lines [16,18], and in the PC-3 androgen–independent prostate cancer bone metastasis cell line [19]. Conversely, convincing prometastatic effects were noted in the benign breast disease MCF10A cell line [14]. Finally, PDEF may function as a transcription activator or repressor, contingent on the cellular context, particularly through cooperation with hormone receptors [11] and the extracellular-regulated kinase/mitogen-activated protein kinase signaling pathway.

In conclusion, the PDEF transcriptional expression profile in clinical samples suggests potential uses in molecular staging, possibly for the heterogeneous subgroup of ER-positive tumors, and for early lymph node metastasis diagnostic purposes. Further findings on PDEF protein biology are needed to conclude if PDEF could represent an appropriate therapeutic target.

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