

Down-regulation of the candidate tumor suppressor gene *PAR-4* is associated with poor prognosis in breast cancer

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Abstract. Substantial experimental evidence indicates that *PAWR* gene (PKC apoptosis WT1 regulator; also named PAR-4, prostate apoptosis response-4) is a central player in cancer cell survival and a potential target for cancer-selective targeted therapeutics. However, little is known about the role of PAR-4 in breast cancer. We investigated the possible role of PAR-4 expression in breast cancer. IHC results on tissue microarrays containing 1,161 primary breast tumor samples showed that 57% (571/995) of analyzable cases were negative for PAR-4 nuclear staining. Down-regulation of nuclear PAR-4 protein expression predicted a poor prognosis for breast cancer patients (OS; P=0.041, log-rank test). PAR-4 down-regulation also correlates with poor survival in the group of patients with luminal A subtype breast cancer (P=0.028). Additionally, in this large series of breast cancer patients, we show that ERBB2/HER2, EGFR and pAKT protein expression are significantly associated with shorter disease-free survival and overall survival, but the prognosis was even worse for HER2-positive, EGFR-positive or pAKT-positive breast cancer patients with tumors negative for nuclear PAR-4 expression. Furthermore, using three-dimensional (3D) cell culture we provide preliminary results showing that PAR-4 is highly expressed in the MCF10A cells inside the acini structure, suggesting that PAR-4 might have a role in the lumen acini formation. Taken together, our results provide, for the first time, evidence that PAR-4 may have a role in the process of the mammary gland morphogenesis and its

functional inactivation is associated with tumor aggressive phenotype and might represent an additional prognostic and predictive marker for breast cancer.

Introduction

Evasion of apoptosis (programmed cell death), which is an active, energy-dependent process involving biochemical and molecular events regulated by a series of distinct genes is a hallmark of cancer (1). Down-regulation of apoptotic rates is associated with tumor pathogenesis and affects chemo- and radioresistance (2). Much experimental evidence indicates that *PAWR* (PKC apoptosis WT1 regulator; also named PAR-4, prostate apoptosis response-4) is one of the central players in cancer cell survival and could be a target for cancer-selective targeted therapeutics (3). The PAR-4 gene is located at chromosome 12q21 and encodes a 38-kDa protein containing two nuclear localization signals (NLS), a leucine zipper domain, and a selective apoptosis induction in cancer cells (SAC) domain (4-6). The PAR-4 was first identified as being up-regulated in androgen-independent prostate cancer cells undergoing apoptosis after treatment with calcium (7).

The PAR-4 protein contains conserved amino acid residues that are the target of phosphorylation by protein kinases A (PKA) and C (PKC), which regulate its sub-cellular localization and possibly its ability to dimerize with other proteins (3,8). The PAR-4 protein binds to and forms complexes with various proteins, including PKC ξ , WT-1, ZIPK, DAXX, and THAP1 through its C-terminal leucine zipper domain to affect cell survival (9-12). There is evidence that PAR-4 displays its pro-apoptotic activity by down-regulating the anti-apoptotic Bcl-2 protein (13,14). In addition, experimental studies showed that PAR-4 induces apoptosis through its ability to activate the pro-death, FasL-Faz-FADD-caspase 8 pathway and by inhibiting the NF- κ B pro-survival pathway, which requires phosphorylation of PAR-4 at T155 mediated by PKA activity (12,15,16). On the other hand, AKT activity, which is elevated in cancer cells through growth factor signaling stimulation, oncogene activation, or loss of the phosphatase and tensin homolog (PTEN) tumor suppressor activity can inhibit PAR-4's

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pro-apoptotic function (17). Goswami *et al* demonstrated that AKT binds to the PAR-4 protein and phosphorylates PAR-4 at residue S249, with subsequent sequestration of PAR-4 in the cytoplasm by the chaperone 14-3-3 proteins, which inhibit PAR-4's apoptotic activity (18).

The PAR-4 gene is expressed in several mammalian cells including mammary-gland epithelial cells (19,20). Expression of PAR-4 may increase the sensitivity of most cancer cells to apoptosis, especially in hormone-independent cancer cell lines, including breast cancer cells (6,15). PAR-4 protein expression is induced by many conditions, such as growth factor withdrawal, *TNF*, ionizing radiation and high levels of calcium (4,20-22). In cultured embryonic rat hippocampal neurons, withdrawal of trophic factors increases the level of PAR-4 mRNA expression (23).

Alterations in PAR-4 mRNA and protein expression were observed in different types of tumors. In hematopoietic cells, this gene exhibits an anti-tumor effect and induces increased sensitivity towards chemotherapeutic agents (24). Decreased PAR-4 protein levels were demonstrated in human renal carcinomas and human renal cancer cells (25). Reduced expression of PAR-4 due to promoter hypermethylation is a frequent event associated with endometrial carcinogenesis (26). PAR-4 down-regulation was associated with K-ras mutation and a poor prognosis in pancreatic tumors (27). The role of PAR-4 in prostate cancer has been well described (15,28).

To date, little is known of the role played by PAR-4 in breast cancer. In the present study, using immunohistochemistry (IHC) on tissue microarrays (TMAs) in a large series of tumor samples, our purpose was to determine whether PAR-4 protein expression has prognostic significance in breast cancer patients. We also attempted to correlate PAR-4, EGFR, ERBB2/HER2, and pAKT protein expression with clinical outcomes in breast cancer patients. In addition, preliminary results with MCF10A mammary epithelial cells using 3D cell culture suggest that PAR-4 might have a role for the lumen acini formation.

Materials and methods

Tissue samples and patients characteristics. Primary breast tumor samples were obtained from 1,161 patients with invasive ductal carcinoma at the Department of Pathology, Hospital AC Camargo, São Paulo, Brasil. All patients were diagnosed at the hospital and treated at same institution by radical mastectomy, modified radical mastectomy or breast-conserving surgery including axillary lymph node dissection. The median age of the patients at the time of diagnosis was 54 (range 24-96) years. The sizes of the tumors ranged from 0.4 to 22 cm (median 4) cm. Table I lists the patient characteristics. The Institutional Ethics Committee approved this study and all patients provided informed consent.

Tissue microarrays (TMA) construction. For TMA construction, formalin-fixed, paraffin-embedded tissue blocks containing invasive breast cancer from 1,161 patients were retrieved from the archives. New sections from each block stained with hematoxylin and eosin (H&M) were made to select and mark representative areas of the tumor specimens, and the arrays were constructed as previously described (29).

Immunohistochemistry of TMA. Immunohistochemistry (IHC) was performed on three slides (3 cores/case) for each marker. Paraffin-TMAs, 3- μ m-thick sections, were deparaffinized and rehydrated as described previously (29). Slides were placed in 3% hydrogen peroxide three times for 5 min, washed in water for 5 min, then incubated for a day in a humidified chamber with one of the primary antibodies. The primary antibodies used in the present study were mouse monoclonal anti-PAR-4 (Santa Cruz, Inc., Santa Cruz, CA, USA; catalog sc-1666; 1:100), anti-HER2 (Dako Cytomation, catalog A0485; 1:2000), anti-EGFR (Novocastra, catalog NCL-EGFR-384; 1:100), anti-phospho-AKT(Ser473) (Cell Signaling, catalog 4051; 1:800), anti-ER (Neomarkers, clone SP1, 1:1000), anti-PR (Dako, clone 636, 1:2000) and anti-CK5,6 (Dako, clone D5/16B4, 1:100) used according to the manufacturer's recommendations. The optimal dilution was defined using well-known positive samples, and tested before staining the TMA slides. The slides were washed in PBS and subsequently incubated with biotinylated anti-IgG for 20 min, then with streptavidin-biotin peroxidase LSAB kit (Dako®, Carpinteria, CA, USA) in a humidified chamber. The immunostaining was performed by incubating slides in diaminobenzidine (Dako) solution containing 1 μ l of chromogen per 50 μ l of buffer substrate for 5 min. After chromogen development, the slides were washed, dehydrated with alcohol and xylene, and mounted with cover slips using a permanent mounting medium.

Evaluation of immunohistochemistry. Immunohistochemical evaluation was semiquantitative for all markers except for pAKT and PAR-4. Only clear staining of the tumor cells were considered positive and scored based on staining intensity and percentage of stained cells, which varied for each marker. Following scores used in the literature ER and PR were evaluated by the percentage and intensity of stained nucleus (30), CK5,6 and EGFR were considered positive when staining was observed in $\geq 1\%$ of the tumor cells (31), tumors were considered positive for HER2 over-expression when $\geq 10\%$ of the tumor cells showed membranous immunostaining (32). A quantitative analysis was performed for the expression of pAKT and PAR-4 using the image capture system, Automated Cellular Imaging System (ACIS® III, Dako, K0690). Two TMA slides for each marker were scanned for image capture. The parameters were established regarding the intensity and area of brown staining captured for two to five areas in each case. A numerical value corresponding to the staining intensity multiplied by the area of brown staining divided by the total area analyzed was obtained for each circular area. For each tissue fragment, the final numerical value corresponding to the mean value of the 2-5 areas analyzed was determined. Tissue cores with less than 50% of the original tissue left on the slides after IHC were not used for the scoring of the stains.

Immunophenotypical classification. Breast tumors were stratified in four subtypes based on the immunohistochemical expression of estrogen receptor (ER), progesterone receptor (PR), HER2 and cyokeratin 5,6 (CK5,6): luminal A (ER-positive, PR-positive, HER2-negative); luminal B (ER-positive and/or PR-positive, HER2-positive); HER over-expression (ER-negative, PR-negative, HER2-positive); and triple-negative

Table I. Sample description.

Variable	Categories	n	%
Stage (TNM)	Stage I	71	6.1
	Stage II	440	37.9
	Stage III	527	45.5
	Stage IV	108	9.3
	Not available	15	1.3
Lymph node metastasis	No	368	31.7
	Yes	762	65.6
	Not available	31	2.7
Scarff-Bloom-Richardson	Grade 1	169	14.6
	Grade 2	670	57.7
	Grade 3	319	27.5
	Not available	3	0.3
Nuclear grade	Grade 1	17	1.5
	Grade 2	374	32.2
	Grade 3	766	66.0
	Not available	4	0.3
Estrogen receptor	Negative	354	30.5
	Positive	716	61.7
	Not available	91	7.8
Progesterone receptor	Negative	572	49.3
	Positive	469	40.4
	Not available	120	10.3
HER2	Negative	840	72.4
	Positive	141	12.1
	Not available	180	15.5

(ER-negative, PR-negative, HER2-negative, and/or CK5,6-positive) (33).

Cell culture conditions. The MCF10A cells were obtained from the American Type Culture Collection (ATCC). The MCF10A cells were cultured at 37°C in an atmosphere of 5% CO₂, 95% air in F12/DMEM supplemented with 5% horse serum, 50 ng/ml epidermal growth factor (EGF), 10 µg/ml insulin, 0.5 µg/ml hydrocortisone, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 0.1 µg/ml cholera toxin.

Morphogenesis assay and immunofluorescence. MCF10A cells were grown in monolayers until they reach 100% confluence, then were trypsinized to obtain a suspension containing single cells. MCF10A cells (2x10³) in media containing 3% of growth factor-reduced Matrigel (BD Biosciences) were plated in 8-well chamber slides onto a bed of 30 µl of growth factor-reduced Matrigel, as described by Debnath *et al* (34). The expression of PAR-4 and activated caspase 3 in the acini structures was analyzed by immunofluorescence on days 3, 5, 7 and 10. The medium containing 3% Matrigel was replaced every 3 days. Immunofluorescence was performed as described by Debnath *et al* (34) with a few modifications, including permeabilization with 0.5% Triton X-100 for 45 min and overnight incubation with primary antibodies at room temperature. The acini

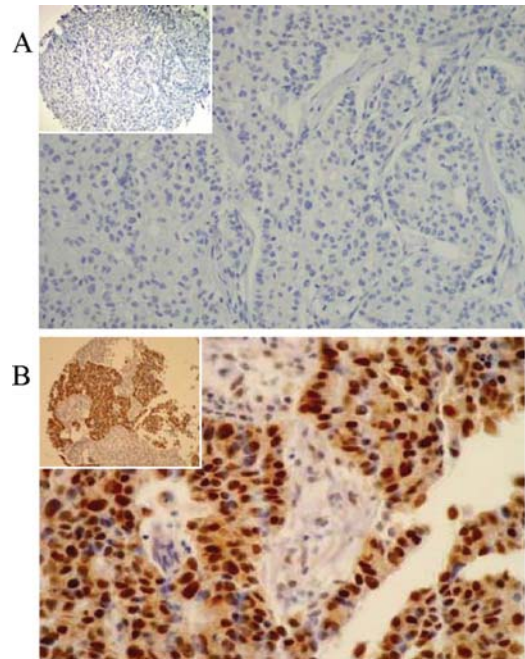


Figure 1. PAR-4 immunohistochemical staining in primary breast tumor samples. (A) Representative photomicrography from a TMA core of negative staining (original magnification x40 and x400 respectively). (B) Representative photomicrography from a TMA core of positive staining (original magnification x40 and x400 respectively).

structures were stained with mouse anti-PAR-4 monoclonal antibody (Santa Cruz, Inc., catalog sc-1666; 1:50) and/or a rabbit anti-activated-caspase 3 polyclonal antibody (Cell Signaling Technology, Inc., catalog 9661). The conjugated secondary anti-mouse-Alexa Fluor 546 and anti-rabbit-Alexa Fluor 488 were purchased from Invitrogen and diluted 1:300. Nuclear staining was performed with Hoechst 33342 (Invitrogen) for 15 min. A Zeiss LSM Meta 510 scanning confocal microscope was used for immunofluorescence analysis and image capture.

Statistical methods. Median values of PAR-4 expression according to clinical and pathological variables were compared by means of Kruskal-Wallis and Mann-Whitney tests. The Spearman's coefficient (rho) assessed correlation between PAR-4 and pAKT expressions. For survival analysis, PAR-4 was classified as negative or positive. ROC curve defined the cut off point and death was set as the event. Ten-year disease-free survival and overall survival rates were calculated based on the Kaplan-Meier method and the curves were compared using the log-rank test. Significance level was set at 5% in all tests. Statistical analyses were performed using SPSS software 15.0 (SPSS Inc., Chicago, IL).

Results

In the present study investigating PAR-4 protein expression in breast cancer, IHC on TMAs containing 1,161 primary breast tumor specimens were performed using an antibody against the PAR-4 protein. The immunoreactivity scoring was based on the number of tumor cells displaying nuclear PAR-4 immunostaining. A representative example is shown in Fig. 1. PAR-4 staining was assessable in 995 cases on

Table II. Nuclear PAR-4 expression according to tumor stage and biomolecular markers.

Variable	Category	n	PAR-4 expression (median value)	P-value
Tumor size	≤2.0 cm	149	31.3	KW: 0.835
	2.1-5.0 cm	510	27.1	
	>5.0 cm	291	26.7	
Lymph node metastasis	No	314	28.4	MW: 0.633
	Yes	652	27.3	
Stage (TNM)	Stage I	54	25.6	KW: 0.145
	Stage II	385	31.0	
	Stage III	451	26.0	
	Stage IV	91	26.8	
Scarff-Bloom-Richardson	Grade 1	134	21.2	KW: <0.001
	Grade 2	574	25.8	
	Grade 3	284	36.2	
Nuclear grade	Grade 1	12	5.2	KW: <0.001
	Grade 2	309	19.3	
	Grade 3	670	32.6	
Estrogen receptor	Negative	315	25.6	MW: 0.526
	Positive	648	28.5	
Progesterone receptor	Negative	491	26.7	MW: 0.087
	Positive	431	31.0	
HER2	Negative	731	25.3	MW: <0.001
	Positive	131	38.5	
EGFR	Negative	790	27.2	MW: 0.069
	Positive	182	35.1	

KW, Kruskal Wallis test. MW, Mann-Whitney test.

the TMA blocks. PAR-4 expression ranged from 0 to 97.4 according to ACIS analysis (median value 27.7).

Table II shows the relationship between PAR-4 protein expression and clinicopathological characteristics. There were no differences in PAR-4 expression according to tumor size, nodal status, or clinical stage. However, there was a statistically significant decrease in PAR-4 expression in low-grade tumors according to the Scarff-Bloom-Richardson system ($P=0.001$) and nuclear grade ($P=0.001$). High expression of PAR-4 protein was observed in positive HER2 ($P=0.001$). However, no associations were observed between PAR-4 expression and estrogen receptor status ($P=0.526$) or progesterone receptor status ($P=0.087$). Tumors positive for EGFR had higher PAR-4 expression when compared with those negative for EGFR. This difference was near the significance level ($P=0.069$). A positive but weak correlation was observed between PAR-4 and pAKT expression ($\rho=0.225$; $P=0.001$).

Table III shows the 10-year overall survival (OS) and disease-free survival (DFS) analysis. There was a significant

difference in OS rates according to tumor stage ($P=0.001$), lymph node status ($P=0.001$), nuclear grade ($P=0.001$), estrogen receptor status ($P=0.001$), progesterone receptor status ($P=0.001$), HER2 status ($P=0.005$), EGFR status ($P=0.002$) and nuclear PAR-4 status ($P=0.041$). The DFS analysis showed differences according to tumor stage ($P=0.001$), lymph node status ($P=0.001$), Scarff-Bloom-Richardson grading ($P=0.001$), estrogen receptor status ($P=0.002$), progesterone receptor status ($P=0.001$), HER2 status ($P=0.032$) and EGFR status ($P=0.001$). However, no differences were observed in the DFS rates according to nuclear PAR-4 expression ($P=0.138$).

Kaplan-Meier survival curves were generated to assess the potential prognostic value of PAR-4 protein expression in breast cancer. Nuclear PAR-4 protein expression was a prognostic predictor of OS, but not of DFS in the group of breast cancer patients analyzed. As shown in Fig. 2, significant differences among survival curves by log-rank was observed for OS (Fig. 2A, $P=0.041$). We also assessed the power of PAR-4 expression to predict OS and DFS in our breast

Table III. Ten-year overall survival and disease-free survival analysis.

Variable	Category	10-year overall survival (cumulative survival - %)			10-year disease-free survival (cumulative survival - %)		
		n	%	P-value	n	%	P-value
Stage	Stage I	71	82.7	<0.001	70	80.0	<0.001
	Stage II	437	70.6		434	70.5	
	Stage III	523	35.8		493	35.3	
	Stage IV	108	1.9		-		
Lymph node metastasis	No	366	69.1	<0.001	355	73.1	<0.001
	Yes	756	39.3		633	43.4	
Scarff-Bloom-Richardson	Grade 1	168	60.9	<0.001	153	66.4	<0.001
	Grade 2	657	48.3		585	52.9	
	Grade 3	312	41.6		256	48.1	
Nuclear grade	Grade 1	16	71.8	0.001	16	65.5	0.103
	Grade 2	368	54.0		330	56.6	
	Grade 3	752	45.1		647	52.2	
Estrogen receptor	Negative	350	41.6	<0.001	291	49.6	0.002
	Positive	700	52.1		626	56.6	
Progesterone receptor	Negative	561	41.6	<0.001	482	47.3	<0.001
	Positive	460	57.7		419	60.6	
HER2	Negative	822	49.2	0.005	722	53.9	0.032
	Positive	140	44.3		120	48.5	
EGFR	Negative	872	51.1	0.002	776	56.7	<0.001
	Positive	202	41.4		164	41.6	
PAR-4	Negative (≤ 35)	566	45.0	0.041	493	50.6	0.138
	Positive (> 35)	409	53.7		361	56.6	
PAKT	Negative (≤ 90)	532	50.8	0.027	477	57.0	0.080
	Positive (> 90)	494	46.2		419	49.8	
PAR-4–PAKT interaction	PAR-4 (-)/pAKT (-)	309	47.6	0.040	276	54.8	0.231
	PAR-4 (-)/pAKT (+)	240	42.7		202	46.6	
	PAR-4 (+)/pAKT (-)	192	53.7		173	58.3	
	PAR-4 (+)/pAKT (+)	214	53.2		185	55.0	
PAR-4–EGFR interaction	PAR-4 (-)/EGFR (-)	459	47.4	0.001	408	53.3	<0.001
	PAR-4 (-)/EGFR (+)	91	34.8		71	34.5	
	PAR-4 (+)/EGFR (-)	311	56.5		280	60.5	
	PAR-4 (+)/EGFR (+)	91	46.3		76	45.2	
PAR-4–HER2 interaction	PAR-4 (-)/HER2 (-)	432	46.3	0.003	379	51.5	0.058
	PAR-4 (-)/HER2 (+)	59	35.7		48	38.1	
	PAR-4 (+)/HER2 (-)	281	55.2		248	56.7	
	PAR-4 (+)/HER2 (+)	71	50.5		64	55.3	

cancer population stratified based on the immunophenotype classification as luminal A, luminal B, HER2 positive and triple-negative tumors. Patients classified as luminal subtype A

with tumors showing PAR-4 negative expression had a significant worse prognosis ($P=0.028$) than patients with luminal subtype A tumors positive for PAR-4 expression

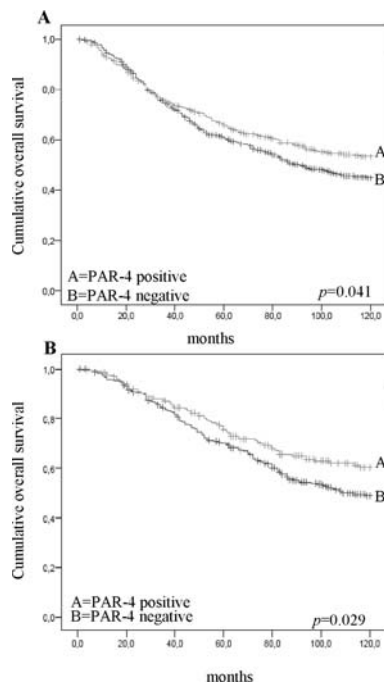


Figure 2. Kaplan-Meier curves for long-term survival in primary breast cancer patients, stratified according to PAR-4 protein expression. (A) Overall survival (all patients); (B) Overall survival (luminal subtype A patients). Tumors were classified as negative or positive for PAR-4 nuclear immunostaining.

(Fig 2B). No associations were observed for PAR-4 nuclear expression and the other breast cancer subtypes.

Since AKT activation, that can be displayed by EGFR and HER2 signaling pathways, has been associated with PAR-4 phosphorylation, cytoplasmic sequestration, and inhibition of its proapoptotic function, we also analyzed the prognostic value of PAR-4 in association with HER2, EGFR and pAKT protein expression. In our analysis, the positive status for HER2, EGFR, and pAKT alone was significantly associated with poor prognosis (Fig. 3A, C and E). In general, PAR-4 protein expression in association with HER2, EGFR or pAKT protein expression had a predictive prognostic value for both DFS and OS in the series of breast cancer patients analyzed. As shown in Fig. 3B, D and F, recurrence or death due to disease occurred earlier in patients with tumors negative for nuclear PAR-4 expression, that over-expressed HER2 (OS, $P=0.003$; DFS, $P=0.058$) or EGFR (OS, $P=0.001$; DFS, $P=0.001$), or exhibited AKT activation (OS, $P=0.04$; DFS, $P=0.231$).

Using three-dimensional (3D) cell culture we also evaluated the expression pattern of the PAR-4 during morphogenesis of MCF10A cells. As shown in Fig. 4, high levels of PAR-4 expression were displayed by the cells within the acini lumen. PAR-4 expression on these cells was only partially co-expressed with activated caspase-3.

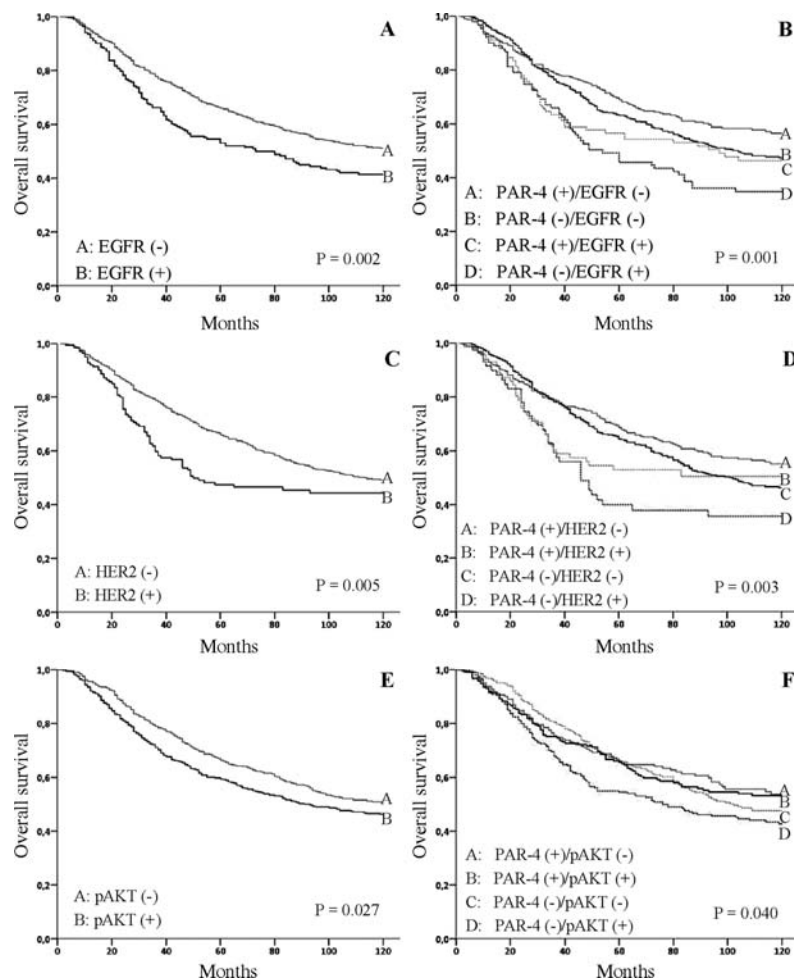


Figure 3. Kaplan-Meier curves for long-term overall survival in breast cancer patients stratified according to: (A) EGFR protein expression; (B) Nuclear PAR-4 and EGFR protein expression; (C) HER2 protein expression; (D) Nuclear PAR-4 and HER2 protein expression; (E) pAKT protein expression; (F) Nuclear PAR-4 and pAKT protein expression.

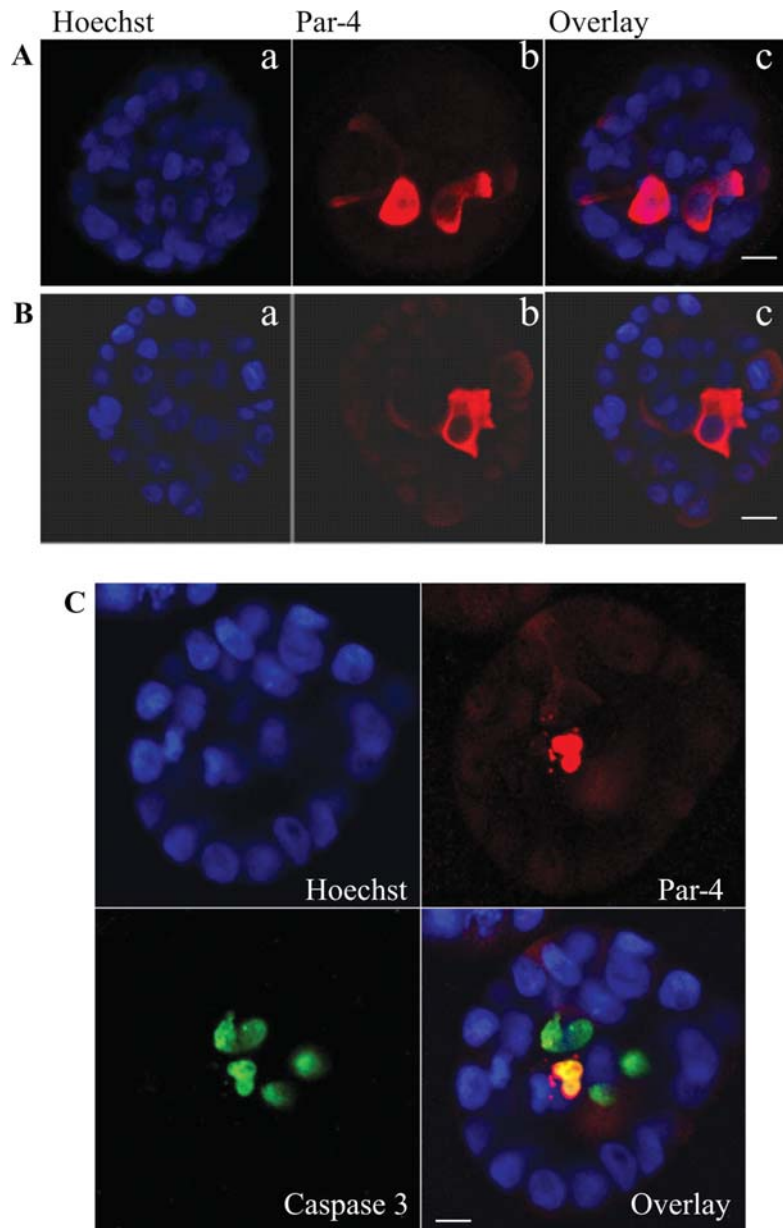


Figure 4. Expression of PAR-4 and activated caspase 3 in MCF10A cells grown in three-dimensional (3D) cell culture. Representative confocal microscopic images of MCF10A cells cultured in growth factor reduced Matrigel for 5 (A) and 7 (B) days and examined for PAR-4 protein expression (red) by immunofluorescence. Nuclei were contraststained with Hoechst (a, blue); overlay (c). (B) Day 7 MCF10A acini were immunostained with anti-PAR-4 (b, red) and anti-cleaved caspase 3 (c, green). Nuclei was contraststained with Hoechst (a, blue); overlay (d). Equatorial cross sections of the acini were obtained using a Zeiss LSM Meta 510 confocal microscope system (scale bar: A, 15 μm ; B, 25 μm ; C, 50 μm).

Discussion

To our knowledge, our study represents the first large, systematic investigation of PAR-4 protein expression in primary breast tumors. In our series of primary breast cancer patients, we observed that nuclear PAR-4 down-regulation was associated with poor breast cancer prognosis. In addition, we found that PAR-4 down-regulation showed to be a prognostic factor for luminal subtype A breast cancer, suggesting a functional association between the presence of ER and PAR-4 expression. PAR-4 has been shown to be ubiquitously expressed in different tissues (19) and PAR-4 knockout mice are prone to develop tumors of the liver, lung, urinary bladder, but with an increased prevalence of

hormone-dependent tumors of the endometrium and prostate (35). Carcinomas of the mammary gland was observed in PAR-4 heterozygous (PAR-4^{+/-}) animals (35). It is interesting to note that estradiol reduces PAR-4 expression in mouse uterus, which corroborates the association between PAR-4 down-regulation and high frequency of endometrial carcinomas in PAR-4 knockout mice (35). Further studies to evaluate the role played by estrogens on the transcriptional regulation of PAR-4 in normal and breast cancer cells are required.

By combining the PAR-4 nuclear status with three prognostic proteins (HER2, EGFR, and pAKT) we also provide information for a more powerful prediction of disease survival in breast cancer patients. Translocation of PAR-4 to the nucleus has been shown to be essential for the PAR-4

apoptotic function (6,16). Gurumurthy *et al* (16) demonstrated that cancer-cell apoptosis elicited by PAR-4 requires its phosphorylation and nuclear translocation. These authors also showed that both Fas/FasL translocation to the cell membrane and inhibition of NF- κ B by PAR-4 is dependent on the phosphorylation of the T155 residue located on the PAR-4 SAC domain. The PAR-4 SAC domain can translocate to the nucleus in both normal and cancer cells; however, it elicits apoptosis specifically in cancer cells through phosphorylation of the T155 residue by PKA. Moreover, cancer cells with deficient nuclear translocation of PAR-4, such as MCF-7 breast cancer cells are resistant to its apoptotic function (6,16). On the other hand, the survival kinase AKT that is known to phosphorylate and inactivate several pro-apoptotic proteins (36) was found to bind and phosphorylate PAR-4 at S249 residue located outside the SAC domain (17,18). In different cancer cell lines established from prostate, cervical and breast cancers, activated AKT leads to PAR-4 phosphorylation and cytoplasmic retention by the chaperone 14-3-3 (18). The PTEN, a dual phosphatase that antagonizes PI-3K function is one of the main negative regulators of AKT (37). AKT activation is a frequent event in cancer cells that harbor genetic alteration leading to PTEN inactivation, which is observed in breast tumors (38). In addition, oncogene activation and growth factor over-expression such as HER2 and EGFR that leads to the activation of downstream signaling components such as MAPK (proliferation) and PI-3K/AKT (survival) are observed and have an established prognostic and predictive value in breast cancer (39). Activated AKT has been observed in HER2-positive breast tumors (40), suggesting that the axis PI-3K/AKT is activated in these tumors. Thus PAR-4 negative nuclear staining due to AKT activation could be associated with ERBB2 or EGFR over-expression in breast tumor biopsies. Our study supports this hypothesis showing a close association between HER2, EGFR over-expression or AKT activation and nuclear PAR-4 down-regulation and the disease outcome of the patients. In the present large series of breast cancer patients, our findings show that HER2, EGFR and pAKT protein expression are significantly associated with short disease-free survival and overall survival, but the prognosis was even worse for HER2-positive, EGFR-positive or pAKT-positive breast cancer patients with tumors showing negative nuclear PAR-4 expression. These findings indicate that PAR-4 down-regulation or functional inactivation may protect breast tumor cells from cell death enhancing the malignant phenotype and suggest a prognostic role for PAR-4 in breast cancer.

Three-dimensional (3D) cell culture of the spontaneously immortalized cell line MCF10A is a well-established model system to study breast epithelial cell biology and morphogenesis (41,42). Using this system, we provide preliminary results showing that PAR-4 is highly expressed in the MCF10A cells inside the acini structure (Fig. 4). During the morphogenesis of MCF10A cells in 3D cell culture, the cells within the lumen show apoptotic activity demonstrated by caspase-3 activation (34,42). Our results show co-expression of PAR-4 and activated caspase 3 in some cells only. Loss of interaction between the cells and the components of the extra-cellular matrix leading to anoikis is required for lumen formation

(42,43). Loss of cell adhesion could be the factor that induces PAR-4 expression, which marks and sensitizes mammary epithelial cells for apoptosis. It is interesting to note that the morphogenesis of MCF10A can be altered by oncogene activation, such as HER2 over-expression that leads to increased proliferation and disruption of lumen formation (42,44). It is possible that the production of filled acinar structures by HER2 over-expression could be, at least in part, due to its effect on PAR-4 inhibition. Although preliminary, our results indicate that PAR-4 may play a role in the process of mammary gland morphogenesis. New studies to investigate the role of PAR-4 in mammary morphogenesis and its connection with HER2 activation are warranted.

The findings presented in this work provide evidence that nuclear PAR-4 down-regulation is a frequent event associated with breast cancer progression. Besides its potential to be a target for anticancer strategies, PAR-4 could be considered an end marker of the functionality of the PI-3K/AKT pathways for therapeutic approaches targeting EGFR, HER2 or AKT in breast cancer.

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