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PDIA3 and *PDIA6* gene expression as an aggressiveness marker in primary ductal breast cancer

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ABSTRACT. Changes in the expression of the protein disulfide isomerase genes *PDIA3* and *PDIA6* may increase endoplasmic reticulum stress, leading to cellular instability and neoplasia. We evaluated the expression of *PDIA3* and *PDIA6* in invasive ductal carcinomas. Using reverse transcription-quantitative polymerase chain reaction, we compared the mRNA expression level in 45 samples of invasive ductal carcinoma with that in normal breast samples. Increased expression of the *PDIA3* gene in carcinomas (P = 0.0009) was observed. In addition, *PDIA3* expression was increased in tumors with lymph node metastasis (P = 0.009) and with grade III (P < 0.02). The *PDIA6* gene showed higher expression levels in the presence of lymph node metastasis (U = 99.00, P = 0.0476) and lower expression for negative hormone receptors status (P = 0.0351). Our results suggest that alterations in *PDIA3/6* expression

levels may be involved in the breast carcinogenic process and should be further investigated as a marker of aggressiveness.

Key words: Protein disulfide isomerases; Metastasis; Stress response; Tumorigenesis

INTRODUCTION

Protein disulfide isomerases (PDIs) are responsible for the formation and isomerization of disulfide bonds, which function in protein folding. In addition, PDIs also act as chaperones, inhibiting the aggregation of unfolding substrates and/or assisting in the refolding of polypeptides (Wilkinson and Gilbert, 2004).

The *PDIA3* gene is highly expressed in response to cellular stress, as its product can function as a chaperone. It has also been linked to DNA repair as a member of the damage recognition complex and as a potential regulator that binds to genes with recognized repair functions (Chichiarelli et al., 2007). Several studies have linked PDIs to different types of cancer, including gastric (Leys et al., 2007), prostate (Pressinotti et al., 2009), esophageal (Ayshamgul et al., 2011), cervical (De Marco et al., 2012; Chung et al., 2013), salivary gland (Müller et al., 2013), bone metastasis (Santana-Codina et al., 2013), and breast (Lee et al., 2012; Gaucci et al., 2013) cancers. Another gene in this superfamily, *PDIA6*, also encodes a product that exhibits chaperone activity by inhibiting misformed protein aggregation. The protein plays a role in platelet aggregation and activation by agonists such as convulxin, collagen, and thrombin. In addition, it interacts with polypeptide-related sequence A of class I major histocompatibility complex on the surface of tumor cells. This leads to a reduction in disulfide bonds with polypeptide-related sequence A of class I major histocompatibility complex, which is necessary for its release from tumor cells (Kaiser et al., 2007).

In our previous study, we analyzed the proteome of ductal and lobular carcinomas, and PDIA3 and PDIA6 showed higher expressions levels in ductal than in lobular carcinomas (Oliveira et al., 2011). Because of the high prevalence of invasive ductal carcinomas (IDC) among breast tumors, the importance of PDIs in protein folding and their relationship with proper cell function, we evaluated the correlation between expression of the *PDIA3* and *PDIA6* genes in patients with IDC and with their clinico-pathological parameters.

MATERIAL AND METHODS

Sample characterization

Forty-five primary IDC samples were collected during surgery at Hospital Nossa Senhora das Graças, Curitiba, south Brazil. Non-compromised tissues from the contralateral breast were obtained from 9 patients who underwent mammoplasty for simultaneous symmetrization. These tissues were analyzed by a pathologist and were considered to be normal. Tumor and normal samples were conserved in an RNA stabilization solution (RNAlater, Ambion Inc., Applied Biosystems, Foster City, CA, USA) immediately after surgery and were stored at 4°C. Clinico-pathological data were obtained from the pathology service of the hospital. None of the patients had any family history of hereditary breast or ovarian cancer. The average age of the patients was 57.53 ± 16.39 years and that of the control group was 49.11 ± 10.43 years ($P > 0.05$). Patients had

received neither chemotherapy nor radiation prior to surgery. Table 1 summarizes the clinico-pathological features of the patients. Informed consent forms were signed by all patients. The study was approved by the Ethical Committee of Nossa Senhora das Graças Hospital and was registered by the CONEP (National Research Ethics Comission) No. 7220-251/2003.

Table 1. Sample characterization.

	Age (years)	Diagnostic	LN	G	TS	ER	PR	*ERBB2*
1	97	Invasive ductal carcinoma (luminal)	-	-	-	POS	POS	NEG
2	56	Invasive ductal carcinoma (NOS) associated with ductal carcinoma *in situ* comedocarcinoma with necrosis	P	II	37	-	-	-
3	33	Invasive ductal carcinoma	A	II	15		NEG	NEG
4	44	Bilateral invasive ductal carcinoma	P	II	50	POS	POS	NEG
5	56	Invasive ductal carcinoma	A	II	27	POS	POS	NEG (1+)
6	62	Invasive ductal carcinoma	A	II	15	NEG	NEG	NEG
7	72	Invasive ductal carcinoma NOS	P	II	35	POS	POS	POS (2+)
8	67	Invasive ductal carcinoma NOS	P	II	5	POS	POS	NEG (1+)
9	66	Invasive ductal carcinoma with relapse	P	I	20	POS	POS	POS (2+)
10	67	Invasive ductal carcinoma NOS	P	III	50	POS	POS	NEG (1+)
11	35	Invasive ductal carcinoma	P	III	-	POS	POS	NEG
12	44	Invasive ductal carcinoma NOS	P	II	18	POS	POS	NEG
13	57	Invasive ductal carcinoma	A	II	20	POS	POS	POS (3+)
14	72	Invasive ductal carcinoma	A	II	24	POS	POS	NEG
15	48	Invasive ductal carcinoma	A	II	23	NEG	NEG	NEG
16	45	Invasive ductal carcinoma associated with ductal carcinoma *in situ*	-	III	23	NEG	NEG	NEG
17	45	Invasive ductal carcinoma	A	I	5	POS	POS	POS (3+)
18	86	Invasive ductal carcinoma	A	III	30	POS	POS	NEG
19	83	Invasive ductal carcinoma	A	II	8	POS	NEG	NEG
20	32	Invasive ductal carcinoma	P	II	25	-	-	-
21	46	Invasive ductal carcinoma	A	I	17	POS	POS	NEG
22	49	Invasive ductal carcinoma	A	II	40	POS	POS	NEG
23	71	Invasive ductal carcinoma	A	I	10	POS	POS	NEG
24	74	Invasive ductal carcinoma	A	III	35	NEG	POS	NEG (2+)
25	81	Invasive ductal carcinoma	A	II	20	POS	POS	NEG (1+)
26	73	Invasive ductal carcinoma	A	II	32	POS	POS	NEG
27	46	Invasive ductal carcinoma, associated with ductal carcinoma *in situ*	P	III	40	POS	POS	NEG
28	39	Invasive ductal carcinoma	P	II	18	POS	POS	NEG
29	49	Multifocal invasive ductal carcinoma	P	III	60	POS	POS	NEG (1+)
30	89	Invasive ductal carcinoma	P	III	60	NEG	NEG	POS (2+)
31	39	Invasiveductalcarcinoma NOS associated with ductal carcinoma *in situ*	P	II	80	NEG	NEG	-
32	61	Invasive ductal carcinoma	A	II	20	POS	POS	NEG
33	58	Invasive ductal carcinoma	A	II	32	POS	POS	POS (2+)
34	84	Invasive ductal carcinoma	P	III	35	NEG	NEG	POS (3+)
35	81	Invasive ductal carcinoma	A	II	40	POS	POS	NEG
36	58	Invasive ductal carcinoma	P	III	17	POS	POS	NEG
37	42	Invasive ductal carcinoma, associated with ductal carcinoma *in situ*	A	II	32	POS	POS	NEG (+2)
38	34	Invasive ductal carcinoma	P	III	45	NEG	NEG	-
39	50	Invasive ductal carcinoma	P	II	80	NEG	NEG	NEG (+1)
40	68	Invasive ductal carcinoma, associated with ductal carcinoma *in situ*	A	II	10	POS	POS	NEG
41	80	Invasive ductal carcinoma	A	II	58	POS	POS	NEG
42	54	Invasive ductal carcinoma	A	II	25	NEG	NEG	NEG
43	51	Invasive ductal carcinoma	A	II	20	POS	POS	NEG (2+)
44	47	Invasive ductal carcinoma	A	II	15	POS	POS	NEG (2+)
45	38	Invasive ductal carcinoma	A	II	14	POS	POS	NEG

LN = lymph node; G = histological grade; TS = tumor size; ER = estrogen receptor; PR = progesterone receptor; *ERBB2* = amplification of the *ERBB2* oncogene; P = presence of metastasis; A = absence of metastasis; NOS = not otherwise specified.

Gene expression analysis

Total RNA was isolated using the RNAeasy extraction kit (Qiagen, Hilden, Germany). Next, 1200 ng total RNA was reverse-transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems).

Primers (Table 2) were designed using the PrimerBlast software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/), using ideal conditions (Bustin et al., 2009) for reverse transcription-quantitative PCR. The *ACTB* and *B2M* genes were used as endogenous controls, selected using the TaqMan Human Endogenous Control Array microfluidic card (Applied Biosystems). Reaction efficiencies were determined using the Miner software (http://www.miner.ewindup.info/). The reverse transcription-quantitative PCR was performed using RealPlex equipment (Eppendorf, Hamburg, Germany). In this experiment, 15 ng cDNA, 2 pmol of each primer, and 5 µL Sybr Green PCR Master Mix (Applied Biosystems) were used in a total volume of 10 µL. The PCR program was as follows: 2 min at 50°C, 10 min at 95°C (initial denaturation), 15 s at 95°C, and 1 min at the annealing temperature, repeated 40 times (amplification). The PCR was evaluated by melting curve analysis following manufacturer instructions.

Table 2. Sequence of primers.

Gene	Direction	Sequence	Size (bp)	Th (°C)
PDIA3	F	AAGGCTCTGGAGAGGTTCCTGC	22	66.4
	R	GGCCCATCATTGCTCTCTGGGA	22	66.4
PDIA6	F	GGAGGTCAGTATGGTGTTCAGGGAT	25	66.2
	R	CTGCCACCTTGGTAATCTTCTGGTC	25	66.2

bp = base pair; Th = hybridization temperature; F = forward; R = reverse.

Statistical analysis

Gene expression analysis was performed using the $2^{-\Delta\Delta Ct}$ method relative to the selected controls, corrected for efficiency of amplification as previously described (Livak and Schmittgen, 2001). The χ^2 test was used to evaluate the homogeneity of the reference gene data. Further, we tested normality between groups. The Student *t*-test was used when the data showed a normal distribution; otherwise the nonparametric Mann-Whitney (U) test was applied. The significance of differences between the means of the expression between groups was analyzed using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA). The significance threshold was considered to be 5%.

RESULTS

Evaluation of *PDIA3* and *PDIA6* gene expression

ΔΔCt values were obtained for each sample tested for the *PDIA3* and *PDIA6* genes and normalized in relation to the reference genes. The normalized value was compared with the expression of a calibrator (1 stable sample chosen from the control group). Data are reported as the fold changes in gene expression ($2^{-\Delta\Delta Ct}$) relative to a calibrator sample (Livak and Schmittgen, 2001). Table 3 and Figures 1-4 summarize the relative expression levels observed.

Our results showed that the *PDIA3* gene contributes significantly to breast carcinogenesis compared with the *PDIA6* gene, showing differential expression between IDC and normal tissue, as well as in most tumor subgroups. *PDIA6* was differentially expressed in 2 tumors subgroups (LN metastasis and HR), suggesting a role in disease progression as well.

Table 3. Relative *PDIA3* and *PDIA6* expression.

Groups		*PDIA3*	N	*PDIA6*	N
IDC	FC	2.293	40	0.730	42
NT		0.923	9	0.677	9
	P	0.0009		0.784	
LN metastasis	FC	2.351	17	1.570	17
LN non-metastasis		1.575	18	0.808	19
	P	0.009		0.047	
Grade I+II	FC	1.977	30	1.067	30
Grade III		3.491	9	1.581	11
	P	0.024		0.157	
ER+/PR+	FC	1.570	17	1.195	30
ER-/PR-		0.08	19	0.585	8
	P	0.388		0.035	

IDC = invasive ductal carcinoma; NT = non-tumoral; LN = lymph node; ER+/PR+ = positive estrogen and progesterone receptors; ER-/PR- = negative estrogen and progesterone receptors; FC = fold change; N = sample number.

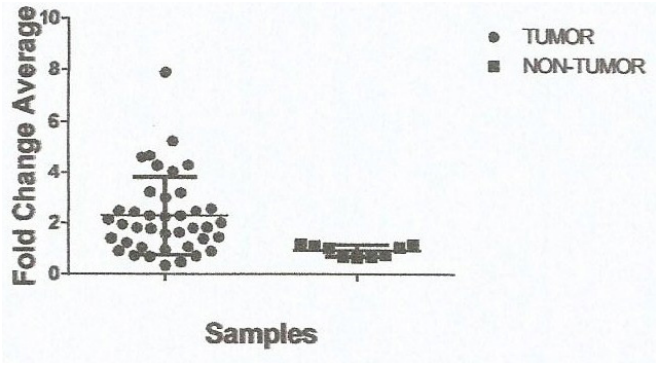

Figure 1. Fold change in *PDIA3* gene in tumor and non-tumor groups.

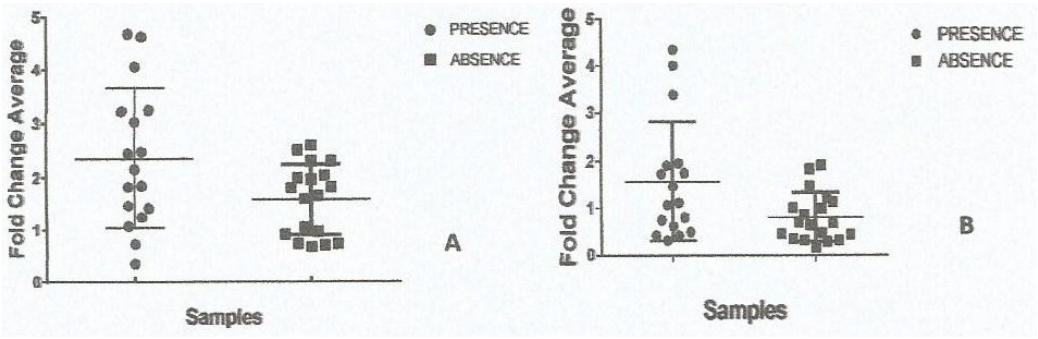

Figure 2. Fold change in *PDIA3* (**A**) and *PDIA6* (**B**) genes in subgroups with and without lymph node metastasis.

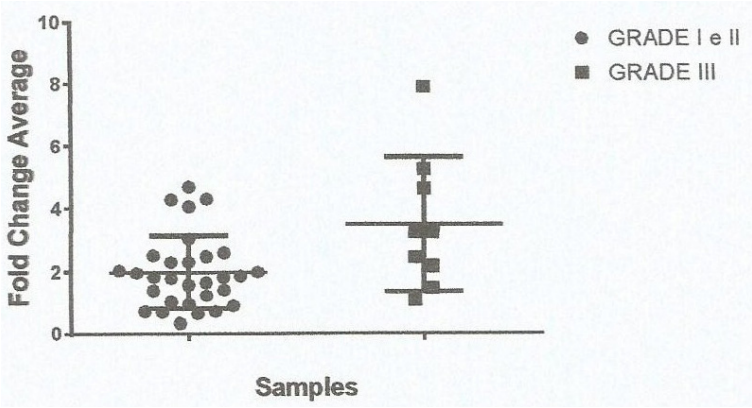

Figure 3. Fold change in *PDIA3* gene for subgroups of histological grade.

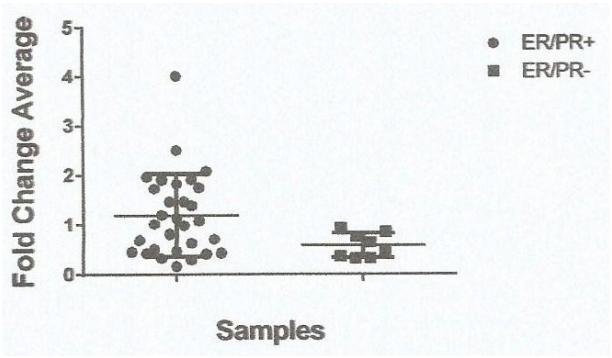

Figure 4. Fold change in *PDIA6* gene and hormone receptors. ER = estrogen receptor; PR = progesterone receptor.

DISCUSSION

PDIs are primarily found in the endoplasmic reticulum (involved in protein folding) and are linked to cancer invasion and migration (Lee et al., 2012). Pressinotti et al. (2009) studied nearly 1200 genes using cDNA microarray in microdissected cell populations of low- and high-risk prostate tumors and found an association between high expression of *PDIA3* and malignant stages. Our results in breast cancer agree with this observation, as overexpression of *PDIA3* was observed in samples from patients with lymph node metastasis and grade III tumors. In addition, *PDIA6* was overexpressed in samples from patients with lymph node metastasis, corroborating the hypothesis that higher expression of PDIs is linked to neoplastic progression (Bernardini et al., 2005). Khan et al. (2004), using a promyelocytic leukemia/retinoic acid receptor α fusion in promyelocytic leukemia, and demonstrated that the resulting aberrant protein is recruited to the endoplasmic reticulum by binding to PDI. This mechanism suggests a link between protein quality control and neoplastic transformation, and indicates that new alternative therapeutic approaches can be developed to promyelocytic leukemia and

other cancers by targeting these molecules (including PDI). Considering the large number of chromosome aberrations in cancer cells, PDIs have an important role in directing aberrant proteins to the endoplasmic reticulum. PDI3 was also associated with chemoprevention, as its expression was up-regulated in chemosensitive ovarian tumors when compared to chemoresistant tumors (Krynetskaia et al., 2009). Krynetskaia et al. (2009) reported an increased resistance of lung and renal carcinomas to several chemotherapeutics agents after knocking down PDIA3 (Ménoret et al., 2012). Finally, PDIs were detected in a proteomic search for proteins altered by resveratrol. This drug was found to decrease the association between PDIA3 and several proteins (including transformation/transcription domain-associated protein, a potent c-myc co-factor) and increase in others (including α, β, and γ catenin) (Xiong et al., 2012). *PDIA6* gene expression was lower in the group that was negative for hormonal receptors. It is well known that estrogen signaling is a contributing factor in the regulation of breast cell proliferation and that estrogen receptor targeting is a successful treatment for breast cancer. PDI shares homology with the estrogen-binding domain of estrogen receptor α and acts as a molecular chaperone required for estrogen receptor α-mediated gene regulation. As an estrogen-binding protein, PDI can modulate the level of estrogen released. Therefore, the level of PDI may be associated with the absence of hormone receptors, as observed in our study, which included a small sample size. Further studies including a larger number of samples should be conducted to confirm this hypothesis.

We observed higher expression of both genes in tumor samples compared with normal tissue, as well as in samples of patients with lymph node metastasis compared with samples of patients with no metastasis. *PDIA3* expression was also increased in grade III tumors, indicating that it was altered in 2 important prognostic parameters. Our results suggest that *PDI3* and *PDI6* can be used as prognostic markers of aggressiveness. In addition, new therapeutic approaches can be developed using PDI inhibitors. Further studies are necessary to determine their functional properties and interactions with substrates and small chemical ligands.

Conflicts of interest

The authors declare no conflict of interest.

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