

Glutathione and glutathione peroxidase expression in breast cancer: An immunohistochemical and molecular study

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Abstract. The use of prognostic markers for breast cancer allows therapeutic strategies to be defined more efficiently. The expression of glutathione (GSH) and glutathione peroxidase (GPX) in tumor cells has been evaluated as a predictor of prognosis and response to cytotoxic treatments. Its immun-expression was assessed in 63 women diagnosed with invasive ductal carcinoma in a retrospective study. The results showed that high GSH expression was associated with tumors negative for the estrogen receptor (ER) ($P < 0.05$), and GPX expression was associated with tumors negative for the progesterone receptor (PR) and patient mortality. Focusing on the 37 patients who received adjuvant chemotherapy/radiotherapy (Group I), high expression of GPX was associated with a high rate of patient mortality ($P < 0.05$). The 19 patients who received only adjuvant chemotherapy (Group II) showed high expression of GSH in relation to metastasis ($P < 0.05$). In addition, high levels of GPX expression were significantly associated with a shorter overall survival ($P < 0.05$). To confirm this, the expression of precursor genes of GSH [glutamate cysteine ligase (GCLC) and glutathione synthetase (GSS)] and the GPX gene was analyzed using quantitative PCR in cultured neoplastic mammary cells treated with doxorubicin. Doxorubicin treatment was able to eliminate tumor cells without alterations in the gene expression of GSS, but led to underexpression of the GCLC and GPX genes. Our results suggest that high levels

of GPX may be related to the development of resistance to chemotherapy in these tumors, response to treatment and the clinical course of the breast cancer patients.

Introduction

Breast cancer is the second most common type of cancer worldwide and the most common in women (1,2). In Brazil, it is estimated that 52,650 new cases of this tumor emerged in 2012 (2), whereas in the USA and western Europe, the estimate is for 500,000 new cases/year (3). Despite the high incidence, early diagnosis and introduction of more effective treatments make it possible to reduce mortality and improve the quality of life of patients with this disease (4).

Every prognostic marker employed for determining global patient survival is capable of generating important information related to the clinical behavior of mammary gland tumors. However, in most cases, they neither predict disease progression nor the response to chemotherapy (5,6). According to van't Veer *et al* (5), women with breast cancer at the same disease stage can have completely different responses to the same treatment.

Systemic treatment of breast cancer includes the use of cytotoxic, hormonal and immunotherapeutic agents. In general, these treatments are active at the beginning of therapy in 90% of primary breast cancers and 50% of metastases; however, after a variable period, the disease tends to progress (1,7).

Better predictive and prognostic markers are needed in clinical practice (8), since studies of clinical and molecular characteristics of tumors allow therapeutic strategies to be designed more efficiently and with less toxicity (1,4,9). In this context, the expression of antioxidant proteins in tumor cells has been assessed as a predictive and prognosis factor of the response to cytotoxic treatments (10-12).

Glutathione (GSH) is a tripeptide comprising of glycine, cysteine and glutamic acid residues (13). Its synthesis requires the participation of two enzymes consecutively. First glutamate cysteine ligase enzyme (GCLC) conjugates glutamic acid and cysteine, producing γ -glutamyl cysteine. This compound with

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Table I. Specifications and manufacturers of the antibodies used for the study.

Antibody	Clonality	Dilution	Buffer	Company
Primary antibody anti-ER	Monoclonal	1:100	Tris EDTA pH 9.0	Dako
Primary antibody anti-PR	Monoclonal	1:100	Tris EDTA pH 9.0	Biocare Medical
Primary antibody anti-HER-2/neu	Polyclonal	1:200	Tris EDTA pH 9.0	Dako
Primary antibody anti-p53	Monoclonal	1:500	Tris EDTA pH 9.0	Zymed
Primary antibody anti-Ki-67	Monoclonal	1:100	EDTA pH 8.0	Spring
Primary antibody anti-GSH	Monoclonal	1:100	Citrate pH 6.0	Millipore
Primary antibody anti-GPX	Polyclonal	1:1,200	Citrate pH 6.0	Abcam

ER, estrogen receptor; PR, progesterone receptor; HER-2/neu, human epidermal growth factor receptor-2; GSH, glutathione; GPX, glutathione peroxidase.

its sulfhydryl group (SH) is responsible for the antioxidant activity of GSH. The second step is the binding of γ -glutamyl cysteine to glycine catalyzed by glutathione synthetase (GSS), producing the tripeptide γ -glutamyl cysteine glycine, known as GSH (14,15).

Glutathione peroxidase (GPX) contains selenium at a catalytic site and uses GSH as an electron donor for the reduction of H_2O_2 to H_2O , converting itself to its oxidized form, glutathione disulfide (GSSG) (16,17). GSH is considered one of the most important agents of the antioxidant defense system of the cell. Because it is conjugated to GPX, GSH plays a role in the removal of xenobiotic and carcinogenic agents (18).

High levels of GSH and/or GPX increase the antioxidant capacity, as observed in many tumor cells. Although the mechanism and the consequences of these changes are not well characterized (17), recent research has demonstrated that an increase in antioxidants in neoplastic mammary tissue provides certain advantages to these cells when compared to healthy tissue. The presence of these enzymes in neoplastic cells may represent a low-grade response to treatments that cause oxidative damage, such as radiotherapy and various chemotherapeutics (19,20).

These previous findings show the importance of GSH and GPX expression in tumor cells, making them potential predictive and prognostic markers for breast cancer. Thus, the aim of this study was to determine the prognostic and predictive values of GSH and GPX expression and their relationship to several clinicopathological parameters, patient overall survival, and response to breast cancer treatment.

Materials and methods

Immunohistochemical study

Sample characterization. In a retrospective study, tumor fragments were selected from 63 women aged 30 to 99 years (mean 58 years) attending the Obstetrics and Gynecology service, Hospital de Base, Faculty of Medicine of São José do Rio Preto from 2000 to 2005. The clinicopathological parameters of the patients were obtained from the service medical records.

Patients with no other malignancy who were subjected to mastectomy/quadrantectomy but not to neoadjuvant chemotherapy/radiotherapy were included in the study. All the patients had been diagnosed with invasive ductal carcinoma.

A panel of classic prognostic markers were evaluated: estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor-2 (HER-2/neu), p53 tumor-suppressor gene and the cell proliferation marker, Ki-67. Furthermore, the patients were divided in to 4 subgroups according to the immunohistochemistry of the following markers: luminal A (ER⁺PR⁺HER-2/neu⁻), luminal B (ER⁺PR⁺HER-2/neu⁺), HER-2/neu⁺, and triple-negative (ER⁻PR⁻HER-2/neu⁻).

The parameters for histopathological evaluation were those recommended by the International Union Against Cancer (UICC) TNM classification of 1989. Among the entire group, 37 patients (59%) who had received the same treatment including surgery and adjuvant chemotherapy/radiotherapy [5-fluorouracil/epirubicin/cyclophosphamide (FEC)] were designated as group I; 19 patients (30%) who had received only adjuvant chemotherapy were designated as group II and 7 patients (11%) who had received no treatment after surgery were designated as group III. The patients underwent clinical evaluation every 3-4 months. Local tumor recurrence and metastasis in such cases were carefully monitored, as well as the date and cause of death in order to record the survival time of each patient.

Patient follow-up ranged from 120 to 2,704 days, with a median of 1,473 days. For analysis of survival curves, the patients were divided into higher and lower enzyme expression groups using a cut-off value established by the receiver operating characteristic curve (ROC). Only patients who died of tumor disease were included in the final analysis. Patients with an incomplete follow-up were excluded. The study was approved by the Research Ethics Committee of the Faculty of Medicine of São José do Rio Preto (protocol no. 25372009).

Immunohistochemical technique. For administration of the technique, blocks of paraffin-embedded tumor fragments were cut to provide 3- μ m sections. The sections were prepared on silanized slides and subsequently deparaffinized, rehydrated through graded alcohol and incubated with 3% hydrogen peroxidase for 30 min to block endogenous peroxidase activity. Antigen retrieval was carried out in a Pan Steam (ARNO, São Paulo, Brazil) at 95°C with buffer for 35 min for each specific antibody (Table I). After cooling, the slides were covered with bovine serum albumin (BSA) solution for 30 min and incubated at 4°C overnight with the antibodies described in Table I.

Subsequently they were washed with phosphate-buffered saline (PBS) for 15 min and incubated with the Easy Path kit (Erviagas, São Paulo, Brazil), which consisted of the secondary antibody biotinylated anti-mouse, rabbit, and goat immunoglobulins for 1 h and the streptavidin-peroxidase complex for 30 min, followed by washing with PBS for 15 min. We applied 0.5% 3,3'-diaminobenzidine tetrahydrochloride (DAB; Signet Laboratories, Dedham, MA, USA) to the slides for 2-5 min at 20-22°C. The slides were counterstained with Harris's hematoxylin for 40 sec. Negative controls were obtained by omitting the primary antibody, and liver or prostate tissue served as an internal positive control in every assay.

Evaluation of immunohistochemical staining. To assess the immunoexpression of GSH and GPX, multiple fields were examined on each slide, particularly demarcated areas with distinct brown staining. The slides were photographed and the enzymes were quantified by AxioVision software and by observation through a x40 lens of a Zeiss Axioskop2 microscope.

For each sample, three regions of tumor tissue were selected and 20 spots of the tumor cells were marked within each region, thereby analyzing 60 different spots of every sample to average the relative intensity of immunoreactivity. The values were obtained as arbitrary units (au), and the mean optical density (MOD) indicated the specific staining intensity in the immunoreactive areas.

The staining of HER-2/neu was considered by counting the number of positively stained cells in the membrane and was expressed as a percentage of the total tumor cells. According to ASCO/CAP guidelines (21) the following categories were defined: 0, no immunostaining; 1+, weak or incomplete staining of the membrane in any proportion of tumor cells; 2+, complete membrane staining or weak staining in 10% of the tumor cells stained; 3+, uniform and intense staining of the membrane in more than 30% of tumor cells. Tumors scored with 0 or 1+ were considered negative and those with scores 2+ or 3+ were considered positive. Tumors were categorized into two groups in relation to cellular proliferation according to the staining of Ki-67 based on the count of the number of positively stained cells and expressed as a percentage of the total: 0 or 1, no cellular proliferation; 2 or 3, the presence of cellular proliferation. The same parameter was used in the classification of the ER, PR and p53 tumor-suppressor gene: 0 or 1, no staining; 2, 3 or 4, presence of staining.

Statistical analysis. The patients were separated into groups according to the clinicopathological variables. The averages of the densitometric analysis referring to the densitometry results and the quantification of the different breast tumor groups were compared by Student's t-test or ANOVA, followed by the Bonferroni test. The values are expressed as means \pm SEM.

The cut-off for the risk of death was determined by ROC. Survival curves were plotted by the Kaplan-Meier method, and the differences between the curves were evaluated by a log-rank test and hazard function. Multivariate logistic regression was performed to evaluate the factors that influenced death. For all tests, $P < 0.05$ was considered to indicate a statistically significant result. All analyses were performed using GraphPad Prism 4 and StatsDirect software.

Molecular study

Sample characterization. Twelve core biopsies or tumor fragments > 2 cm were collected by surgery from the breast cancer patients who were diagnosed with ductal carcinoma, who had not undergone chemotherapy and/or radiation therapy prior to the point of collection. The tumor fragments were washed in PBS containing 1% penicillin/streptomycin, cut into microfragments with a scalpel and incubated at 37°C in 5% CO₂ with RPMI-1640 supplemented with 20% BSA, 1% penicillin/streptomycin and 1% L-glutamine. The cells were cultivated until they reached 80% confluence and were subjected to immunocytochemistry using the primary antibodies anti-cytokeratin, anti-vimentin and anti-calponin to confirm epithelial origin.

Treatment of the cells in vitro. Cells were divided into two groups: control (no treatment), and cells treated with 0.2 mg routine chemotherapy (doxorubicin) for 24 h. At the end of the treatment, cell viability was verified by cell counting in a Neubauer chamber (LaborOptik, Bad Homburg, Hessen, Germany) with trypan blue dye (0.4%).

Quantitative PCR. Total RNA was extracted from the cell culture with TRIzol (Invitrogen Life Technologies, São Paulo, Brazil) as recommended by the laboratory and the total RNA from each sample was reverse transcribed to complementary DNA (cDNA) using a High Capacity cDNA kit (Applied Biosystems, Foster City, CA, USA).

Quantitative real-time polymerase chain reactions were performed in triplicate using StepOnePlus™ System (Applied Biosystems). Briefly, the reactions were performed in a 20 μ l volume with 10 μ l of Power SYBR®-Green PCR Master Mix (Applied Biosystems), 250 nM of each primer, and 10 ng of cDNA. PCR conditions consisted of 50°C for 2 min, 95°C for 10 min, followed by 35 cycles of 95°C for 15 sec and 60°C for 1 min. Following PCR, dissociation curve analysis was performed to confirm the desired single gene product: one cycle of 95°C for 15 sec, 60°C for 1 min, and 95°C for 15 sec was carried out.

Each transcript level was normalized by division with the expression values of *HPRT1* used as an internal control. Gene expression stability was analyzed by geNorm software. The transcript level was calculated using the 2^{-Ct} method (22). The Ct was the difference between the threshold cycles of the target and the internal control and Ct was the difference between the average Ct of the sample and the average Ct of the calibrator sample. The fold difference (relative abundance) was calculated using the 2^{- $\Delta\Delta$ CT} formula and plotted as mean \pm SD of the triplicate reactions. At least 3-fold differences were considered significant.

A negative control was included in each reaction, and one sample was chosen for reaction calibration. Experiments were repeated when the coefficient of variation was higher than 5%. After each reaction, the products were analyzed on 2% agarose gel stained with ethidium bromide.

The gene was searched, selected in PubMed (<http://www.ncbi.nlm.nih.gov/entrez>), and synthesized from human messenger RNA already sequenced and confirmed. Its design was made using the program PRIMER 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The *HPRT1* gene

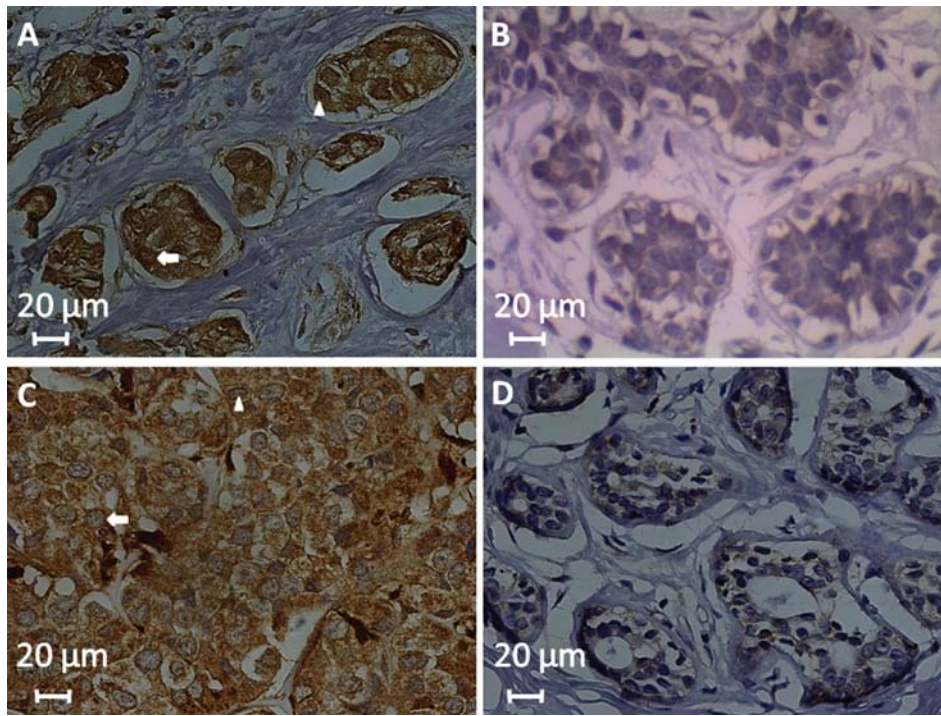


Figure 1. Images of immunohistochemical staining for GSH and GPX (original magnification x400). (A) Immunostaining for GSH in breast cancer. Immunostaining was evident in the cytoplasm (arrow) and nucleus (arrowhead) of neoplastic cells. (B) Immunostaining for GSH in normal breast tissue of patients, with weak immunoreactivity in the cytoplasm of cells. (C) Immunostaining for GPX in breast cancer. Immunostaining was evident in the cytoplasm (arrow) and was focally present in the nucleus (arrowhead) of neoplastic cells. (D) Immunostaining for GPX in normal breast tissue, with weak immunoreactivity in the cytoplasm of cells.

was used as an endogenous control. Primers used for amplification include: GSS forward, 5'-TGCTAAAGCCCCAGAGAG AG-3' and reverse, 5'-AGCAGGCAATTCTCAAAAGG-3'; GCLC forward, 5'-GCACAACGTTCTCAAGTG-3' and reverse, 5'-TGGTTTGGGTTTGTCTTTC-3'; GPX forward, 5'-ATGGCGCAATTGTCCAAG-3' and reverse, 5'-CTGGCC TCCCCTTACAGTG-3'; HPRT1 forward, 5'-TTATAGTCAAG GGCATATCC-3' and reverse, 5'-AGCTTGCTGGTGAAA AGGAC-3'.

The genes were classified as underexpressed (samples with less than $-1 \log_3$ measurement) and overexpressed (samples with quantification greater than 1 \log_3).

Results

All tumors were histological grade II invasive ductal carcinoma, with a preponderance of tumors of clinical stage II (76.2%), in agreement with the TNM (tumor, node and metastasis) classification.

Immunohistochemical procedure. Immunostaining of GSH and GPX was evident in the cytoplasm and focally in the nuclei of the neoplastic cells, whereas the stroma showed no reactivity (Fig. 1).

Regarding the classical markers, GSH expression was correlated with tumor ER-positivity, and GPX correlated with tumor PR-negativity ($P=0.03$) (Table II). Only 13 (20.6%) of the patients had tumors with luminal expression A (ER⁺PR⁺HER-2/neu⁻), whereas 22 (35%) of the patients had tumors with luminal expression B (ER⁺PR⁺HER-2/neu⁺);

41 (65%) patients were HER-2/neu⁺ independently of the hormone receptors, and 9 (14.2%) patients had a triple-negative (ER⁻PR⁻HER-2/neu⁻) phenotype. Nevertheless, there was no correlation between GSH or GPX expression and the different clinical groups ($P>0.05$).

Immunohistochemical expression of GSH and GPX was assessed in relation to the clinical course of the patients, taking into account local recurrence, metastasis and/or rate of mortality (Table II). High expression of GPX was significantly correlated with a high rate of patient mortality ($P=0.03$) (Table III). Group I showed higher GPX expression in patients that succumbed to disease ($P=0.02$) and Group II showed higher GSH expression in patients with metastasis ($P=0.03$) (Table III).

ROC was calculated to explore the performance, and the threshold values for GSH and GPX expression were used to predict the risk of mortality in breast cancer patients. The ROC graph indicated calculations for sensitivity/specificity of the patients. Thus, the best cut-off value for GSH to discriminate high risk of death in all patients was MOD = 170 au (sensitivity = 30% and specificity = 88%). For GPX the best cut-off in the total population was: MOD = 200 au (sensitivity = 60% and specificity = 67%).

Multivariate logistic regression showed that clinical stages III or IV and metastasis were associated with an increased risk of mortality in the breast cancer patients ($P<0.05$) (Table IV). In addition, in patients >50 years of age, high levels of cell proliferation (Ki-67 positivity), local recurrence and higher GPX expression showed a significant trend towards an increased risk of mortality ($P=0.05-0.06$) (Table IV).

Table II. Mean expression of GSH and GPX and its correlation with clinicopathological characteristics of the breast cancer patients.

Clinicopathological factors	No. of patients n (%)	MOD of GSH	MOD of GPX
Patient age (years)			
≥50	16 (25.4)	187.8±3.517	192.6±2.234
<50	47 (75.6)	184.3±3.253	194.1±4.240
P-value		0.54	0.74
Smoker			
Yes	11 (17.5)	188.0±5.177	196.7±5.951
No	52 (82.5)	184.3±2.887	192.2±2.041
P-value		0.58	0.38
Hormone therapy			
Yes	11 (17.5)	179.7±4.918	195.9±4.863
No	52 (82.5)	186.3±2.842	192.3±2.164
P-value		0.32	0.49
Lymph node involvement			
Positive	13 (20.6)	186.2±4.546	193.7±4.464
Negative	50 (79.4)	184.7±2.992	192.5±2.249
P-value		0.81	0.80
Histological grade			
I	3 (4.7)	175.5±21.50	191.0±5.000
II	50 (79.4)	184.0±2.837	192.2±2.301
III	10 (15.9)	190.9±5.360	194.9±3.650
P-value		0.30	0.51
Clinical stage			
I	4 (6.3)	175.3±8.686	204.0±13.320
II	48 (76.2)	185.4±3.112	192.6±2.207
III	9 (14.3)	185.4±5.373	188.9±5.832
IV	2 (3.2)	195.5±0.500	199.5±0.500
P-value		0.75	0.51
Median tumor diameter (cm)			
>3 cm	13 (20.6)	186.9±4.190	196.8±3.211
≤3 cm	50 (79.4)	184.5±3.022	192.0±2.329
P-value		0.69	0.32
Estrogen receptor			
Positive	46 (73)	188.1±2.089	191.4±2.484
Negative	17 (27)	176.4±7.285	197.2±2.671
P-value		0.03 ^a	0.19
Progesterone receptor			
Positive	37 (58.7)	188.3±2.330	189.4±2.870
Negative	26 (41.3)	180.2±5.106	198.0±2.160
P-value		0.11	0.03 ^a
HER-2/neu			
Positive	41 (65)	188.1±2.199	193.1±2.447
Negative	22 (35)	178.8±6.180	192.6±3.389
P-value		0.08	0.90
p53			
Positive	44 (69.8)	184.4±3.353	194.8±2.322
Negative	19 (30.2)	186.4±3.747	188.7±3.606
P-value		0.73	0.16
Ki-67 cell proliferation index			
High	30 (47.6)	185.3±4.526	194.2±2.878
Low	33 (52.4)	183.9±2.680	191.8±2.724
P-value		0.78	0.54

^aSignificant value as determined by Student's t-test. GSH, expression of glutathione; GPX, glutathione peroxidase; MOD, mean optical density; HER-2/neu, human epidermal growth factor receptor-2.

Table III. Association of GSH and GPX expression and treatment and clinical outcome of the breast cancer patients.

Group	Clinical outcome	No. of patients n (%)	MOD of GSH	MOD of GPX
Total Group	Local recurrence			
	Yes	6 (9.5)	179.7±3.412	196.3±9.315
	No	57 (90.5)	185.5±2.778	192.3±2.004
	P-value		0.50	0.56
	Metastasis			
	Yes	23 (36.5)	186.8±3.030	196.5±3.379
	No	40 (63.5)	183.9±3.617	190.9±2.389
	P-value		0.59	0.17
	Death due to disease			
Yes	20 (32)	183.5±3.549	199.1±2.867	
No	43 (68)	185.7±3.354	190.1±2.459	
P-value		0.68	0.03 ^a	
Group I	Local recurrence			
	Yes	5 (13.5)	180.4±4.082	203.2±6.111
	No	32 (86.5)	184.1±4.326	192.3±2.705
	P-value		0.74	0.14
	Metastasis			
	Yes	14 (38)	181.6±4.223	196.3±4.908
	No	23 (62)	184.8±5.556	192.3±2.808
	P-value		0.68	0.44
	Death due to disease			
Yes	13 (35)	179.3±4.033	201.6±3.362	
No	24 (65)	185.9±5.393	189.5±3.163	
P-value		0.41	0.02 ^a	
Group II	Local recurrence			
	Yes	1 (5)	176.0±0.000	158.0±0.000
	No	18 (95)	185.1±4.195	193.1±4.009
	P-value		-	-
	Metastasis			
	Yes	6 (31.5)	196.8±3.311	200.2±5.382
	No	13 (68.5)	179.9±4.954	187.2±5.384
	P-value		0.03 ^a	0.15
	Death due to disease			
Yes	5 (26)	189.2±8.789	190.0±5.030	
No	14 (74)	183.0±4.555	191.7±5.532	
P-value		0.51	0.86	
Group III	Local recurrence			
	Yes	0 (0)	-	-
	No	7 (100)	193.3±2.168	195.3±4.412
	P-value		-	-
	Metastasis			
	Yes	3 (43)	191.0±2.309	195.0±11.060
	No	4 (57)	195.0±3.391	195.5±2.630
	P-value		0.41	0.96
	Death due to disease			
Yes	2 (28.5)	196.0±1.000	205.0±12.00	
No	5 (71.5)	192.2±2.956	191.4±3.641	
P-value		0.47	0.18	

^aSignificant value as determined by Student's t-test. GSH, expression of glutathione; GPX, glutathione peroxidase; MOD, mean optical density.

Table IV. Results of the multivariate logistic regression analysis.

Variables	OR	95% CI	P-value
Age \geq 50 years	918.55	0.99-851,872.18	0.05 ^a
Smoker	2.30	0.00-6,851.12	0.83
Large tumor size (>3 cm)	1.32	0.05-33.00	0.86
Lymph node involvement	31.62	0.39-2517.38	0.12
Staging III or IV	460.42	1.33-15,8371.26	0.03 ^b
Histological grade III	0.01	0.00-86.94	0.32
ER positivity	14.97	0.42-525.45	0.13
PR positivity	0.007	0.00-1.73	0.07
HER-2/neu negativity	11.26	0.33-378.73	0.17
Cell proliferation (Ki-67 positivity)	66.57	0.75-5,905.44	0.06 ^a
p53 positivity	0.018	0.00-1.56	0.07
Chemotherapy	15.22	0.05-4,337.16	0.34
Radiotherapy	0.12	0.00-5.31	0.27
Metastasis	1,397.97	7.90-247,365.34	0.006 ^b
Local recurrence	57,817.26	0.98-3,390,813,161.68	0.05 ^a
High GSH expression	0.16	0.001-14.42	0.43
High GPX expression	117.26	0.68-19,969.60	0.06 ^a

^aTrend toward significance; ^bstatistically significant value. OR, odds ratio; CI, confidence interval; ER, estrogen receptor; PR, progesterone receptor; HER-2/neu, human epidermal growth factor receptor-2; GSH, glutathione; GPX, glutathione peroxidase.

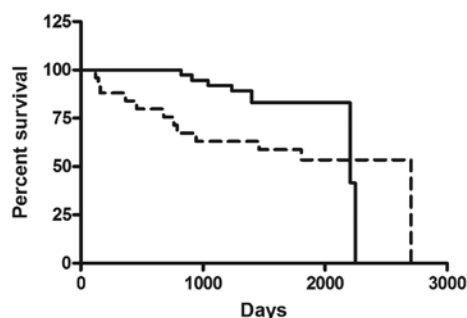


Figure 2. Overall survival of the patients with high (dotted line) and low (continuous line) GPX expression. (Cut-off selected was MOD = 200 au; P=0.03; OR, 2.63; 95% CI, 1.05-7.01). OR, odds ratio; CI, confidence interval.

Patient follow-up ranged from 144 days (0.4 years) to 2,704 days (7.4 years) with a median of 1,542 days (4.2 years). For analysis of the survival curves, the patients were divided into higher and lower enzyme expression groups using a cut-off value established from the ROC curve. There was no correlation between GSH expression and overall survival in groups I, II and III ($P>0.05$). High GPX expression was correlated with a lower overall survival rate in the entire group ($P=0.03$) (Fig. 2).

Quantitative PCR. Samples collected for the *in vitro* study were from patients diagnosed with invasive ductal carcinoma; 3 patients with histological grade I (25%), 6 with grade II (50%) and 3 with grade III (25%). Only one patient had local recurrence and 1 patient died of metastasis. Following cell culture, the epithelial origin was confirmed by immunocytochemistry,

and GCLC, GSS and GPX gene expression was evaluated after treatment with doxorubicin.

The GCLC gene, responsible for the first step in the synthesis of GSH, was underexpressed in 7 (58.3%) of the culture samples following treatment with chemotherapy. This gene was overexpressed in one (8.3%) of the samples, and 4 (33.3%) samples failed to reach the minimum level of expression in the log₃ range required to be considered indistinguishable from the controls (Fig. 3). Only in 1 (8.3%) culture sample treated with chemotherapy was the GSS gene overexpressed. Eleven (91.6%) samples fail to reach the minimum level of expression in the log₃ range required to be considered indistinguishable from the controls (Fig. 4).

The GPX gene was underexpressed in 6 (50%) of the culture samples treated with chemotherapy. Only one (8.3%) sample showed overexpression and 5 (41.6%) samples showed no significant difference in expression compared to the control cells (Fig. 5).

Discussion

Studies concerning the association between immunohistochemical expression of GSH, as well as GPX, the clinicopathological parameters of breast cancer patients are sparse. The majority of previous studies have used biochemical methods to quantify the activity of these proteins, comparing patients with breast cancer and healthy control patients (19,20,23,24).

In this study, tumors considered ER-positive presented higher expression of the GSH protein when compared to those that were ER-negative. In addition, PR-negative tumors presented higher expression of GPX compared to those that were PR-positive. According to Fernandes *et al* (25), indi-

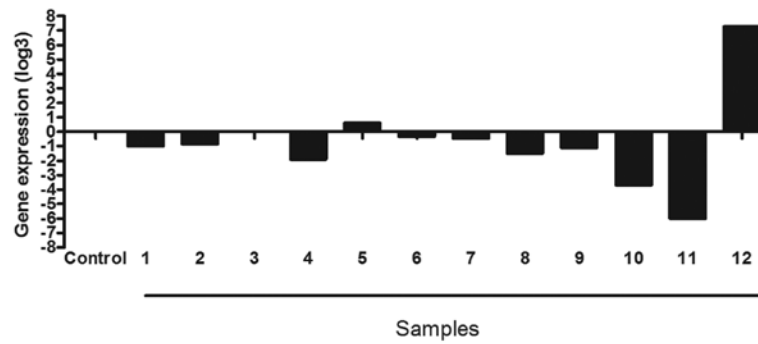


Figure 3. Quantitative gene expression of GCLC in the breast cancer cells. Quantitative gene expression in cells exposed to doxorubicin in relation to the pool of unexposed cells (control). Value of gene expression in log3.

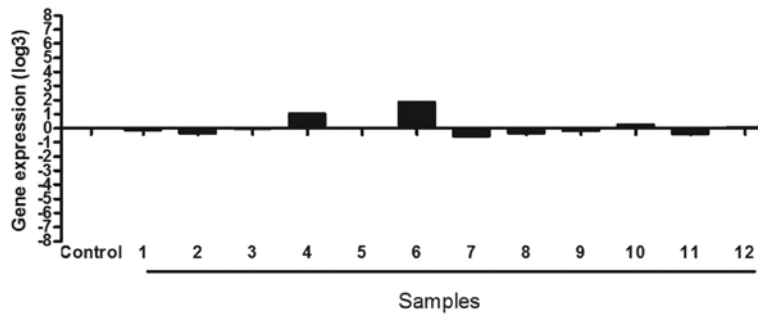


Figure 4. Quantitative gene expression of GSS in the breast cancer cells. Quantitative gene expression in cells exposed to doxorubicin in relation to the pool of unexposed cells (control). Value of gene expression in log3.

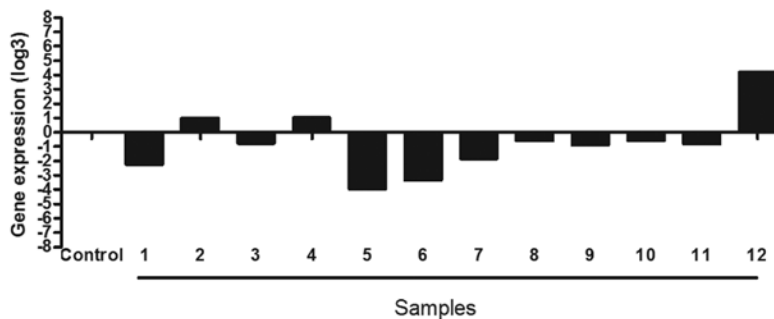


Figure 5. Quantitative gene expression of GPX in the breast cancer cells. Quantitative gene expression in cells exposed to doxorubicin in relation to the pool of unexposed cells (control). Value of gene expression in log3.

vidual analyses of the hormonal receptors are not conclusive. With the combined evaluation of hormonal receptors and the HER-2/neu protein, mammary carcinomas can be grouped into 4 main subtypes that provide important information related to the degree of malignancy and therapeutic response to certain drugs (25,26). Triple-negative carcinomas are considered more aggressive than the luminal A or B subtypes, or even those overexpressing HER-2/neu. There was no statistical correlation between GSH and GPX expression and these carcinoma subtypes.

The immunohistochemical expression of GSH and GPX was also related to the clinical progression of the breast cancer patients. Patients that received only adjuvant chemotherapy (Group II) and had metastases showed higher GSH expres-

sion. Ballatorri *et al* (17) demonstrated that a high level of GSH increased the antioxidant capacity of neoplastic cells, making them more resistant to chemotherapy. Based on this, high expression of GSH can be characterized as an indicator of low response to chemotherapy in those analyzed patients in this study, and may have contributed to the development of metastasis.

High expression of GPX was associated with a high rate of mortality, upon univariate and multivariate analyses. In addition, patients with lower GPX expression had a lower overall survival time. The association between high GPX expression and mortality remained significant when evaluated only in the patients restricted to the group of 37 women treated with adjuvant chemotherapy and radiotherapy (Group I). The

correlations found in this study may be explained on the basis of enzymatic reactions catalyzed by GPX. Some chemotherapeutic and radiotherapeutic protocols potentially increase the already existent oxidative stress in neoplastic processes, causing damage to DNA and cell death (27). High levels of GPX are known to correlate with cellular responses to oxidative stress. In this way, cytotoxic treatments can reduce intracellular GPX concentrations, based on the high concentration of GSSG in the environment, or, on the other hand, cytotoxic treatments can induce GPX expression as a cellular response to a high concentration of H₂O₂ (17,20,28,29). A high level of GPX helps prevent oxidative damage that would otherwise lead to tumor cell death due to the applied treatments (18,19).

In vitro studies corroborate the participation of GSH and GPX in cellular resistance to treatments. In this study, there was no significant expression of GSS after treatment with doxorubicin, whereas the GCLC and GPX genes were under-expressed in 58.8 and 50% of the samples, respectively.

Many studies have demonstrated that alterations in expression of genes responsible for the synthesis of GSH or GPX usually occur after *in vitro* treatment with doxorubicin or similar drugs. It is suggested that a large production of reactive oxygen species (ROS) following treatment with doxorubicin is responsible for the cytotoxicity noted in neoplastic cells, and as a consequence, these cells overexpress genes responsible for the synthesis of antioxidants, such as GSH and GPX, making them more resistant to oxidative damages (30). Ozkan and Fiskin (31) found that the application of epirubicin (analogous to the structure of doxorubicin) in mammary neoplastic cells reduced GSH and GPX activity within 24 h of *in vitro* exposure. More unlikely, a study by Ilvsova *et al* (32) showed that the total GSH concentration in the blood of breast cancer patients increased significantly 24 h after doxorubicin administration.

Han *et al* (33), using the MCF-7 breast cancer cell line, found a high sensitivity to doxorubicin when the levels of GSH decreased. Vibet *et al* (34) using docosahexaenoic acid, known to increase the oxidative mechanism of chemotherapeutics in mammary neoplastic cells when combined with doxorubicin, showed that a high concentration of ROS, due to this treatment, inhibited GPX activity. The same finding was noted in animal models of breast cancer. In this way, Sun *et al* (35) observed that high H₂O₂ concentrations increased the sensitivity of tumor cells *in vitro* and *in vivo* not only to doxorubicin, but to ionizing radiation. In contrast, Di *et al* (36) demonstrated that GSH overexpression did not prevent apoptosis in tumor cells after treatment with doxorubicin, suggesting that the cytotoxicity of this drug is not directly correlated with ROS production.

In conclusion, GPX was highly expressed in breast cancer cells of patients with a worse clinical outcome and reduced overall survival who underwent chemotherapy and radiotherapy. Thus, it is suggested that GPX has an important role in the progression of this disease, especially as a possible prognostic marker for these patients. In addition, there was a relationship between application of the chemotherapeutic drug doxorubicin and reduced expression of the GPX gene, making it a candidate marker for predicting therapeutic responses in breast cancer cases, yet this needs to be confirmed in larger studies.

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