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ORIGINAL ARTICLE

Glutathione S-transferase Pi expression in invasive breast cancer and its relation with the clinical outcome

R.L. Franco[1], N.G.M. Schenka[2], A.A. Schenka[2], L.F. Rezende[1], M.S.C. Gurgel[1]

[1]Department of Obstetrics and Gynecology, [2]Department of Pharmacology, Universidade Estadual de Campinas, Campinas, Brazil

Summary

Purpose: *Glutathione S-transferase (GST) is a cytosolic enzymatic system involved in cellular detoxifying process. In vitro studies have shown that the presence of this enzymatic system in breast carcinoma cells can accelerate the elimination of drugs commonly used in chemotherapy, thereby decreasing its efficacy. The aim of the present study was to evaluate the association between GST Pi expression by breast carcinoma cells and disease-free and overall survival.*

Methods: *Ninety-five female patients with invasive breast carcinoma submitted to surgical treatment and adjuvant chemotherapy from January, 1995 to June, 1997 and followed until August, 2006 were evaluated. The expression of GST Pi in breast carcinoma cells, determined by immunohistochemistry, was correlated with several clinical and pathological parameters of prognostic significance.*

Results: *There were 36 (37.9%) GST Pi-positive cases. GST Pi immunoexpression was not significantly correlated with patient's age, histological tumor type, clinical stage, hormone receptor status and survival. On the other hand, GST Pi positivity showed a significant correlation with a lower histological grade/C-erb-B2 negative breast carcinoma phenotype.*

Conclusion: *The findings suggest that GST Pi expression does not constitute a satisfactory prognostic factor in breast cancer.*

Key words: breast cancer, glutathione S-transferase, immunohistochemistry, prognosis, survival

Introduction

Breast cancer is the second most common neoplasia affecting women in Brazil. The incidence for 2011 is expected to be 49,240 new cases [1]. In the United States the estimate for 2008 is 182,460 new cases with 40,480 deaths due to this disease [2]. Despite the high incidence, mortality is decreasing due to screening with mammography and advances in treatment [3]. Nowadays, the treatment is performed under a multidisciplinary approach and combines different strategies including surgery, radiation therapy, hormone therapy and chemotherapy.

Patients with breast cancer have heterogeneous response to drugs used in chemotherapy [4] and this is partially related to certain biological features of the tumor, most of which are yet to be established [5]. The expression of several molecules has been tested as possible prognostic/predictive factor in breast cancer. Recently, it has been suggested that the GST system may be one of the most promising molecules in this respect [6].

The human GST is a multigene, isoenzyme family. Cytosolic GST isoenzymes can be classified by their substrate specificities, isoelectric points and amino acid sequence homologies into major classes termed Alpha, Mu, Pi, Theta and Zeta, which are encoded by a superfamily of genes located at different loci [7, 8]. These enzymes are responsible for blocking the deleterious action of toxins over cellular DNA [9], by catalyzing the conjugation of electrophilic reactive molecules with glutathione. By acting so, GST enhances toxin metabolism and excretion, therefore preventing cellular DNA mutation [10].

Since the GST system can increase cellular detoxification, a hypothesis was proposed, firstly through *in vitro* studies, in which the presence of this system in breast cancer cells could accelerate the conjugation

Correspondence to: Ricardo Laier Franco, MD. Department of Obstetrics and Gynecology, Universidade Estadual de Campinas, Rua Dr. Antonio Hossri 695, Cidade Universitaria, Barão Geraldo, Campinas, SP, 37701316, Brazil. Tel: +55 35 37153281, Fax: +55 35 37216343, E-mail: laier.franco@gmail.com

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and elimination of the chemotherapy drugs and consequently decreasing their efficacy [11, 12]. These *in vitro* studies demonstrated that breast cancer cells expressing GST were more resilient to anti-neoplastic drugs such as cyclophosphamide [11] and doxorubicin [12], commonly used in breast cancer adjuvant treatment.

To the best of our knowledge, only two studies have evaluated the expression of GST Pi and disease-free survival in breast carcinoma [6,13]. Of notice, not only are they based on short follow-up, but also, their results are contradictory. Huang et al. [6], studying 116 cases of breast cancer, demonstrated that patients with GST Pi positive cancers had a significantly worse disease-free survival. On the other hand, Peters et al. [13], evaluating 139 cases of breast cancer, showed no difference in disease-free survival between patients with GST Pi positive and negative cancers.

Considering that GST Pi expression is demonstrated by immunohistochemistry in about 50% of breast carcinomas [14] and that it may represent a useful prognostic and/or predictive factor, it could be of great value to solve the controversy concerning the actions of this enzyme in breast cancer management and outcome.

The present study has evaluated the expression of GST Pi in breast cancer cells by immunohistochemistry and its correlation with several clinical and pathological variables of prognostic relevance (including overall and disease-free survival) in a longer follow-up period than former studies.

Methods

Case selection

The medical records of 95 women diagnosed with invasive breast carcinoma, who underwent surgical treatment at the Universidade Estadual de Campinas (UNICAMP), Brazil, between January 1995 and June 1997 were evaluated. Only patients ranging from 18 to 70 years old, without any other malignancies, and subjected to mastectomy/quadrantectomy with free surgical margins, axillary dissection and adjuvant chemotherapy/radiotherapy were included. Moreover, the corresponding paraffin blocks had to be available for evaluation. Patients with distant metastasis at diagnosis, submitted to neoadjuvant chemotherapy or who received incomplete adjuvant chemotherapy, radiotherapy or hormone therapy were excluded. Patients with an incomplete follow-up were also excluded.

Follow-up

The patients' follow-up was performed according to the local Institutional protocol which is summarized as follows: clinical examination was carried out every 3 months post-surgery in the first 2 years, twice a year between the 3rd and 5th year and annually thereafter. At least once a year, patients were subjected to mammography, chest x-ray, abdominal ultrasonography and bone scintigraphy.

Immunohistochemical technique

All H&E-stained and immunostained slides were reviewed by two experts in breast pathology and immunohistochemistry, the tumors were re-classified according to the Scarf, Bloom and Richardson grading system modified by Elston and Ellis [15-17] and the score was agreed by consensus between the two pathologists.

All tissue samples were formalin-fixed and paraffin embedded blocks selected from each case, containing representative tumor samples and, if possible, normal breast tissue. Deparaffinized 4- to 5-μm sections of the selected blocks were rehydrated and either stained with H&E or subjected to antigen retrieval (10 mM citrate buffer, pH 6.0, at 100° C, 30 min) optimized for anti-GST Pi monoclonal antibody (NCL-438, 1:600, Novocastra Laboratories, Newcastle upon Tyne, U.K.). Positive and negative controls were respectively liver and a tumor sample with the omission of the primary antibody. Other primary antibodies included anti-estrogen receptor (ER) (M7047, 1:300, Dako, Carpenteria, CA, USA), anti-progesterone receptor (PR) (M3569, 1:800, Dako) and anti-C-erb-B2 (A0485, 1:600, Dako). All primary antibodies were incubated overnight at room temperature, thoroughly washed, and treated for 30 min with ready-to-use Novolink (Novocastra Laboratories, Newcastle upon Tyne, U.K.) using standard manual procedures. The reactions were developed with diaminobenzidine (DAB) as chromogen. In addition, the sections were counterstained with hematoxylin, dehydrated in a series of ethanols and mounted with Enthelan® (Merck, Darmstadt, Germany).

Scoring of immunoreactivity patterns

GST Pi expression was assessed in terms of frequency of positive cells and staining intensity. The frequency of positive cells was graded and scored as follows: negative= 0, 1-10%= 1 point, 11-50%= 2 points and ≥51%= 3 points. The intensity was estimated and scored as follows: negative= 0, weak= 1 point, moderate= 2 points and strong= 3 points. A final GST Pi score was obtained by adding the points achieved on each parameter. The cases were considered positive for GST Pi with a final score of at least 4 points. All the GST Pi positive cases showed nuclear and cytoplasmic immunostaining. Hormone receptors and C-erb-B2 immunoexpression was scored as described elsewhere [18,19].

Statistical analysis

Statistical analysis was performed using the SPSS software for Windows (release 8.0). Association between GST Pi expression and other clinicopathological variables (local, regional and distant recurrences, as well as death from disease) was determined by using the Fisher exact test or the x^2 test as appropriate. Survival curves were plotted by the Kaplan-Meier method and the differences between the curves were evaluated by the log-rank test and hazard function. A p-value of <0.05 was considered significant.

Results

The age of the 95 patients ranged between 33 and 70 years (median 50.33). Forty-six of the patients (48.42%) were ≤ 50 years old and 49 > 50 years at the time of diagnosis. All axillary dissections were performed without prior sentinel node dissection. In 12

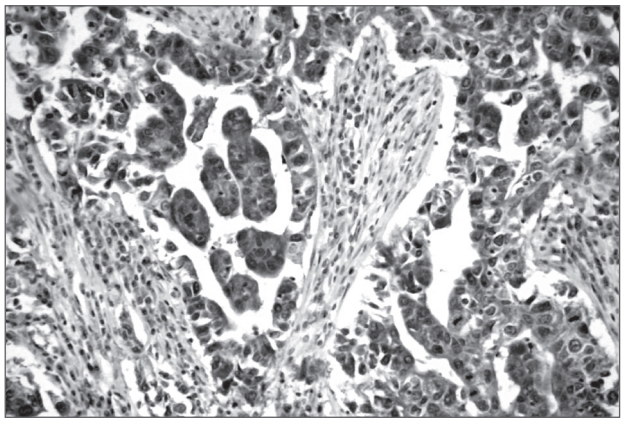

Figure 1. Breast carcinoma showing positive nuclear and cytoplasmic immunostaining for GST Pi (original magnification ×100).

cases (12.63%) the disease was diagnosed in stage I, in 38 (40%) in stage II and in 45 (47.37%) in stage III. The positivity rates for ER and PR were 77 and 75%, respectively. Eleven cases (11.58%) were positive for C-erb-B2 (3+), 10 (10.52%) were 2+ and 74 (77.90%) were negative (0/1+).

There were 36 (37.9%) GST Pi-positive breast tumors (Figure 1) and 59 (62.1%) negative. The cases were well balanced in relation to age, pathological stage and scheme of chemotherapy, hormone therapy and radiotherapy. GST Pi-positive tumor cells were characterized by nuclear and cytoplasmic positivity. Clinical and pathological data are summarized in Table 1.

No correlation was found between GST Pi expression and the histological type or the hormone receptor status. On the other hand, GST Pi positivity was significantly associated with grade I ductal carcinoma (p= 0.041). Ten out of 17 grade I tumors (59%) were positive for GST Pi, while only 19 out of 60 grade II/III tumors (32%) were GST Pi positive.

Seventy-four tumors (78%) were negative for C-erb-B2 (0/1+) and 21 tumors (22%) were either 2+ or 3+ (positive). A significantly higher frequency of GST Pi-positive cases were found among C-erb-B2 negative cases (89%) in contrast to 2+/3+ positive tumors (11%) (p=0.044).

Patient follow-up ranged from 9.16 to 11.66 years (median 10.68). In a preliminary analysis the GST Pi positivity was significantly correlated with regional (p= 0.024) but not with local/distant recurrence. All 3 regional recurrences in this study occurred in patients with GST Pi-positive tumors.

Kaplan-Meier overall and disease free survival curves revealed no significant difference between GST Pi-positive and negative cases (Figure 2). The lack of difference in terms of overall survival and disease-free survival persisted even when analyzing stages II and III separately (Figures 3 and 4). Analysis of stage I patients was hampered by the small number of cases (6 GST Pi-positive and 6 GST Pi-negative) and because of the small number of events (data not shown).

Table 1. Distribution of GST Pi expression according to clinical data and tumor histological type, grade, hormone receptor and C-erb-B2 status

Clinicopathological characteristics	*GST Pi expression*	
	Positive (%)	*Negative (%)*
Age (years)		
≤50	17 (17.89)	29 (30.53)
>50	19 (20)	30 (31.58)
Stage*		
I	6 (6.32)	6 (6.32)
II	14 (14.74)	24 (25.26)
III	16 (16.84)	29 (30.52)
Therapeutic modalities		
Chemotherapy		
CMF	9 (9.48)	23 (24.21)
FAC/FEC	27 (28.42)	36 (37.89)
Hormone therapy	5 (5.26)	4 (4.21)
Radiotherapy	33 (34.74)	51 (53.68)
Histological type		
Ductal	27 (28.42)	45 (47.37)
Lobular classic	2 (2.11)	8 (8.42)
Lobular pleomorphic	1 (1.05)	2 (2.11)
Tubular/colloid	4 (4.21)	1 (1.05)
Mixed ductal and lobular	1 (1.05)	2 (2.11)
Mixed ductal and colloid	1 (1.05)	1 (1.05)
Histological grade		
I	10 (10.53)	7 (7.37)
II	8 (8.42)	25 (26.31)
III	11 (11.58)	16 (16.84)
Hormone receptors		
Estrogen +	28 (29.47)	45 (47.37)
Estrogen –	8 (8.42)	14 (14.74)
Progesterone +	25 (26.31)	46 (48.42)
Progesterone –	11 (11.58)	13 (13.69)
C-erb-B2		
0/1+	32 (33.68)**	42 (44.21)
2/3+	4 (4.21)	17 (17.90)

*American Joint Committee [33], **Statistically significant (p<0.05)
CMF: cyclophosphamide/ methotrexate/ 5-fluorouracil, FAC: 5-fluorouracil/ adriamycin/ cyclophosphamide, FEC: 5-fluorouracil/ epirubicin/ cyclophosphamide

Discussion

In the present study GST Pi expression was detected in 37.9% of breast invasive carcinomas. This percentage is slightly lower than those found in previous studies in which they ranged from 47-52% [6,14]. This may be due to methodological differences, especially those concerning the quantification of GST Pi staining and the definition of GST Pi positivity (cutoff point). The GST

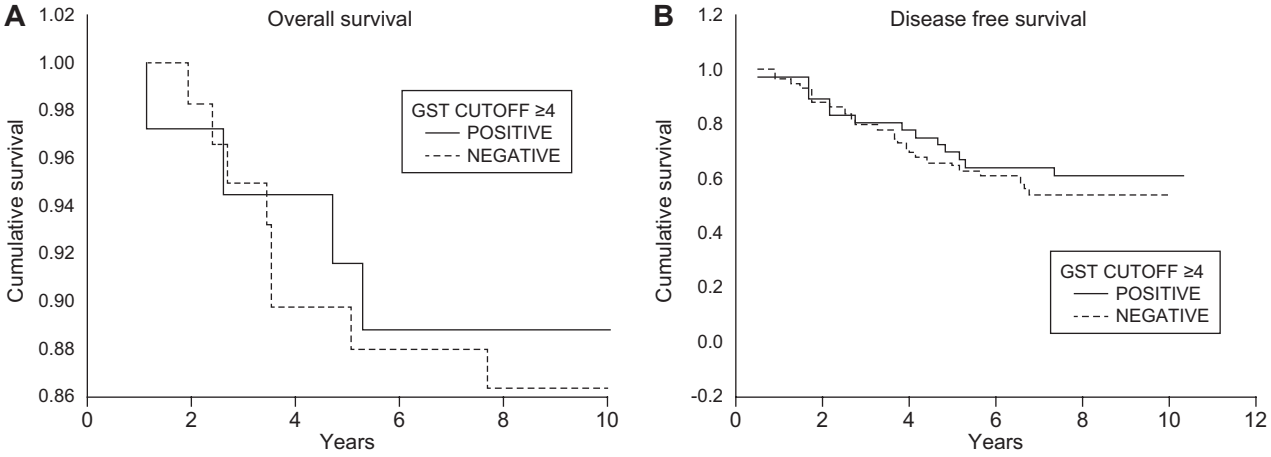

Figure 2. Patients with GST Pi positive and negative breast carcinomas (N = 95) in relation to overall (A) and disease free (B) survival (p = nonsignificant).

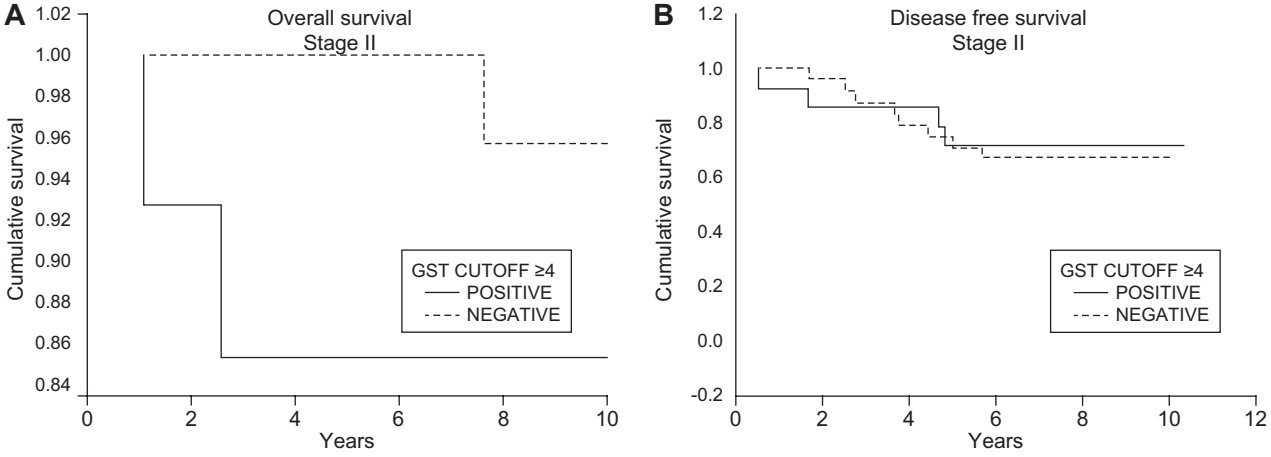

Figure 3. Patients with stage II GST Pi positive and negative breast carcinomas (N = 38) in relation to overall (A) and disease free (B) survival (p = nonsignificant).

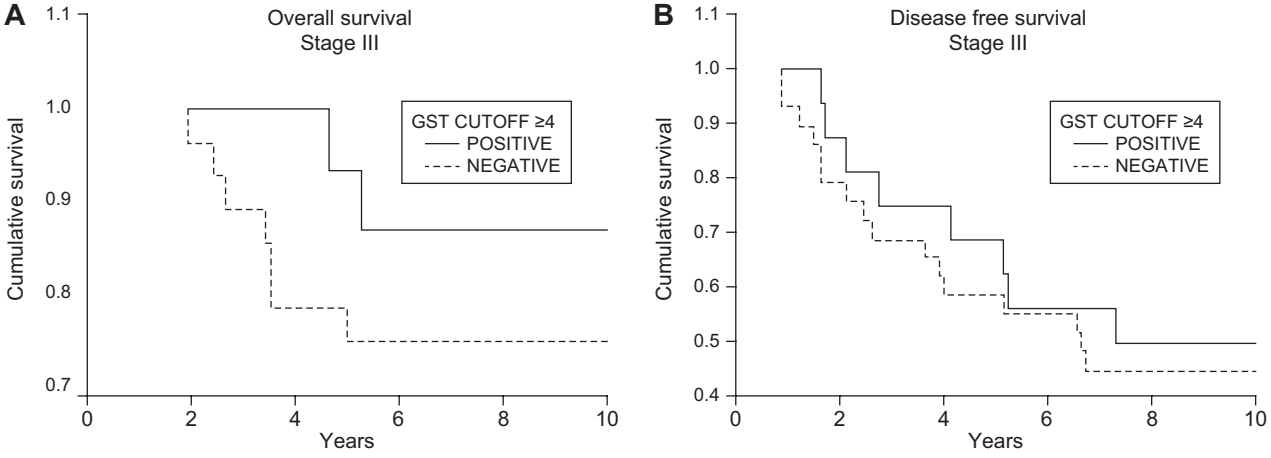

Figure 4. Patients with stage III GST Pi positive and negative breast carcinomas (N = 45) in relation to overall (A) and disease free (B) survival (p = nonsignificant).

family has long been implicated in chemotherapeutic drug resistance [11,12], so not only the number of cells with GST Pi expression but also the intensity of this expression must be important in terms of drug chemotherapy detoxification. For that matter, in this study we combined the assessment of the percentage of positive cells and staining intensity in a single score.

The results of this study on 95 breast cancer patients with more than 10 years follow-up on average showed that GST Pi expression bears no relationship with neither disease free-survival nor overall survival. These results are in agreement with Peters et al. [13] who evaluated 139 patients with an average follow-up of 4 years. On the other hand, our study and the latter are in contrast to Huang et al. [6] who evaluated 116 patients and found a significantly worse disease-free survival, with a mean follow-up of 3 years. Of note, these two contradictory studies are the only ones that have evaluated this matter so far.

It is important to mention that, to the best of our knowledge, our findings are not only based on a homogeneous sample but also on the longest follow-up ever to be published.

Breast cancer is a clinically heterogeneous disease; individuals with the same stage and similar pathological diagnoses can experience very different clinical outcomes [4]. During the last years an effort has been made to try to identify a molecular fingerprint for a group of individuals with breast cancer that could benefit from specific treatment [20,21]. Lately, an increasing number of molecular markers have been shown to predict resistance to drugs commonly used in chemotherapy and this fact has intensified research towards the development of targeting drugs. A good example of such a molecular marker would be the expression of C-erb-B2 in breast cancer cells, which is a well-known predictor of response to trastuzumab [22, 23].

The expression of GST Pi in cancer cells has been advocated as a predictor of chemotherapy resistance in several solid tumors [24,25]. Nevertheless, this matter is still extremely controversial. For ovarian cancers, some studies have shown shorter survival among patients with GST Pi-positive tumors [26,27], while another study has shown no prognostic significance [28]. In gastric cancers, the controversy remains with studies showing contradictory results [29,30]. In lung cancer GST P1 polymorphisms predicted worse outcome [31].

The hypothesis that tumor cell resistance could be increased by the expression GST Pi enzymatic system is supported by the conjugation of the chemotherapeutic with glutathione leading to its faster elimination [32]. The fact that, in our study, the tumor GST Pi expression was significantly associated with low histological grade/C-erb-B2 negative cancers, which are well-known good prognostic factors [33], probably explains the lack of association between GST Pi positive breast cancers and worse overall and disease free survival.

Despite the fact that our study is retrospective, it had the longest average follow-up (10.68 years) and showed that there was no correlation between GST Pi positivity in breast cancer cells and disease free survival. In this study the only proven data in terms of prognosis is that GST Pi expression was positively correlated with regional recurrences in a preliminary univariate analysis. Nevertheless, the significance of this finding should be better evaluated in larger series.

In conclusion, although the expression of the enzyme GST Pi in breast cancer cells is associated with a low grade/C-erb-B2 negative phenotype, which is usually associated with better prognosis, we could not demonstrate association between this molecular marker and longer disease free/overall survival. Prospective studies with a longer follow-up and larger number of patients should be conducted to better evaluate the matter of the GST Pi expression in breast cancer and chemotherapy resistance.

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