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Glutathion S Transferase π Indicates Chemotherapy Resistance in Breast Cancer

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Background. **Breast cancer is the most common malignant disease of women. Pathologic response of breast cancer to chemotherapy has a great prognostic importance. Glutathion S Transferases (GSTs) might detoxify chemotherapeutic drugs within the cancer cells, thus contributing to chemotherapy resistance. The pi isoenzyme of GSTs seems to be of great relevance. Thus, we hypothesized that GSTpi expression in cancer biopsy can be a prognostic indicator for resistance to chemotherapy. To test this hypothesis, we evaluated before and after chemotherapy, tumor size, apoptosis of tumor cells with TUNEL assay, and proliferation of tumor cells by determining PCNA expression in biopsy samples, or in the surgically removed tumor tissue of GSTpi (−), and GSTpi (+) cases.**

Materials and methods. **GSTpi immunoreactivity was determined in 42 female patients with breast cancer. Patients were divided into two groups according to the expression of GSTpi in the pre-treatment biopsy specimen: (+) (n = 22) and (n = 20) samples were analyzed. Surgery was performed 2 weeks after a single intravenous injection of the chemotherapeutic drugs [5-fluorouracil, adriamycin, mitomycin (FAM protocol)].**

Results. **Pre-chemotherapy values of tumor size, apoptosis, or proliferation did not differ between GSTpi (−) and (+) samples. Chemotherapy significantly inhibited tumor growth, and cell proliferation, and induced apoptosis in GSTpi (−) cases. However, these effects were significantly reduced in GSTpi (+) patients.**

Conclusion. **These results suggest, that the presence of GSTpi in breast cancer tissue is a bad prognostic indicator, and these tumors are largely resistant to chemotherapy. Thus, GSTpi might be important in inactivating one or more of the chemotherapeutic agents used in this treatment.**

Key Words: **breast cancer; chemotherapy resistance; MDR; GSTpi; apoptosis; FAM.**

INTRODUCTION

Data from major studies indicate, that the pathological response of breast cancers following preoperative chemotherapy is of far greater prognostic importance than the clinical response [1]. Multidrug resistance (MDR) is the major mechanism of drug resistance in malignant tumor cells [2].

Glutathion S transferases (GSTs) are enzymes detoxifying many potentially carcinogenic agents. Three major classes include alpha, mu and pi (α, μ, π) [3]. The pi isoenzyme (GSTpi) is of great relevance in these detoxifying effects [4]. Loss of GSTpi expression is a phenotype associated with malignant transformation [5].

However, GSTpi also inactivates chemotherapeutic substances by conjugating them to glutathion. Though well established from cultured cancer cell lines, its involvement in resistance to chemotherapy is still unclear in tumors in vivo [6]. Increased expression of GSTpi—detected as strong immunoreactivity—has been documented to contribute to drug resistance of ovarian carcinomas [7], head and neck cancer [8], or lung squamous-cell carcinoma [9]. On the other hand,

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Abbreviations used: GSTs, Glutathion S Transferases; FAM, 5-fluorouracil, adriamycinandmitomycin therapy; MDR, multidrug resistance; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling; PCNA, PC10 nuclear antigen.

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no relation was found between variations of GSTpi contents and efficiency of tamoxifen hormone therapy in human breast carcinomas [10]. GSTpi immunoreactivity was reported not to correlate with response to chemotherapy in cervical carcinoma [11]. Finally, antibody staining for GSTpi in 45 cases of primary breast tumors was associated with poor prognosis, however, resistance to chemotherapy was not investigated in this study [12], and it has been shown previously, that GSTpi expression is an important predictor of early recurrence, and bad prognosis [13]. So far, there is no evidence regarding the relationship of GSTpi expression, and outcome in breast cancer patients preoperatively treated according to the FAM protocol.

Based on these previous findings we hypothesized, that GSTpi immunoreactivity could be a prognostic indicator for chemotherapy resistance in breast cancer patients. To test this hypothesis, we evaluated chemotherapy efficacy in patients with or without GSTpi expression.

PATIENTS AND METHODS

All patients in this prospective study were females diagnosed with primary breast cancer at the Department of Surgery, Sun-Yat-Sen Memorial Hospital, Guangzhou, China The study was carried out in accordance with the Helsinki Declaration of 1975. Age at diagnosis was recorded. Pathological examinations included conventional histopathologic studies and immunohistochemical identification of GSTpi. Stage, differentiation, and response to chemotherapy of the tumor, based on biopsy specimen, as well as tumor size were evaluated. Percutaneous core needle biopsy of the breast [14] was performed, which does not influence tumor size significantly. In addition, all of our patients were in stage B or C, thus the tumor size was 5 cm or more in diameter, making core needle biopsy appropriate to perform histological examination. Pre-chemotherapy and post chemotherapy tumor size was determined by type B ultrasound and by measurement upon the Halsted mastectomy.

Forty-two patients with primary breast cancer were treated according to the FAM protocol: 5-fluoruracil [5-Fu: 500 mg/square meter body surface (SqM)], adriamycin (A: 30 mg/SqM), and mitomycin C (M: 10 mg/SqM) intravenous injection once, after diagnosis. A modified Halsted mastectomy was performed 2 weeks after chemotherapy.

Conventional Histopathologic Analysis

Diagnosis was based on biopsy specimen. For conventional histopathologic examination, 4-μm paraffin sections stained with hematoxylin and eosin were examined. Tumors were graded (TNM) according to morphologic differentiation: grade 1 is well differentiated, grade 2 is moderately differentiated, and grade 3 is poorly differentiated or undifferentiated [15].

Determination of Chemotherapy Effectivity

Pre-chemotherapy biopsy specimen, and postoperative samples were evaluated in vitro with immunohistochemistry. Conventional histopathologic and immunohistochemical examinations were performed by a pathologist without knowing the clinical outcome of the patients.

Immunohistochemical Examinations

For immunohistochemical detection of GSTpi, and PCNA in biopsy specimen, 4-μm-thick sections obtained from paraffin blocks were mounted on glass slides [16]. The slides were air-dried, deparaffinized, and rehydrated with phosphate-buffered saline solution. The slides were then incubated with methanol containing 0.3% hydrogen peroxide for 5 min at room temperature, to block the endogenous peroxidase activity, and treated with normal horse serum to block the nonspecific proteins. After washing with phosphate-buffered saline (PBS) solution, the slides were incubated with the specific monoclonal antibody.

GSTpi Staining

To determine GSTpi expression, biopsy samples were incubated (overnight at 4°C, in humidity chambers) with primary rabbit polyclonal anti-GSTpi antibody (Signet Laboratories Inc, Dedham, MA) and after washing in PBS, with secondary, peroxidase labeled, goat anti-rabbit IgG (60 minutes, room temperature) (Sigma Immunochemicals, St. Louis, MO). The peroxidase reaction was developed using 3-3′-diaminobenzidine tetrahydrochloride (DAB) as substrate (Sigma) diluted in 1% PBS (incubation: 5 min). The GSTpi polyclonal antibody stained primarily the cytoplasma of the cancer cells (Fig. 1a). In the case of GSTpi positivity, it was nearly an "all or none phenomenon": Either most cells were positive, or none of the cells were positive. Patients were divided into two groups based on GSTpi immunoreactivity of the tumor biopsy specimen: staining was negative for GSTpi (GSTpi−) in 20 and positive (GSTpi+) in 22 patients.

PCNA Staining

In the PCNA assay antibody against PC10 (DAKO, Glostrup, Denmark) was incubated for 60 min at room temperature. The slides were washed again with PBS solution and finally stained with avidin-biotin peroxidase complex (Vectastain, Vector Laboratories, Burlingame, CA) method (Fig. 1b). Appropriate positive and negative control tests were done.

Evaluation of immunohistochemical staining was performed by counting at least 1000 cells in at least 5 different regions of the tumor. The ratio of cells stained for PCNA to the total number of cells was recorded as the proliferative index (PI).

TUNEL Assay

The number of apoptotic cells in frosen sections of the tumor biopsy specimen was determined by, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining (Boehringer Mannheim GmbH, Mannheim, Germany) (Fig. 1c), as previously described [17]. Tumor cells were permeabilized with 0.1% Triton X-100 for 5 min and incubated with the TUNEL reaction mixture. The reaction was terminated with rinse buffer after 60 min. Incorporated bromodeoxyuridine (Br-dUTP) was detected after the addition of fluorescein-labelled anti Br-dUTP antibody (5.0 μl) and incubation for 30 min at room temperature in the dark. Cells were counted on an occular grid. The percentage of apoptotic cells was determined as TUNEL positive cells/total number of cells counted.

Statistics

Statistical comparison of the 2 groups was performed with student's t-test. Values are given as average of each group $\pm$ standard deviation (SD). A $P < 0.05$ was considered significant. Correlations were analyzed with the Fischer's least square test, and the Spearman's test [18].

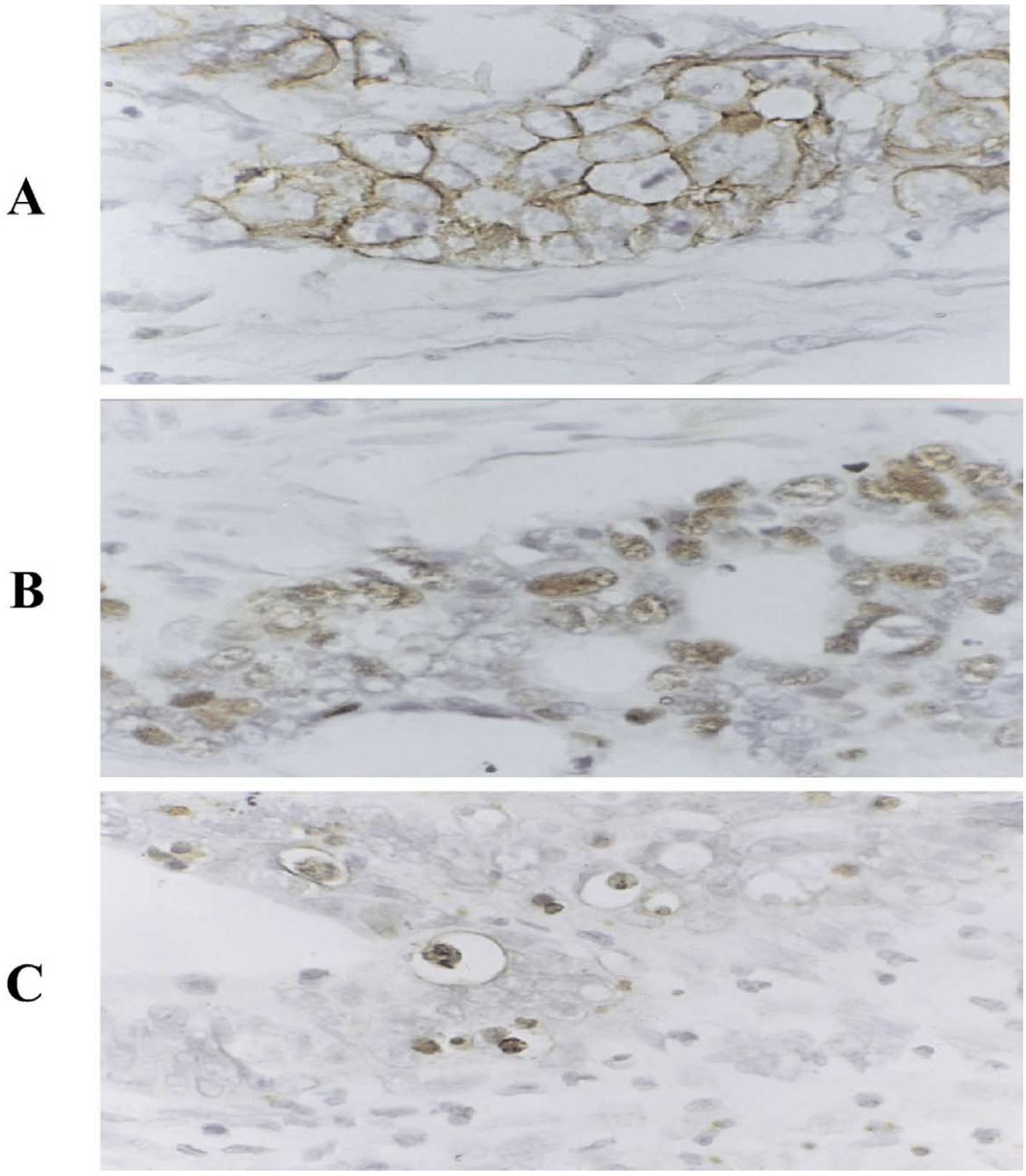

FIG. 1. Immunhistochemical staining. (A) GSTpi staining. Breast cancer cells showing GSTpi immunhistochemical expression, as indicated by diffuse, brown cytoplasmic staining, using 3-3′-diaminobenzidine (DAB). Anti- GSTpi, immunoperoxidase, 200 × magnification. (B) PCNA staining. Breast cancer cells showing high proliferating cell nuclear antigen (PCNA) expression, as indicated by brown nuclear staining. Anti-PCNA immunoperoxidase, 200 × magnification. (C) TUNEL staining. Breast cancer cells showing TUNEL positivity, as indicated by brown nuclear staining. 200 × magnification.

RESULTS

Patient and Tumor Characteristics

Age at diagnosis was 55 ± 12 years ranging from 31 to 77 years in the GSTpi (−) group ($n = 20$), and 60 ± 10 years (range: 37–79 years) in the GSTpi (+) group ($n = 22$).

There was no significant difference between the 2 groups in TNM state. In the GSTpi (−) group average TNM score was 2.1 ± 09 versus 2.3 ± 0.8 in the GSTpi (+) group.

At the time of diagnosis, there was a positive correlation between age of the patient and tumor differentiation ($r = 0.46$, $p < 0.05$), older patients had less

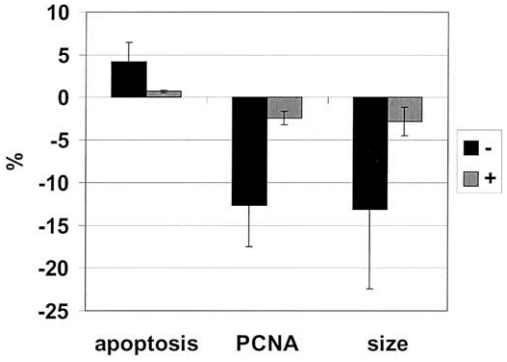

FIG. 2. Change in tumor characteristics before and after chemotherapy in GSTpi + and − cases: apoptosis rate increased, whereas PCNA positivity, and tumor size decreased after chemotherapy as expressed in percentage of the initial apoptosis rate, PCNA positivity and tumor size.

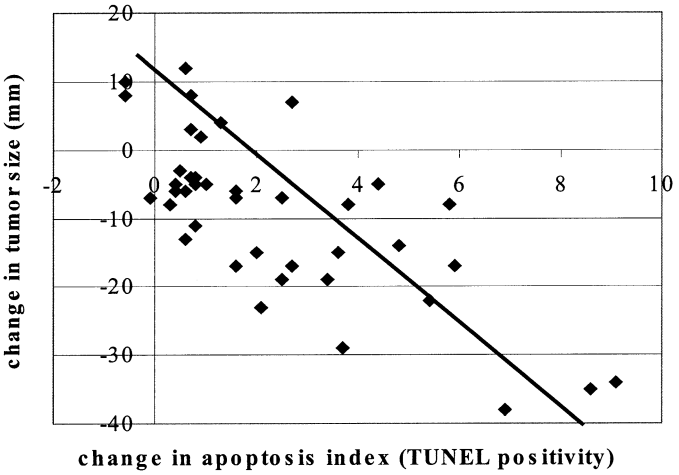

FIG. 3. Linear correlation between the change in PCNA index and TUNEL positivity in the 42 patients included in this study, before and after chemotherapy ($r = 0.58$) ($P < 0.001$).

differentiated tumors. These less differentiated tumors were larger at diagnosis (correlation between tumor size, and differentiation: $r = 0.71, p < 0.01$). These less differentiated tumors were also more apoptotic and less proliferative as demonstrated by a positive correlation between TUNEL positivity ($r = 0.36, p < 0.05$) or PCNA index ($r = 0.41, p < 0.05$) and tumor differentiation.

GSTpi (−) cases were less differentiated to some extent, than the GSTpi (+) cases, however, this difference was not statistically significant. In the GSTpi (−) group, 5 patients had well differentiated (25%), 7 had moderately (35%), and 8 had poorly differentiated (40%) carcinoma, vs. 9 well differentiated (41%), 9 moderately (41%), and 4 poorly differentiated (18%) carcinomas in the GSTpi (+) group.

Response to Chemotherapy

Pre-chemotherapy apoptosis rate did not differ significantly between the groups (GSTpi (−): 5.2 ± 1.6 versus 4.4 ± 1.4 in GSTpi (+) samples) (ns). Apoptosis rate increased in both groups following chemotherapy. Average increase in the GSTpi (−) group was 4.2 ± 2.2 (to 9.4 ± 2,9), whereas in the GSTpi (+) group average increase in apoptosis was only 0.7 ± 0.7 (to 4.8 ± 1.7) ($p < 0.001$) (Fig. 2).

Pre-chemotherapy PCNA index was also similar in the 2 groups (32.2 ± 6.3 in the GSTpi (+) versus 32.9 ± 6.7 in the GSTpi (−) group). PCNA index decreased by the time of surgery, however, this decrease in the GSTpi (+) group was 2.4 ± 3.3 (to 29.8 ± 6.3), significantly less compared to 12.7 ± 4.8 (to 20.2 ± 7.3) in the GSTpi (−) samples ($p < 0.001$) (Fig. 2).

Tumor size was also similar in the groups before chemotherapy: 38.3 ± 17.9 mm in the GSTpi (−) group versus 39.3 ± 13.8 in GSTpi (+) patients. Size of GSTpi (−) tumors decreased significantly with 13.2 ± 9.3 mm to 25.1 ± 12.7 mm versus 2.8 ± 6.5 mm decrease (to 36.4 ± 10.3 mm) of GSTpi (+) tumors ($p < 0.001$) (Fig. 2).

Chemotherapy sensitive and resistant phenotypes distinguished clearly from each other. Strong negative correlations were observed between the changes in TUNEL positivity (Δ-apoptosis index), and changes in cell proliferation (Δ-PCNA) index (Fig. 3) or tumor size decrease (Fig. 4): more apoptosis was accompanied by less proliferation after chemotherapy, resulting in a more expressed tumor size reduction.

DISCUSSION

In the present study we show, that chemotherapy according to the FAM protocol was effective against

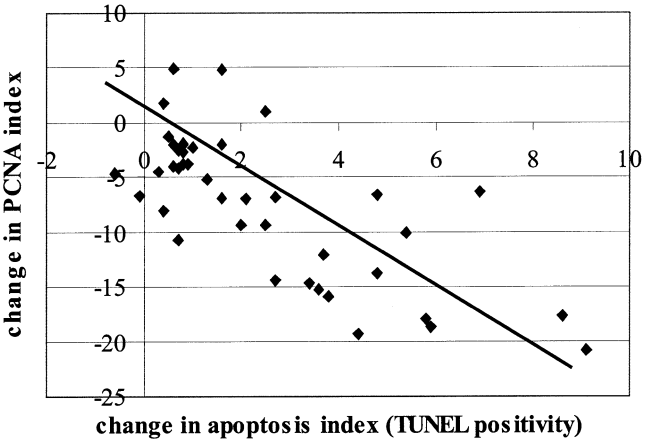

FIG. 4. Linear correlation between the change in tumor size and TUNEL positivity in the 42 patients included in this study, before and after chemotherapy ($r = 0.78$) ($P < 0.001$).

breast cancer, as cell proliferation (PCNA) index decreased, apoptosis rate (TUNEL positivity) increased, and tumor size was reduced after chemotherapy. However, there was a significant difference between GSTpi (+) and (−) tumors. The GSTpi (+) phenotype seems to be resistant to this treatment protocol.

There are contrary reports on the contribution of GST genotypes to breast cancer risk [19]. A number of studies excluded a role for the μ type GSTM1 genotype as a prognostic factor in breast cancer [20, 22]. Clinical response to chemotherapy did not correlate with GSTM1 genotype either [21]. On the other hand, GSTpi polymorphism seemed to be a significant risk modifier [21]. In 1998 a study on 115 blood donor women with incident breast cancer, and 115 control subjects, in the National Cancer Institute concluded that genetic variability in GST M1, P1, and T1 genotype may be associated with an increased susceptibility to breast cancer [23]. Contrary to this, the Carolina Breast Cancer Study of 1341 cases, as well as an analysis of 258 Australian women concluded, that GSTM1, T1, and P1 genotypes do not play a strong role in susceptibility to breast cancer either independently or in combination [24, 25]. The phenotype of breast cancer tissue has also been investigated previously. Neither GST-alpha nor GST-mu immunopositivity in tumor or non-neoplastic breast was found to correlate with overall survival [26]. In the present study, GSTpi (−) tumors were somewhat less differentiated than GSTpi (+) tumors but the difference was not significant. Thus GSTpi genotype does not seem to be crucial in breast cancer development.

A recent study has suggested, that inherited metabolic variability due to GSTp1 polymorphism may influence chemotherapeutic treatment outcome [27]. GSTpi conjugates certain types of anti cancer chemotherapeutic agents, such as cisplatin (CDDP) [28] or docetaxel (DXT) [29] in vivo. In vitro, in different cancer cell lines GSTpi expression correlated with resistance to CDDP [30]. Testing a number of chemotherapeutic agents, only CDDP resistance was found to correlate with GSTpi expression in human lung squamous cell carcinoma [9]. On the other hand, a human lung carcinoma cell line resistance to doxorubicin was not influenced by GSTpi expression [31], and high expression of GSTpi was not associated with resistance to 4-hydroxy-ifosfamid (IFOS) or daunorubicin (DNR) [3]. There is no consensus regarding the involvement of GSTpi in resistance to the FAM protocol: *mitomycin C* was conjugated to glutathion in vitro [32], however, a HPLC analysis of *adriamycin* resistant human breast cancer cell content and culture broths found no glutathion conjugates and ruled out significant biochemical transformation of *adriamycin* [33]. On the contrary, in GSTpi antisense gene transfected cancer cells *adriamycin* sensitivity increased, but sensitivity to, *mitomycin C*, and *5-fluoruracil* remained unchanged [34].

In our study, there was no difference in apoptosis rate, cell proliferation index, or tumor size before chemotherapy, between the two groups. Thus, the observed differences in these parameters after chemotherapy were not due to a different pre-treatment state of the GSTpi (+), and (−) tumors, but due to different sensitivity to the applied chemotherapy. Similarly to our findings, GSTpi immunoreactivity was tested in primary human squamous-cell lung carcinoma. Fifty-two percent of the cases were GSTpi (+), whereas 48% was (−). No significant correlation between the expression of GST-pi and clinicopathologic factors was observed, while no significant difference in the survival of the two groups was found either. Similarly to our results, this study demonstrated a strong relationship between resistance to chemotherapy, and GSTpi expression in human lung carcinoma [9].

Although, GSTpi immunohistochemistry was reported not to correlate with response to chemotherapy in cervical carcinoma, those patients were treated with a chemotherapy combination different from the FAM protocol used in our study: 5-fluoruracil, and mitomycin C, plus cisplatin and doxorubicin, but not adriamycin [35]. Furthermore, hypermetilation of the GSTp1 promoter region, is common in breast cancer, and has been reported to significantly contribute to GSTpi expression. This control at the transcription level—besides genetic polymorphism—is a possible mechanism for the observed difference in GSTpi expression between the two groups in our study [36, 37].

Our results suggest, that the pi isoenzyme of glutathion S transferase might be important in detoxifying one or more of the used drugs in the FAM protocol in breast cancer patients.

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