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Original article

Breast cancer: Pretreatment drug resistance parameters (GSH-system, ATase, P-glycoprotein) in tumor tissue and their correlation with clinical and prognostic characteristics

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Summary

Background: The identification of new factors predicting relapse, outcome and response to systemic therapy in breast cancer is warranted. The measurement of biological markers such as drug resistance parameters (DRPs), which are part of the phenotype of malignant cells and contribute to resistance to anti-cancer drugs may be a possibility, which may ultimately lead to improvement of therapeutic results.

Patients and methods: The level of glutathione (GSH), activities of glutathione-S-transferase (GST), glutathione-peroxidase (GPx), 06-alkylguanine-DNA-alkyltransferase (ATase), and P-glycoprotein (PGP) were measured in tumor and adjacent tumor free tissue samples from 89 consecutive, untreated females with breast cancer and correlated with clinical and prognostic factors. Early breast cancer (EBC) was diagnosed in 56 patients, 22 patients had locally advanced (LABC) and 11 patients metastatic breast cancer.

Results: All DRPs showed significantly higher expression in tumor than in tumor free tissues. GPx was positively correlated with GST ($r = 0.3$, $P = 0.0048$) and with GSH ($r = 0.5$, $P = 0.0001$) in tumor as well as in normal tissue. GST activity was significantly higher in EBC than in LABC or metastatic breast cancer ($P = 0.02$). GSH level was significantly higher in grade 1 than in grade 2 or grade 3 tumors ($P = 0.01$). When clinical characteristics were related to the level of DRP, 'high' GSH was associated with age > 60 years ($P = 0.01$) in EBC, and with grade 1–2 tumors ($P = 0.05$) in LABC. No differences in OS were apparent between groups of 'high' and 'low' DRP-expression. However, the four-year estimated disease-free survival of EBC tended to be higher in patients with 'high' GST ($P = 0.10$) and of LABC in patients with 'high' GPx levels ($P = 0.06$).

Conclusion. We conclude that 'high' levels of DRP in tumor tissue of breast cancer patients are part of the initial phenotype of the malignant cells. Due to its high prevalence (83% in EBC, 100% in primarily metastatic breast cancer), PGP did not add to prognostic information. High levels of GSH, GST and and GPx were associated with favorable clinical characteristics and good prognosis, whereas low levels of GSH and GST activity were associated with more aggressive or more advanced disease.

Key words: ATase, breast cancer, drug resistance, PGP, GPx, GSH, GST, prognosis

Introduction

Breast cancer has become the second leading cause of cancer deaths in women after lung cancer [1]. Currently, none of the known prognostic factors in breast cancer are capable of fully defining the patients with highest risk for relapsing disease [2]. Therefore, the identification of new factors predicting relapse, outcome and response to systemic therapy is warranted. The measurement of biological markers, which are part of the phenotype of malignant cells and contribute to resistance to anti-cancer drugs, may be a possibility, which may ultimately lead to improvement of therapeutic results.

Current clinical investigations try to circumvent resistance pathways, by adding resistance modulators, previously identified as modulators *in vitro*, to chemotherapeutic regimens. So far, results remain behind expectations [3], suggesting that therapy response in most cases may depend on more than one parameter and that the modulators may not be specific and/or effective enough. While most studies focus on one parameter, the aim of this study was to measure P-glycoprotein (PGP), glutathione (GSH), GSH-S-transferases (GST), glutathione-peroxydase (GPx) and O^6-alkylguanine-DNA alkyltransferase (ATase) simultaneously in tumor samples of patients with newly diagnosed early, locally advanced and metastatic breast cancer as well as normal breast tissue of the same patients.

The best understood drug resistance parameter is PGP, a 170-kd protein coded for by the MDR1 gene, which works as an energy-dependent efflux pump to decrease intracellular accumulation of a number of cytostatic drugs [4]. PGP expression was found in a majority of tumor specimens in patients with breast cancer. Although high PGP expression appears to correlate with poor response to chemotherapy and short disease-free survival in some instances [5], its role is still controversial. Some evidence suggests that PGP may

also be a marker of 'biological maliciousness' of cancers [6].

Subsequently, other biochemical parameters, which play an important role in the detoxification or cell repair systems, were identified. Although many of them were shown to contribute to drug resistance *in vitro*, a direct connection, between these parameters and drug resistance *in vivo* has not yet been established. GSH plays an important role in detoxification and repair of cellular injury caused by cyclophosphamide, nitrosoureas and quinone antibiotics [7]. Elevated levels of GSH have been described in different human tumor tissue samples when compared with normal tissue [8, 9].

GSTs are a family of enzymes which catalyse the conjugation of electrophilic substrates with GSH, thereby detoxifying them. Increased levels of these enzymes have been found in human breast cancer when compared with benign lesions [10]. GST-π expression, one of the four different classes of GST known in humans, was reported to be inversely correlated with hormone receptor status in breast cancer [11]. GPx, an enzyme removing toxic oxygen intermediates, is also associated with GSH. GPx activity was found at increased levels in breast cancer when compared to normal tissue [12].

ATase is an enzyme involved in DNA repair and is present in all human tissues [13]. Many human tumor tissues show higher activity than corresponding normal tissue. However, so far no differences in ATase activity between breast cancer and corresponding normal breast tissue have been reported [14].

Despite the great amount of information about GSH, GST, GPx, and ATase, and their involvement in the cellular defense against toxic substances, association with either clinical behavior or prognosis has yet to be established.

We attempted to evaluate whether the simultaneous assessment of these parameters would allow detection of a distinct pattern of distribution in tumor tissues and whether an association between any of these parameters and established prognostic factors and/or clinical outcome could be found.

Patients and methods

Patient characteristics

From May 1988 until November 1991, 89 nonselected caucasian female patients with histologically proven breast cancer, but no other cancer, were evaluated. None of the patients had received any systemic treatment. The patients characteristics are presented in Table 1 The median follow-up from diagnosis is 45 months. Relapse or disease progression was observed in 34 patients, 30 patients died during follow-up and of these six patients died without relapse or progression (one cerebrovascular insult, three cardiovascular disease, one influenza, one pneumonia).

Tissue collection

Tumor tissue and tumor-free tissue samples were obtained from the operating theater at first surgery. Tissue samples were snap frozen and maintained at −70 °C until assayed.

Pathologic examination

The pathology reports and the histological slides of the tumors were evaluated by a board-certified pathologist. The histological determination of the tumor type was performed according to the WHO classification. Histological tumor grading was performed according to Elston and Ellis [15]. Tissue samples from tumor and tumor-free tissue samples were used for pathologic review It was confirmed by the pathologist that the tissue samples examined for histology were directly adjacent to the sample used for the biochemichal assays.

The tumor mass, estimated on the corresponding histological slides, ranged between 15% and 90%, the remaining tissue consisted of desmoplastic stromal fibrosis and fat tissue. None of the tumor samples contained relevant necrosis and none of the samples from tumor-free breast tissue revealed any breast cancer cells. For the steroid hormone receptor determination the dextrane coated charcoal method (DCC) was used [16]. Patients were designated as hormone-receptor positive (progesterone receptor ⩾20 fmol/mg protein and/or estrogen receptor ⩾10 fmol/mg protein) or hormone-receptor negative (progesterone receptor <20 fmol/mg protein and/or estrogen receptor <10 fmol/mg protein).

Tissue preparation

Techniques of sample preparation and assays have been described in a previous report [8]

Table 1. Patient characteristics.

	No. (%)
Number of patients	89 (100%)
Median age (years)	68
Range	(33–89)
Clinical stage (TNM)	
Early primary breast cancer (T1–T2, N1, M0, T3aN0M0)	56 (63%)
Advanced breast cancer (T3–T4, N2 or N3, M0)	22 (25%)
Primarily metastatic breast cancer (T1–T4, N0–N3, M1)	11 (12%)
Histology	
Invasive ductal carcinoma	71 (80%)
Lobular invasive carcinoma	9 (10%)
Medullary carcinoma	6 (7%)
Mucinous carcinoma	3 (3%)
Grading	
Grade 1	3 (3%)
Grade 2	60 (68%)
Grade 3	26 (29%)
Hormone-receptor status	
Positive	78 (88%)
Negative	11 (12%)
Axillary lymph node status	
Positive	31 (35%)
Negative	58 (65%)
Menstrual status	
Premenopausal	19 (21%)
Postmenopausal	70 (79%)
Adjuvant therapy	
Chemotherapy ± radiotherapy	17 (19%)
Hormones ± radiotherapy	41 (46%)
Chemotherapy + hormones ± radiotherapy	8 (9%)
Radiotherapy alone	1 (1%)
None	22 (25%)

Enzyme assays and Western blotting

GSH content was measured according to Tietze's recycling assay. Overall GST activity was measured according to the method of Habig. GPx activity was assayed with the improved method of Günzler. For enzyme assays several data points in the linear response range were used and the results were calculated per mg of protein in the cytoplasmatic fraction as determined using Bradford's reagent with bovine serum albumin as standard.

ATase was measured according to the method of Morten and Margison and calculated as fmol methyl transferred to protein per μg DNA.

PGP was determined semi-quantitatively by Western blot after separation of membrane proteins by SDS-PAGE [8]. The antibody used was a polyclonal rabbit antiserum raised against amino acids 1205–1224 of the human mdr protein. This peptide (ALDTESEKVV-QEALDKAREG) was made for us by Multiple Peptide Systems, Inc., San Diego, CA The antibody used recognizes the gene products of both MDR1 and MDR3. In the tissue samples analyzed, however, MDR3 is not ecpected to contribute the the signals detected, since no MDR3 expression has been found in human breast tissue. Positive and negative controls were crude membrane pellets from the doxorubicin-resistant lung cancer cell line SW 1573 IR 500-0 and its drug sensitive parent SW 1573, generously provided by Dr. H. Joenje, Amsterdam. All gels included internal standards consisting of three different amounts of membrane protein from the positive control. The signals produced by PGP were compared to the internal standards and three categories were arbitrarily defined: 1) no detectable signal = 0 (negative); 2) weakly positive signal = + (PGP-signal weaker than one produced by 1.25 μg protein of the positive control) and 3) strongly positive signal = ++ (PGP-signal equal to or stronger than the one produced by 1.25 μg protein of the positive control). For practical reasons, in the result presentation, we grouped together weakly and strongly positive PGP (labeled as 'positive').

All analyses were performed with the investigators unaware of the patients' characteristics and outcome.

Statistical methods

Differences between expression/activities of GSH, ATase, GST, and GPx in normal breast and tumor tissue were calculated for each patient, and the 'paired' Wilcoxon signed rank test was used to test the hypothesis of no difference. Correlation between different DRPs was measured with the Spearmann rank correlation coefficient The Wilcoxon rank-sum test or the Kruskal–Wallis test were used to compare the distribution of the DRPs according to clinical and prognostic parameters. In case of ordered groups (clinical stage and grade) a non parametric test for trend was performed [17]. The relationship between PGP levels and other parameters was determined with the chi-square or Fisher's exact test where appropriate Values of DRPs except for PGP expression/activity were divided into 2 groups taking the median values as cutting point ($\leqslant$ median: 'low', $>$ median: 'high'). This decision was made *a priori*, before examination of the results for outcome and prognosis. The Kaplan–Meier method was used to estimate distributions of disease-free survival (DFS), time to progression (TTP), and overall survival (OS) [18]. The estimates are reported with standard errors. Differences in time distributions were evaluated by the log-rank test [19]. P-values < 0.05 were considered to be statistically significant. All P-values were derived from two-sided tests for significance. No adjustment for multiple comparison was performed.

Results

DRP expression in normal breast and tumor tissue

Significantly higher levels of all tested DRPs were found in tumor tissue compared to normal breast tissue. Moreover, in normal tissue GPx activity significantly correlated with GST activity ($r = 0.48$, $P = 0.0001$) and with GSH levels ($r = 0.51$, $P = 0.0001$). Similarly, in tumor tissue a significant correlation of GPx activity with GST ($r = 0.3$, $P = 0.0048$) and GSH ($r = 0.52$, $P = 0.0001$) was observed.

Biological behavior and outcome is different in patients with EBC as compared to patients with LABC or primary metastatic breast cancer. These three groups of patients were therefore separated for further analyses. GST activities were high in early and decreased in locally advanced and metastatic breast cancer (median values: 62.6 vs. 46.9 vs. 35.9 nmol/min/mg protein respectively; test for trend P-value = 0.02; Figure 1a). Similarly, GSH-levels were higher in grade 1 than in grade 2 and 3 tumors (median values: 40.9 vs. 36.9 vs. 22.4 nmol/mg protein, respectively; test for trend P-value = 0.01; Figure 1b).

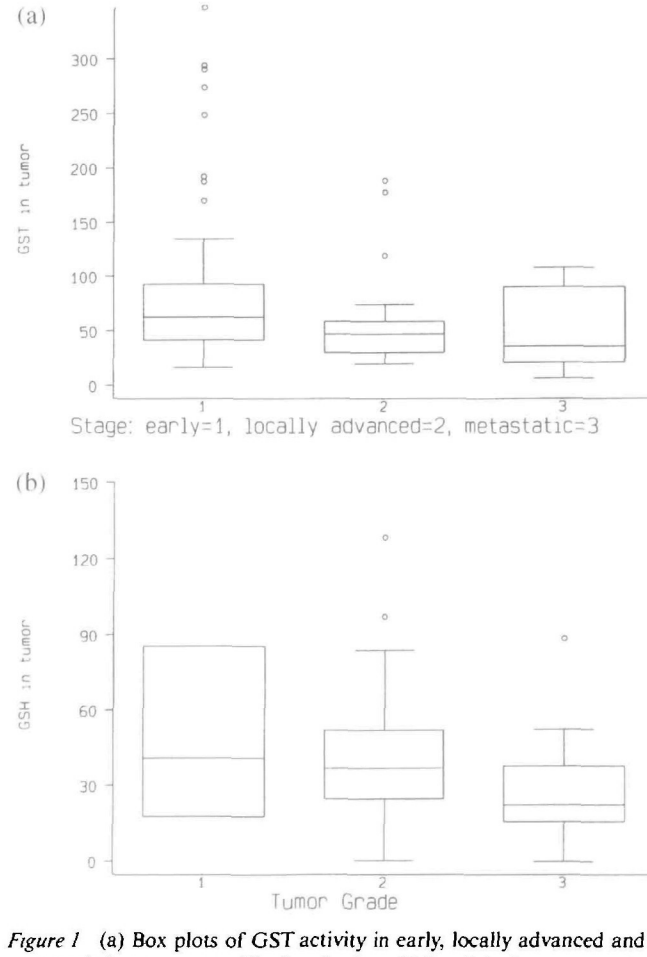

Figure 1 (a) Box plots of GST activity in early, locally advanced and metastatic breast cancer. The line in the middle of the box represents the median. The box extends from the 25th to the 75th percentile. The lines emerging from the box extend to the upper and lower 'adjacent values' Points more extreme are individually plotted. (b) Box plots of GSH activity in grade 1, 2 and 3 breast cancer. The line in the middle of the box represents the median. The box extends from the 25th to the 75th percentile. The lines emerging from the box extend to the upper and lower 'adjacent values'. Points more extreme are individually plotted.

DRP expression and clinical characteristics in early breast cancer

The median levels of DRPs in tumor tissue of EBC-patients calculated separately for the different clinical and pathological characteristics (age, histology, grading, hormone-receptor status, axillary node involvement and menopausal status) are shown in Table 2a. With one exception none of the DRPs appeared to be associated with any of these characteristics. Only the median GSH level was significantly elevated in patients older than 60 years as compared to younger patients (P = 0.01). PGP was expressed in the tumors of 44 of 53 patients (83%) and the frequency of expression was similar for all tested characteristics (Table 2a). We were interested to see whether any of the DRPs analyzed influenced OS or DFS in these patients. No significant differences in OS or DFS were apparent. However, a trend was found for GST, the four-year estimated DFS being 74% (SE 8%) for 'high' and 57% (SE 9%) for 'low' GST activity (log-rank P = 0.10).

DRP expression and clinical characteristics in locally advanced breast cancer

Median levels of GSH, ATase, GST, GPx and PGP activity/expression in tumor tissue of patients with LABC for the different clinical and pathological characteristics are shown in Table 2b. Again with one exception none of the DRPs appeared to be associated with any of these characteristics. Only the median GSH level was higher in patients with low-grade tumors (P = 0.05) compared to high grade. PGP expression was found in 15 of 20 patients (75%). The analysis of OS and DFS in this subgroup of patients revealed no significant differences but a clear trend for GPx, the four-year estimated DFS being 75% (SE 16%) for 'high' and 46% (SE 16%) for 'low' GPx activity (log-rank P = 0.06).

DRP expression in primarily metastatic breast cancer

Eleven of our patients had primarily metastatic breast cancer. This group was therefore too small to allow a meaningful correlation between DRPs and prognostic characteristics. Median values in this group were 29.2 nmol/mg protein for GSH (range 7.16–62.62), 35.9 nmol/min/mg protein for GST (range 6.5–108.2), 8.38 mU/mg protein for GPx (range 1.07–50.5) and 3.85 fmol/μg DNA for ATase (range 0.74–14.76). All 11 patients expressed PGP, without any difference in TTP for levels of positivity. All patients with metastatic disease progressed, and all, except one, died during the observation time.

Table 2a Drug resistance parameters in tumor tissue of early breast cancer by prognostic characteristics. Median values (% positive for PGP).

	n	GSH nmol/mg protein	GST nmol/min/mg protein	GPx mU/mg protein	Atase fmol/μg DNA	PGP % positive (n = 53)
Overall	56	35 3	62.6	9.3	4.6	83%
Range		(0–128 6)	(16 5–347.2)	(3.5–32 2)	(0.9–11 7)	
Age						
≤ 60 years	20	**23.7**[a]	75 3	7.9	3.3	89%
> 60 years	36	**38.1**[a]	60.3	9 9	5.5	79%
Histology						
Invasive ductal	48	36 8	62.6	9.4	4.4	80%
Other	8	28 7	83 1	7.3	6 4	100%
Grading						
1–2	43	36 8	63 2	9.1	4 5	78%
3	13	22.9	57 3	10 9	5.4	100%
Hormone-receptor status						
Negative	7	21.4	73 1	9.1	5 7	100%
Positive	49	36.8	62.1	9 3	4.5	80%
Axillary node involvement						
Negative	26	34.9	65	7 7	4.7	78%
Positive	30	35 3	61 7	10.1	4.4	87%
Menopausal status						
Premenopausal	12	26 1	73 1	8.1	3 7	100%
Postmenopausal	44	36.9	61.2	9.3	5 1	78%

[a] Significantly different values are in bold (P-value = 0 01)

Table 2b Drug resistance parameters in tumor tissue of locally advanced breast cancer by prognostic characteristics. Median values (% positive for PgP).

	n	GSH nmol/mg protein	GST nmol/min/mg protein	GPx mU/mg protein	Atase fmol/μg DNA	PGP % positive (n = 20)
Overall	22	37 6	46 9	8.9	3 3	75%
Range		(2 5–85 5)	(19.9–188 6)	(2–21.5)	(0 5–10 1)	
Age						
≤ 60 years	6	42 3	41 5	11 6	4.4	67%
> 60 years	16	35	49.4	7 3	3.3	79%
Histology						
Invasive ductal	15	34.3	43.7	7 9	3 2	79%
Other	7	52.7	58 5	11 9	4 6	67%
Grading						
1–2	14	**40.1**[a]	51.4	10 9	3 3	67%
3	8	**24.6**[a]	41 5	7 2	3.3	88%
Hormone-receptor status						
Negative	2	25.2	84.9	10 1	3.6	100%
Positive	20	39.8	44 8	8.9	3 4	72%
Axillary node involvement						
Negative	4	46.5	37.1	6.1	2 8	50%
Positive	18	35	49.4	8 9	3.4	78%
Menopausal status						
Premenopausal	4	40.3	41.5	11	4 3	100%
Postmenopausal	18	37.6	37 6	8.5	3.3	69%

[a] Significantly different values are in bold (P-value = 0.05)

Discussion

Breast cancer is a disease, which can be treated with variable success. More valid criteria for defining patients at risk of poor response to chemotherapy would be helpful. Our study was an attempt to define additional criteria by measuring the level of expression of several pretreatment parameters thought to be involved in cytostatic drug resistance. GSH, GST, GPx and ATase were all found to be expressed at significantly higher levels

and PGP was significantly more frequently expressed in tumor, when compared to tumor-free tissue. Similar observations have been made for other cancer types [20] and support the hypothesis that these biological markers are part of the intrinsic, metabolic pattern of malignant cells.

The comparison of DRPs in tumor tissue and corresponding normal tissue by biochemical methods has to be interpreted with caution because the amount of epithelial cells within the normal tissue fragments is about 5%–10% whereas the amount of neoplastic cells in tumor tissue fragments vary from 10%–90%. The biochemical measurements in normal tissue should only give an impression on the natural arsenal of DRP in normal breast tissue. Therefore, the results of the DRP measurements of normal tissue fragments has not been included for the correlation with clinical and prognostic characteristics of the investigate breast cancer.

While PGP is the best studied of all putative DRPs, its role as prognostic factor is still controversial. Most authors agree that PGP is present in many cases of untreated breast cancer. It is not clear, however, whether its expression may influence treatment outcome. Eighty-three percent of our patients had detectable levels of PGP in their tumor tissue. No association with prognostic factors was apparent, a finding that matches the results of other investigators [21, 22]. Interestingly, however, all patients with primarily metastatic disease expressed PGP. This is more extreme than in Linn et al. [23] where 58% of the samples were PGP positive. PGP was highly prevalent (83% in EBC, 100% in primarily metastatic breast cancer), and therefore not adding further to prognostic information. The semiquantitative assessment of PGP did not improve its prognostic value. There was no apparent influence of the level of PGP-expression on OS, DFS or TTP. Recently, a similar lack of correlation between PGP-expression and chemotherapy response or survival was reported by Decker et al. [24]. Other authors, however, found high PGP-expression to be associated with a poor response to chemotherapy and short DFS [5]. These discrepancies suggest that more studies are needed in order to clarify the role of PGP in breast cancer. A major obstacle in comparing the results from different studies is the use of variable techniques and reference standards. Thus, while many of the recently published studies used histochemistry to detect PGP, we have chosen to assess PGP expression by Western blot. This was done so as to be able to perform the measurement of all DRPs on material derived from the same sample. With this method, however, it was not possible to localize PGP within the tissue. Nevertheless, contamination of analyzed tissue samples e.g., with white blood cells expressing MDR1 and/or MDR3, which are both recognized by our antibody, can be neglected, because the level of expression of PGP in these cells is below the detection limit of our assay [25].

The overexpression of GSH, GST, GPx and ATase in the tumor tissue show, that potential drug resistance mechanisms other than PGP are present in tumor cells of untreated patients. The high interindividual variability of expression/activity indicate, that the measured parameters may contribute to resistance to variable degrees in different tissues. The relationship between DRPs and clinical as well as pathological characteristics in breast cancer has been studied by several authors, so far with contradictory results [11, 22, 23, 26, 27]. In the present study ATase activity was increased in tumor tissue, a finding which to our knowledge is new for breast cancer, but matches the findings reported in non small cell lung cancer [9]. An association of ATase activity with any of the other characteristics considered was not apparent in our untreated patients. We think, however, that the role of ATase in breast cancer merits further investigation, e.g., it would be interesting to know whether ATase is upregulated in tumor tissue of women treated with DNA-damaging agents. This would, however, require follow-up tumor samples, which is clinically rarely justified.

Most authors agree that GSH and its associated enzymes are overexpressed in human breast cancer in comparison with tumor-free breast tissue [28, 12]. Furthermore we observed a correlation of GPx with GSH and GST in tumor as well as in tumor-free tissue. The significant correlations between these three related parameters may point out the presence of common regulatory elements [29].

In our patients GSH levels were higher in grade 1 when compared to grade 2 or 3 tumors. In the subgroup of EBC 'high' GSH levels were associated with age > 60 years and in LABC with grade 1 and 2 tumors. GST was significantly higher in EBC when compared to LABC or metastatic disease. In contrast, low levels of GSH or GST activity seemed to be associated with a more malignant phenotype of tumor cells or more advanced disease. When analyzed for prognosis, 'high' GST in EBC and 'high' GPx in LABC showed a borderline association with longer disease-free survival. This finding was unexpected but similar to observations made in leukemias [30]. So far 'high' GST-levels in tumor tissue have been assumed to be associated with poor prognosis, although a significant correlation has been rarely found [11]. One reason why our observations differ from those of Gilbert et al. may be that our population consisted mainly of elderly women (median age: 68 years). Furthermore it may be that the expression of GSH and its associated enzymes is hormone dependent as it has been shown in endometrium cancer [31]. Such a hormone dependent modulation, which is not taken into account in any of these studies, may influence the interpretation of the results [32].

We conclude that high levels of potential DRPs such as PGP, GSH, GST, GPx and ATase in tumor tissue of newly diagnosed, untreated breast cancer are part of the phenotype of the malignant cells and are a reflection of their constitutive characteristics. Overexpression/activity of potential DRPs does not necessarily explain if and how they function as DRP modulators in clinical drug

resistance. In addition EBC and LABC as well as primarily metastatic disease may have their own specific pattern of potential DRP composition. Our findings illustrate the difficulty to associate single biological parameters with prognosis. DRPs appear to be a complex system of partly interdependent parameters, most likely with none of them being exclusively responsible for treatment outcome. Studies which aim at circumventing drug resistance by adding only one modulating agent to standard chemotherapy may therefore ultimately fail [3, 33]. If significant impacts on clinical outcome are expected from future studies on human tumor tissue samples so that 'bench work' can be translated into therapy recommendations for the patient, it will be necessary to standardize laboratory assays and to study a representative variety of markers prospectively in larger and more homogeneous patient groups.

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