Variability of glutathione S-transferase isoenzyme patterns in matched normal and cancer human breast tissue

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The determination of GST levels in blood has been proposed as a marker of tumour burden in general, whereas level of the P1 isoenzyme has been identified as a prognostic factor for breast-cancer patients receiving no adjuvant chemotherapy. Particular glutathione S-transferase (GST) isoenzymes differ in their substrate specificity, however, and their presence or absence might therefore account for the resistance of tumours to particular chemotherapeutic drugs, as already established for cultured cell lines. Determination of the GST isoenzyme profile of a cancer tissue could have prognostic value in the selection of treatment if the levels of expression/activity show a degree of variation comparable with that exhibited by actual patient responses. Using reversed-phase h.p.l.c. to quantify affinity-isolated GSTs, we have analysed full isoenzyme profiles in the first large sample of matched normal and cancer human tissues (18 breast-cancer patients). In no patients did the tumour tissues express any isoenzymes that were not found in normal breast tissue. In addition to the GSTs, another enzyme, identified as enoyl-CoA isomerase, was regularly found in breast tissue cytosol following elution from a hexyl-glutathione affinity column. In most cases, the average level of GST was substantially elevated in the cancer tissues above the levels in normal breast tissue from the same patient. Furthermore, the relative levels of the isoenzymes were substantially more variable in the cancer samples than in the normal breast tissue, providing a plausible mechanism for the well established variable response to treatment.

INTRODUCTION

Glutathione S-transferases (GSTs, EC 2.5.1.18) constitute a multi-gene superfamily of cytosolic enzymes that catalyse conjugation of electrophilic compounds with the scavenger peptide glutathione [1]. These reactions can detoxify xenobiotics including cancer chemotherapeutic agents of the alkylating and antibiotic classes, for example chlorambucil and doxorubicin, as has been extensively reviewed [2]. There are four major sequence-defined classes of cytosolic GSTs: α (A), μ (M), π (P) and θ (T), which exhibit broad, overlapping substrate specificities [3]. In humans, two α and five μ isoenzymes, but only one π isoenzyme, have been firmly identified at the protein level [4]. So far, two θ isoenzymes have been positively identified [5,6]. Based on DNA hybridization data and sequence similarity to other animal species, additional forms of all classes probably exist [1]. GST is active as a dimer, composed of monomers from a single class with each subunit functioning kinetically as an independent catalytic site [7].

GSTs are normally present at $\sim 1\%$ of total cell protein as part of the phase II defensive system against electrophilic toxins, such as mitochondia-derived free radicals. As such, GSTs are also well positioned to be major contributors to the multifactorial process of tumour resistance to many chemotherapeutic drugs. The most direct evidence for such a role has come from the transfection of GSTs into yeast [8] or mammalian cells [9], which increased resistance to chlorambucil and doxorubicin. Use of the generalized GST inhibitor ethacrynic acid potentiates the cytotoxicity of alkylating agents in numerous cell lines [10]. Further, ethacrynic acid treatment of patients with advanced cancer inhibited cellular GST activity and increased the plasma levels of the alkylating agent thio-TEPA; no severe collateral toxicities were observed in this Phase I trial [11]. In one anecdotal case study from a similar protocol, a major remission was achieved [12]. Inhibitors of GST which are more isoenzyme-specific than ethacrynic acid have recently been developed [13], and their ability to potentiate cytotoxicity of alkylating agents in cell culture is proportional to their inhibitory potency for isoenzymes prominent in the cells [14].

Normal human tissues exhibit considerable variation in isoenzyme content. For example, liver and kidney tissue contain primarily α-class GSTs, although the level of P1-1 is higher in kidney than in liver [3]. By contrast, P1-1 is the primary isoenzyme in other normal tissues such as placenta, lung, heart and spleen. Evaluation of the μ-class isoenzymes in most tissues has primarily focused upon the M1 locus, expressed in lung, colon, stomach, breast, kidney and liver [15]. The M1 locus has attracted special attention because its hereditary absence in approx. half of the population correlates with an increased risk of lung cancer for smokers [16]. Other μ isoenzymes have been identified in specific tissues: M2 in muscle [17], M3 and M5 in brain [18,19], and M4 in lymphoblastoid cell lines [20], with other tissues not yet having been fully explored. The presence of θ-class isoenzymes in human tissues has not been well documented as these isoenzymes do not bind to the GSH or hexyl-GSH affinity sorbents typically used as the first step in the isolation of GSTs.

Human tumours from a variety of tissues, including colon and breast [15], lung [21] and stomach [22], have previously been shown to express high concentrations of P1-1 in a majority of the samples, with the other more complex isoenzyme families having

Abbreviations used: CDNB, 1-chloro-2,4-dinitrobenzene; GST, glutathione S-transferase; HxGSH, S-hexyl-glutathione; r.p., reversed-phase.

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received less attention. Significantly, minor isoenzymes can contribute greatly to the detoxification of particular substrates, as shown for aflatoxin B1 epoxide [23].

Identification and quantification of the full spectrum of human GST isoenzymes in tissue samples has been limited for methodological reasons. Many studies have relied upon assays of enzymatic activity using a convenient substrate such as 1-chloro-2,4-dinitrobenzene (CDNB) [24,25], resulting in considerable uncertainty about absolute levels of isoenzymes with different specific activities towards this substrate. SDS/PAGE following an affinity chromatography step has allowed improved identification of isoenzyme class (molecular-mass range 23–28 kDa), but has not been able to distinguish among isoenzymes within a class. Procedures relying upon antibody reactions have also been largely limited to class identification. The most effective high-resolution analysis reported so far is a reversed-phase (r.p.)-h.p.l.c. procedure that has achieved separation of essentially all known isoenzyme subunits, although under denaturing conditions. Only a small number of samples analysed at this level of resolution have been reported [26], and even fewer in which tumour and matched normal tissue have been analysed in parallel. No previous attempts have been made to determine breast-tissue GST isoenzyme profiles by a method with resolution comparable to the r.p.-h.p.l.c. method.

Analysis of breast tissue is of special interest because treatment of breast-cancer patients with alkylating agents is a standard therapy following surgery [27]. GST isoenzyme variability in breast tumours may play a role in the well known variability of clinical efficacy of standard therapy. In this report, we have used an improved r.p.-h.p.l.c. methodology, supplemented by other methods as appropriate to validate the method, to identify and quantify GSTs in matched normal and cancer human breast tissue.

MATERIALS AND METHODS

Reagents

Epoxy-activated Sepharose-6B was obtained from Pharmacia LKB Biotechnology, Inc. (Uppsala, Sweden). *S*-Hexyl-GSH (HxGSH) was synthesized by the method of Vince et al. [28]. HxGSH was coupled to the Sepharose-6B via the free NH_2 group on glutamic acid in 33% (v/v) ethanol adjusted to pH 11 [29]. Silent Monitor 96-well membrane-bottomed microplates were purchased from Pall Biosupport (East Hills, NY, U.S.A.). To prevent proteins sticking to the plastic, standard microplates (COSTAR, Cambridge, MA, U.S.A.) were routinely soaked in 0.1% (v/v) Tween, rinsed, and dried before use. The protein assay Dye Reagent Concentrate was obtained from Bio-Rad Laboratories (Richmond, CA, U.S.A.). All other chemicals were reagent-grade commercial products.

Tissue handling

Samples of cancerous breast tissue at lump resection, along with a portion of surrounding normal tissue removed as a precaution against local metastases, were obtained from the Cooperative Human Tissue Network (Columbus, OH and Birmingham, AL, U.S.A.), and stored at −80 °C. A portion of each tissue was examined by a pathologist at the source facility and confirmed as being cancerous or normal. All cancer samples selected for this study were infiltrating ductal carcinomas. Samples were slightly thawed, minced with scissors and homogenized at 4 °C in TED buffer (containing 10 mM Tris/HCl, pH 7.8, 1 mM EDTA, 1 mM dithiothreitol and 100 μM phenylmethanesulphonyl fluoride) using an OMNI stator-generator homogenizer (Marietta, GA, U.S.A.). Cytosol was prepared by ultracentrifugation at 105000 *g* for 35 min at 4 °C in a Beckman Optima TL-100 tabletop ultracentrifuge.

Affinity purification of GSTs

Affinity chromatography performed in membrane-bottomed 96-well microplates has been described earlier [30]. Briefly, 400 μl of a 50% (v/v) slurry of HxGSH–Sepharose-6B sorbent was pipetted into appropriate wells in the membrane-bottomed microplate, and the resulting micro-affinity columns were equilibrated with the homogenization TED buffer by two cycles of centrifugation at 650 *g* for 30 s in a Beckman TJ-6 centrifuge equipped with microplate carriers. Cytosolic protein (2 mg, ~ 200–400 μl of crude breast cytosol) was applied to the semi-dry sorbents in three equal aliquots and incubated with continuous shaking in a Minimix vibrator (Fisher, San Francisco, CA, U.S.A.) for 30 min at 4 °C. The sorbent–microplate was placed above a recipient Tween-treated standard microplate and centrifuged to recover the flow-through fractions. The sorbents were washed with the same TED buffer and the retained proteins were eluted with 10 mM HxGSH in TEN buffer (TED buffer supplemented with 200 mM NaCl). The variability of GST isoenzyme levels in elutions prepared by this method was < 5% in our previous extensive studies of rat-liver cytosol. Eluted samples were stored frozen until use; no difference in recovery was observed between frozen and fresh samples as long as fresh dithiothreitol was used in the buffer.

Gel electrophoresis

SDS/PAGE was performed according to standard methods [31] using a 12.5% (w/v) acrylamide gel. Proteins were visualized after electrophoresis by silver staining [32]. Molecular mass (kDa) was calibrated against a standard mixture of proteins purchased from Bio-Rad (Richmond, CA, U.S.A.). Western blot analysis was performed by the method of Castro et al. [33] following SDS/PAGE separations of the samples. The proteins were transferred to poly(vinylidene difluoride) membrane (Millipore, Bedford, MA, U.S.A.) for 20 h. Isoenzymes were visualized by treatment with rabbit antisera (prepared by Terrapin) raised against each of recombinant P1-1, A1-1 and M1a-1a, as well as rabbit serum (gift of I. Listowsky, Mount Sinai Medical School, New York, NY, U.S.A.) against an internal peptide from M3-3 [19].

R.p.-h.p.l.c.

Analysis of a 25 μl sample of affinity eluate was performed by a modification of the method of Kelley and Bjeldanes [34]. Using either a Dionex Bio-LC (Sunnyvale, CA, U.S.A.) or a Rainin Rabbit h.p.l.c. system (Woburn, MA, U.S.A.), with a 0.46 cm × 25 cm r.p. column (Dynamax 300 Å C-4, Rainin Instruments; or Vydac 218ATP54 C-8) and acetonitrile/0.1% (v/v) TFA and water/0.1% TFA as solvents, a gradient from 41 to 51% acetonitrile over 55 min at a flow rate of 0.6 ml/min was delivered. The 25 cm column length improved the separation of the GST isoenzymes over that seen with a 5 cm column used previously. The elution was monitored at 214 nm, and isoenzyme levels were determined by integration of the u.v. peak areas. Purified recombinant GST A1-1, P1-1, M1a-1a, and M2-2 used as standards were provided by B. Mannervik (Uppsala University, Uppsala, Sweden). For aliquots of the same specimen run on both systems, the peak areas across the chromatograms agreed to within experimental error, following normalization to account for different instrument parameters.

RESULTS

Source of tissue and processing

Pathology data for the 18 patients analysed are summarized in Table 1. All were classified by on-site pathologists as infiltrating ductal carcinoma. Most specimens were received from patients at primary presentation, but three patients relapsing following surgery with no additional treatment are also included, as noted. Following homogenization, the cytosolic fraction was collected. The GSTs under consideration in this study are all cytosolic enzymes; a distantly related microsomal GST has been reported but is not considered here [35]. To determine the recovery of GSTs from the preparation procedures, recombinant human P1-1 was chosen as representative and a fixed amount added into both human breast and liver samples. In both tissue types, the recovery of the P1-1 as determined by increases in u.v. peak area was > 93%.

Table 1 Pathological characteristics of patients with breast cancer

Patient number is not sequential as additional unmatched tissues were also received from the Cooperative Human Tissue Network. IDC: infiltrating ductal carcinoma. Metastases: entry represents number of lymph nodes positive for cancer/number of nodes examined; NA, data not available. OR: oestrogen-receptor status as determined by the centre providing the tissue. PR: progesterone-receptor status as determined by the centre providing the tissue.

Patient	Age	Diagnosis	Metastases	OR	PR	Recurrence
1	79	IDC	0/23	−	−	
2	69	IDC	0/22	+	+	
3	53	IDC	0/9	−	−	
4	88	IDC	0/7	+	+	
5	43	IDC	10/30	+	+	
6	41	IDC	1/2	+	+	
8	70	IDC	7/11	+	−	
9	61	IDC	0/7	+	+	
10	63	IDC	1/19	−	−	
11	34	IDC	6/11	NA	NA	Yes
12	70	IDC	NA	−	−	
13	51	IDC	0/13	NA	NA	
14	48	IDC	0/7	−	−	Yes
18	50	IDC	NA	+	−	
24	66	IDC	3/4	−	−	
27	47	IDC	14/17	NA	NA	Yes
29	87	IDC	0/16	+	+	
33	67	IDC	2/4	+	+	

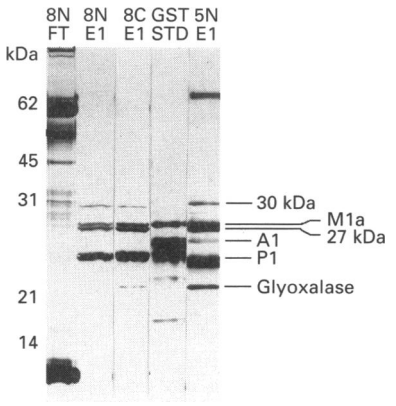

Figure 1 SDS/PAGE analysis of affinity-purified GST isoenzymes in normal and cancer human breast tissues

Flow-through (FT) and elution (E1) fractions from normal (N) and cancer (C) breast tissue were analysed on 12.5% (w/v) polyacrylamide gels with recombinant human GST M1a (28 kDa), A1 (26 kDa) and P1 (23 kDa) as standards (GST STD). Samples from patient 5 and patient 8 were run on two separate gels. A band at 62 kDa in the 5N E1 lane has been seen in all flow-through fractions from the affinity column, and is variably present in the elution fractions, presumably because of incomplete washing.

Isoenzyme identifications

HxGSH–Sepharose affinity sorbents have been widely used to purify GSH-dependent enzymes. Previous work has shown that proteins of monomer molecular mass 23–28 kDa purified on these sorbents have GST activity [36]. Another GSH-dependent protein, glyoxalase I, also binds to HxGSH, but has a lower molecular mass (21 kDa); because of its acidity, it migrates as if it were somewhat larger on SDS/PAGE, but is still well resolved from the higher molecular mass GST bands [37]. SDS/PAGE analysis shows that the four major affinity-purified monomers detected in breast tissue are in mass all near the molecular-mass range of GSTs (Figure 1), with some specimens also showing a band migrating at the glyoxalase I position. In addition to these four major bands, very low levels of proteins co-migrating with α-class isoenzyme standards were detected by SDS/PAGE in the normal breast tissue of six patients. Of these patients, only two had detectable levels of the corresponding proteins in the matched cancer tissue.

Two of the major proteins show very similar gel electrophoretic mobilities as recombinant P1 and M1 monomers respectively, although the π-class isoenzyme in breast tissue occasionally migrated slightly faster than a recombinant P1 standard. In Western blot analysis, the bands comigrating with P1 and M1a were stained intensely by polyclonal antibodies that recognized the corresponding recombinant GST (results not shown). With regard to identifying the 27 kDa protein, only weak and variable immunoreactivity was observed with the rabbit antisera to P1, M1 and A1. Strong staining was observed, however, with a serum raised against a peptide from M3 [19].

The chromatographic methods developed for this study provide near base-line separation of the GST isoenzymes in breast tissues (Figure 2), superior even to the advanced methods previously published [26]. Because the methods can be used with tissue samples as small as 200 mg, we were able to analyse a variety of samples. Peaks from all four proteins observed by SDS/PAGE resolved within the range of known human GSTs. In breast tissue, the proteins identified as P1 and M1a co-eluted with the corresponding recombinant human P1 and M1a. The 27 kDa protein, eluting immediately following A1, matches the position reported recently for the M3 monomer found in drug-resistant HeLa cells [38]. A sample of recombinant M3 [39] gave several peaks, none of which matched the position of the 27 kDa protein, a finding which we attribute to changes occuring during transport.

Preparative-scale isolation of the 27 kDa protein yielded enough material for partial sequencing. The protein appears to have a blocked N-terminus, but enough of an internal tryptic fragment was obtained to provide a partial sequence of 19 amino acids: HNMXGTXTTTXIRVDIIXN (X, ambiguous assignment). The only catalogued sequences matching this sequence were all μ-class GSTs, in particular M3, for which residues 84–102 have a 63% confirmed sequence identity. The triplet TTT corresponds in position to a triplet EEE reported from DNA sequencing of the M3 gene. Because of high background signals in the peptide sequencing chromatography, we cannot be confident of the assignments for these positions; for the reliable

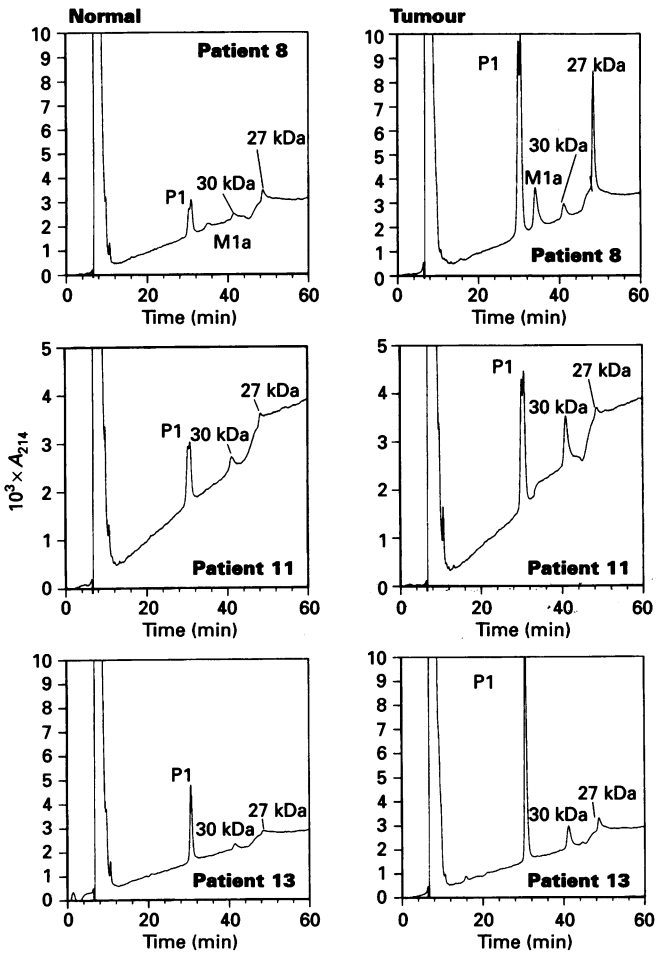

Figure 2 R.p.-h.p.l.c. separation of affinity-purified GST isoenzymes in normal and cancer human breast tissues

HxGSH–Sepharose eluates were separated using a gradient from 41 to 51% (v/v) acetonitrile in water plus 0.1% (v/v) TFA over 55 min. The elution was monitored via A_{214}.

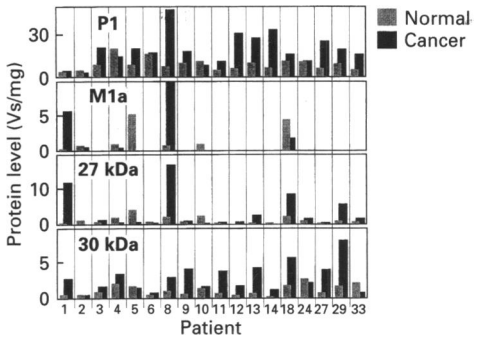

Figure 3 HxGSH-affinity-purified proteins in normal and cancer breast tissue

Protein levels were determined by integration of detector output (V), measuring u.v. peak areas (214 nm) from r.p.-h.p.l.c. analysis, and normalized to the protein in the original tissue extract; units are thus Vs/mg of cytosolic protein. The isoenzyme profiles of patients 1, 3, 8, 11, 18, 24, 29 and 33 showed two P1 forms, which were pooled for quantification.

sequence positions, therefore, the identity of sequence with M3 is 100%. Sequencing of a second peptide fragment yielded more unambiguous results: LLLEFTDTSYEEKR, a sequence which matches 100% with M3 beginning at position 18.

For the remaining major protein, migrating on gels at 30 kDa, identification with known GST isoenzymes was not immediately available by comparison with purified material. We have observed an apparently identical protein in lung tissue as well. Howie et al. [15] also found a protein in breast tissue at the same position as the 30 kDa protein, relative to M1 on SDS/PAGE, which was not reactive to an anti-M1 antibody. We have also seen only low reactivity with antibody prepared against human M1a protein, and at higher tissue concentrations with antibodies to A1 and P1 isoenzymes. As discovered by Listowsky and co-workers [47], a 30 kDa protein with similar properties, isolated from human liver, has been identified as Δ^3,Δ^2-enoyl-CoA isomerase (EC 5.3.3.8). A sample of this enzyme was co-injected on our r.p.-h.p.l.c. system with tissue containing our 30 kDa protein, and found to co-chromatograph. This protein is thus presumed not to be a GST.

Glyoxalase I does not resolve on the r.p.-h.p.l.c. column under conditions for GST separation. R.p.-h.p.l.c. analysis detected low levels of the A1 isoenzyme in two breast tissues but no other previously described α-class isoenzymes. In eight of the 18 patients, the P1 isoenzyme was split into two peaks (Figures 2a and 2b), a finding that was consistent in the normal and cancer tissue of all eight patients. Allelic variants of the P1 isoenzyme have been tentatively identified by Mannervik et al. [4]. The two peaks, which overlap during migration on SDS/PAGE (Figure 1), were typically of equal quantities, consistent with the presence of a heterodimer of the two alleles. Dimerization has only been reported for monomers within a class [40]. For quantification, the u.v. peak areas were combined to represent total P1 present in the tissue. The second peak of the doublet corresponds with recombinant human P1.

On the basis of these data, we believe that the four major peaks detected in cytosols from breast tissues following affinity chromatography on HxGSH correspond to GSTs P1, M1a, M3 and enoyl-CoA isomerase. Isoenzymes of the θ class, which do not bind to the HxGSH affinity sorbent, would not have been detected in this analysis.

Isoenzyme profiles

Figure 3 summarizes the levels of the four major GSH-binding proteins detected in normal and breast cancer tissues of 18 women with infiltrating ductal carcinoma. Consistent with more limited data from other studies [15,41,42], P1 was the predominant isoenzyme in the normal and cancer tissues of all patients (note the expanded scale for P1 in the Figure), and it was elevated in most cancer tissues relative to the surrounding normal tissue. The M1 isoenzyme occurred in the breast tissue of 40% of these patients, consistent with the frequency of a null mutation at the M1 locus in the general population [16].

Scanning across the histogram for each of the four proteins, it is clear that our sample of breast-cancer tissues contained a higher average level of protein than the normal tissues. None of the breast-cancer tissues contained any isoenzymes that were not found in the matched normal breast tissue. Scanning down the columns representing individual patients, it is apparent that P1 was not always the protein with the largest proportional elevation. Further, the S.D. for each of the four proteins analysed

was 2–4-fold higher in the tumour than the normal breast tissue. Multivariate statistical analysis did not show any clear correlation between high levels of one isoenzyme and any of the others, resulting in overall variability for the tumours being higher than for the normal specimens. Patient number 8 showed an unusually high level of most of the detected proteins, in contrast to the rest of the samples for which it appears that elevation of one isoenzyme may compete with elevation of others; no other information is available to suggest why patient 8 is so extreme.

DISCUSSION

Breast cancer is a major cause of morbidity and mortality in women. At this time, the primary treatment for breast cancer is surgery to remove the cancer and any involved axillary lymph nodes [27]. Many patients are also given systemic treatments of hormones or adjuvant chemotherapeutic drugs to increase their chances for long-term survival. Treatment with alkylating agents has provided good clinical response for some patients. However, the rate of success of these chemotherapeutic treatments is not predictable by the markers that are currently available. In a major longitudinal study of node-negative patients receiving no further therapy, prognosis had a negative correlation with increased levels of GST P1-1 that was more informative than oestrogen-receptor status [43]. Given the established role of other GST isoenzymes in drug detoxification *in vitro* and in cell culture, it is important to consider them as possible prognostic markers.

We have found that in infiltrating ductal carcinoma tissue, both the levels and the variability of the GST isoenzymes are elevated relative to normal breast tissue. P1, the predominant isoenzyme in normal breast tissue, was elevated in matched cancer tissue of the majority of patients. Our findings of M1 in the tissues of 40% of patients, consistent with the frequency of the M1-null phenotype in the general population, suggests that its absence does not confer a greater risk for this tumour type, as seen for example in lung [16].

A second peak of the P1 isoenzyme, which may represent a distinct allelic or post-translationally modified form of this isoenzyme [44], was also consistently detected in tissue of some patients. Minor modifications of the GST isoenzymes, such as single amino acid changes or glycosylation, are known to affect p*I* and may also cause shifts in the substrate specificity of the isoenzymes [45], in the overall levels of activity, or in stability. In addition to P1 and M1a, the r.p.-h.p.l.c. procedure used in this study has detected two additional GSH-binding proteins, identified in this work as M3, a μ-class GST and enoyl-CoA isomerase, which were present in breast tissue of all patients.

The majority of patients studied showed an absence of known α-class GSTs in normal or breast cancer, although a small number of patients did display low levels of A1. Increased resistance to alkylating agents has been associated with α-class GSTs in resistant cell lines [10], as well as in cells transfected with α GST genes [9]. The relatively low levels of α isoenzymes in these patients does not completely eliminate the potential role of these isoenzymes in detoxifying the chemotherapeutic drugs, however. Small increases in α isoenzymes have been linked to a 90% decrease in the levels of DNA-adduct formation with aflatoxin B1, a liver carcinogen that is specifically detoxified by these isoenzymes [23].

The known variability in clinical utility of alkylating agents, combined with the variability in overall profile observed here, provides a firm foundation for planning clinical correlation studies. Although clinical outcomes for the patients studied here are not yet available, the results presented here argue strongly for collecting such data in a controlled prospective clinical trial. Retrospective studies would also be of interest if sufficient tissue is available for analysis, which is not usually the case. The findings of a limited number of GST isoenzymes expressed in breast tissue and the absence of new isoenzymes in tumours improves the feasibility of such studies. This work also provides a foundation for developing therapeutic strategies directed to proteins elevated in tumours, irrespective of their function; for example, we have reported synthesis of a cytotoxin activated by high levels of a particular GST isoenzyme [46]. In this regard, it is intriguing to have discovered variability in tumour levels of the lipid-metabolizing enzyme enoyl-CoA isomerase that is comparable to that of GSTs. The net effect of this enzyme is to accelerate chain shortening of membrane lipids, which should affect the fluidity of the membrane, which in turn may affect the function of a variety of growth regulatory receptors.

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