Glutathione Transferase Activity and Isoenzyme Composition in Primary Human Breast Cancers

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ABSTRACT

The human glutathione transferases (GSTs) are a multigene family of detoxication enzymes patterns with expression that are both tissue specific and genetically determined. Changes in the levels of one or more GST isoenzymes have been associated with the development of anticancer drug resistance in cultured cell lines. In this study, total GST activity and GST isoenzyme composition have been determined for 45 primary human breast carcinomas using a 1-chloro-2,4-dinitrobenzene substrate assay and Western blotting, respectively. The GST activity ranged from 5–208 mU/mg protein with a mean of 67 mU/mg protein (±44 SD). GST-α isoenzyme protein was detectable on Western blots in 44 of 45 samples. Mu Class GST protein was detected in 18 of 38 samples and undetectable in 20 of the 38 samples tested. By polymerase chain reaction analysis of genomic DNA, the absence of mu class GST in breast tumors was determined to be due to the deletion of the gene for GST-μ in the DNA of those tumors. None of the 43 primary human breast cancer samples tested contained detectable alpha class GST protein. Neither the total GST activity of tumor samples, the quantity of GST-α protein, nor the presence or absence of mu class GST correlated with other factors known to have a high level of prognostic significance including tumor size, nodal status, estrogen receptor protein positivity, or progesterone receptor protein positivity. Substantial differences exist among primary breast carcinomas in both the amount of GST activity and GST isoenzyme composition. However, these are not tightly linked either to tumor stage or to hormone receptor status. Whether the levels of these enzymes are independent predictors of either risk of recurrence or response to anticancer therapy has yet to be tested directly.

INTRODUCTION

The human GSTs are a multigene, isoenzyme family. The human cytosolic GST isoenzymes can be classified by their substrate specificities, isoelectric points, amino acid sequence homologies, and immunological relationships into three major classes termed alpha, mu, and pi (1). Within the alpha and mu classes are multiple isoenzymes [e.g., GST-α, GST-β,…GST-γ (alpha class), GST-α, GST-σ (mu class)]. Only one GST isoenzyme, GST-π, has been identified in the pi class. These isoenzymes are expressed in a tissue-specific pattern and one or more GST isoenzymes are present in a variety of normal and malignant human tissues including breast carcinomas (2–5). The GST isoenzymes are believed to play an important role in the cellular metabolism and detoxication of electrophilic compounds by their conjugation to glutathione (6). Recent investigations have revealed that these enzymes are capable of utilizing several clinically active alkylating agents as substrates for conjugation to glutathione (7, 8).

Increased GST activity has been reported in a number of rodent (8–11) and human (12–16) tumor cell lines selected in vitro for resistance to anticancer drugs. There are also reports of elevated GST activity in cell lines derived from tumors in patients who had developed clinical resistance to either cisplatin or chlorambucil (17, 18). However, the exact role of the GST elevations in the resistance of these cells to antineoplasics remains to be established (16).

In addition to the roles of GST in drug metabolism, detoxication, and resistance, there are several reports suggesting that a particular GST isoenzyme, GST-π, may serve as a biochemical marker for neoplastic transformation. This was suggested by work using a rat tumor model and by measurement of GST-π in primary human tumor samples (19–21). Recent reports indicate that levels of GST-π mRNA (22) and protein (23) are negatively correlated with ERP content in primary human breast cancer specimens, suggesting that increased GST-π activity is correlated with a marker (i.e., ERP negativity) that indicates a worse prognosis in this disease.

The current study was undertaken to determine the levels of GST activity and isoenzyme composition of primary human breast cancers from untreated patients. The long-term goal of these studies is to determine whether the levels of the various GST isozymes might serve as predictors of either prognosis or response to therapy. The results obtained have been compared with prognostic variables of known significance for these patients to determine whether GST activity or isoenzyme content might be linked to known prognostic variables (24). The current report demonstrates that primary breast tumors vary widely as to both total GST activity and GST isoenzyme content and have no strong linkage to known prognostic variables.

MATERIALS AND METHODS

Tumor Samples. All tumor samples were selected from a bank of cryopreserved tumors at the hormone receptor laboratories of St. Elizabeth’s Hospital (Boston, MA) or Oregon Health Sciences University (Portland, OR) which had been frozen within 2 h of surgery and from which samples had been obtained for estrogen and progesterone receptor analysis. Estrogen and progesterone receptor data were obtained by standard methods utilizing freshly prepared cytosol extracts of tumor specimens using a modification of the dextran charcoal technique (25).

Tumors are defined as ERP or PRP positive if they contain >10 fmole/mg protein of ER or PR, respectively. Histological classification, estrogen and progesterone receptor determination, and GST analysis were performed independently without knowledge of the results of the other analyses. All studies were performed with the approval of the Institutional Review Boards of the contributing institutions.

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3 The abbreviations used are: GST, glutathione transferase; PCR, polymerase chain reaction; CDNB, 1-chloro-2,4-dinitrobenzene; ERP, estrogen receptor protein; PRP, progesterone receptor protein; HPRT, hypoxanthine-guanine-phosphoribosyltransferase.
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GST Analysis. Cytosol extracts from tumor samples of approximately 100 mg dry weight were obtained as previously described (2). GST activity was based on enzyme-dependent conjugation of reduced glutathione with CDNB and expressed as mU conjugate formed/mg protein (1 mU = 1 nmol conjugate formed/min) (2, 26). All results represent the means of three separate assays for each individual tumor extract. For analysis of samples by Western blotting, aliquots of cytosolic extract containing 150 μg protein were resolved by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose by standard techniques (27). Replicate blots were probed with a 1:500 dilution of antisera raised as previously described (2) in rabbits immunized with purified human GSTs, either GST-α (2), GST-μ (Medlabs, Dublin, Ireland), or GST-α (28). Second antibody binding and color development were performed as previously described (2).

PCR. Primers for amplifying the GST-μ gene segment corresponding to exon 4, intron 4, and exon 5 were 5'-CTGCCCCTACTTGG-GATGGG-3' and 5'-CTGGATTGTAGCAGATCATGC-3' and were obtained from Midland Certified Reagent Co. (Midland, TX). Primers for amplification of exon 2 of the HPRT gene were 5'-CTGGTCCA-TAATTAGTCCATG-3' and 5'-ATTAGTGATGATGAACCAG-GTT-3' (29). Genomic target DNA for PCR was prepared from human breast carcinoma samples by standard procedures (30). The polymerase chain reaction was carried out as previously described using 1.0 μg of target DNA (31). Amplification of exon 2 of the HPRT gene was used as a control for the target DNAs that were negative for the GST-μ gene by PCR. Eighteen DNA samples were effective targets for amplification of exon 2 of the HPRT gene (data not shown). PCR products were resolved by electrophoresis on 2.1% agarose gels, stained with ethidium bromide, and photographed under UV light.

Statistical Methods. Correlation coefficients were determined for total GST activity as a function of either estrogen or progesterone receptor content (n = 45). Student's t tests of significance were applied to mean GST activity levels as a function of primary tumor size (T1, <2 cm; T2, 2–5 cm; T3, >5 cm), lymph node involvement (0–4, >4 nodes involved), age (<50 years or >50 years, the presence or absence of estrogen (>10 fmol receptor/mg protein) and progesterone (>20 fmol receptor/mg protein) receptors, and the presence or absence of mu class GST. The x² analysis was used to compare the presence or absence of mu class GST with the other categorical (nonlinear) variables listed above. The Wilcoxon-Mann-Whitney test for two independent variables was used to compare the GST activity and GST-α levels of the ERP-positive group versus the ERP-negative group.

RESULTS

First Tumor Set: GST Activity and GST Isozyme Composition. Two sets of cryopreserved breast cancer specimens were obtained from the hormone receptor laboratories of two different institutions. The first set of 58 samples was randomly selected from the bank of cryopreserved tumor specimens that had been assayed for estrogen and progesterone receptor content at St. Elizabeth's Hospital. Following review of histological material and medical records for these patients, 13 samples were excluded from this analysis for the following reasons: recurrent disease, 6 samples; non-breast carcinoma, 4 samples; insufficient tumor in specimen, 3 samples. Forty-five samples with invasive primary breast carcinoma were analyzed (Table 1). Forty-two specimens were composed of infiltrating ductal carcinoma. One sample contained predominantly intraductal carcinoma with a small area of focal invasion and two samples were diagnosed as infiltrating lobular carcinoma. The small number of carcinomas with histology other than infiltrating ductal carcinoma precluded subset analysis by histology. Thirty-four tumors contained significant estrogen receptor (>10 fmol/mg protein), while 11 did not. Seventeen tumors contained significant progesterone receptor (>20 fmol/mg protein) and 28 did not. Clinical information concerning tumor size and nodal status was available for 29 and 26 of the patients, respectively. Six tumors were <2 cm in size (T1), 14 were 2–5 cm (T2), and 9 were >5 cm (T3). Eighteen tumors were associated with >4 involved lymph nodes and 8 tumors were associated with >4 involved nodes. The median age of these 45 patients was 57 years with 29 patients >50 years and 16 patients ≤50 years.

GST Analysis. Forty-five samples were assayed for GST activity utilizing the universal GST substrate CDNB. The mean GST activity was 67 mU/mg of extract protein (SD, 44 mU/mg; range, 5–208 mU/mg). We determined the extent to which the variation in GST activity among different tumors was due to variations in GST activity in different portions of the same tumor sample. A subset of the tumors was randomly selected, and three portions of each tumor were separated for individual preparation of cytosolic extracts. Each extract was assayed in triplicate for total GST activity (see "Materials and Methods"). The mean coefficient of variation for individual extracts prepared from different regions of the same tumor was 36% (Fig. 1). Seven samples of normal breast tissue from reduction mammoplasties were analyzed and found to contain a mean GST activity of 65 mU/mg protein (SD, ±27 mU/mg; range, 32–109 mU/mg protein).

Sufficient tumor extract was available to evaluate GST-α, alpha class GST, and mu class GST content by Western blotting in 45, 43, and 38 specimens, respectively (Fig. 2). Although the classification of samples as positive or negative for each GST isozyme class on Western blotting was qualitative, the difference between positive and negative samples was always clear and the classification of the samples was reproducible among multiple independent observers. Forty-four of 45 samples contained detectable levels of GST-α isoenzyme. The one lacking GST-α was found to contain the lowest total GST activity of all primary specimens evaluated (5 mU/mg protein). The GST-α-negative tumor did contain mu class GST on Western blot. None of the 43 samples evaluated contained detectable alpha class GST protein despite strongly positive liver control samples. Two of the samples that had been excluded from this analysis because they were found on review of histology and medical records to represent metastatic tumor deposits in the

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>n</th>
<th>GST activity (mU/mg)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>45</td>
<td>67 ± 44</td>
<td></td>
</tr>
<tr>
<td>Er- (&gt;10 fmol/mg)</td>
<td>34</td>
<td>66 ± 49</td>
<td>0.78</td>
</tr>
<tr>
<td>Er-</td>
<td>11</td>
<td>70 ± 25</td>
<td></td>
</tr>
<tr>
<td>Pr- (&gt;20 fmol/mg)</td>
<td>17</td>
<td>64 ± 56</td>
<td>0.80</td>
</tr>
<tr>
<td>Pr-</td>
<td>28</td>
<td>68 ± 37</td>
<td></td>
</tr>
<tr>
<td>Mu Class +</td>
<td>18</td>
<td>69 ± 51</td>
<td>0.67</td>
</tr>
<tr>
<td>Mu Class -</td>
<td>20</td>
<td>60 ± 29</td>
<td></td>
</tr>
<tr>
<td>T1 (&lt;2 cm)</td>
<td>6</td>
<td>58 ± 40</td>
<td>0.63</td>
</tr>
<tr>
<td>T1 (2–5 cm)</td>
<td>14</td>
<td>67 ± 36</td>
<td></td>
</tr>
<tr>
<td>T1 (≥5 cm)</td>
<td>9</td>
<td>60 ± 59</td>
<td></td>
</tr>
<tr>
<td>&gt;4 nodes</td>
<td>8</td>
<td>71 ± 51</td>
<td>0.25</td>
</tr>
<tr>
<td>≤50 yr</td>
<td>16</td>
<td>60 ± 51</td>
<td>0.41</td>
</tr>
<tr>
<td>&gt;50 yr</td>
<td>29</td>
<td>71 ± 41</td>
<td></td>
</tr>
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a Determined as described in "Materials and Methods" or from clinical records.

b GST activity was measured by enzyme-dependent conjugation of reduced glutathione with CDNB, as described in "Materials and Methods."

c Student's t tests of significance for differences between GST activities within each category.
d Not significant (P > 0.10) by Wilcoxon-Mann-Whitney test.
liver and skin contained strong signal for alpha class GST (data not shown), presumably from one or more of the alpha class GSTs known to be present in liver and skin (28, 32). Of the 38 samples evaluated for mu class GST content, 18 contained the mu class protein and 20 did not.

We determined whether GST activity correlated with known prognostic factors in breast cancer. There was no correlation between total GST activity and hormone receptor status regardless of whether the relationship was examined as a function of ERP ($r = 0.12, P > 0.5$) (Fig. 3) or PRP ($r = 0.21, P > 0.4$) levels (data not shown) or hormone receptor positivity or negativity (Table 1). Nor was there significant correlation of tumor GST content with primary tumor size, lymph node involvement, age, or primary tumor size (Table 2).

Second Tumor Set: Relationship of GST-μ Protein to Hormone Receptor Levels and Studies of GST-μ Gene Deletion. As described above, no evidence for correlation between total GST activity (as assessed by CDNB conjugation) and ERP level was noted in the first set of breast carcinomas. In view of the recent report by Moscow et al. (22) that GST-μ mRNA levels are negatively correlated with the level of ERP in human breast carcinomas, we tested the specific hypothesis of a GST-μ to ERP correlation. A second set of cryopreserved breast tumor specimens ($n = 20$) was obtained from the hormone receptor laboratory at Oregon Health Science University. Extracts were prepared and analyzed for total GST activity (by CDNB conjugation) and levels of GST-μ protein were quantitated by Western blotting. Levels of GST activity with CDNB were similar to the first set of tumor samples (average, 100.9 mU/mg protein; SD, ±60.5; range, 0–254 mU/mg), 10 of 20 were estrogen receptor positive, 6 of 20 were progesterone receptor positive, and 7 of 20 were mu class GST positive on Western blotting. All of the samples contained detectable GST-μ on Western blots. GST-μ protein levels varied widely among the tumors. Levels of GST-μ protein for a particular tumor were reproducible (experiment to experiment coefficient of variation 30%). The levels of GST-μ protein in the tumor extracts were correlated with the level of total GST activity determined for each tumor ($r = 0.88, P < 0.01$) (Fig. 4). However, no significant relationship between the level of GST-μ protein levels and quantitative ERP ($r = 0.33, P > 0.5$) (Fig. 5) or PRP ($r = 0.33, P > 0.5$) (data not shown) could be demonstrated. Likewise, comparison of the mean GST-μ levels in ERP-positive and -negative samples demonstrated no significant difference ($P > 0.2$, Student’s $t$ test). Use of the Wilcoxon-Mann-Whitney rank order test for comparison of two groups revealed a trend toward higher GST-μ levels in ERP-positive samples (the opposite of that previously reported), but this trend did not reach statistical significance ($P > 0.13$).

GST-μ and GST-ψ are both alleles of the human GST1 locus,
one of several human genes that code for human mu class GSTs (33, 34). However, GST-μ or GST-ψ are expressed in the lymphocytes or liver of only 31–66% of humans (35–37). In individuals who lack GST-μ and GST-ψ, the deficiency appears to be due to homozygous deletion of the GST1 gene that codes for GST-μ or GST-ψ (38). The frequency of mu class GST in breast carcinomas was similar to that of GST-μ/ψ in the total human population, suggesting that the mu class GST, detected on Western blotting of breast carcinoma extracts, was GST-μ/ GST-ψ. This, in turn, suggested that the presence or absence of this enzyme in breast carcinomas might be determined by the presence or absence of the gene for GST1 in the particular individual developing the breast carcinoma.

To test this possibility, a PCR-based assay for the presence of the GST-μ gene in human DNA samples was used (31). In this assay, primers hybridizing to the 5' region of exon 4 and the 3' region of exon 5 amplify a 273-base pair product from target DNA derived from lymphocytes of individuals who carry the GST1 gene. This PCR product consists of the DNA sequence of exon 4, intron 4, and exon 5 of GST1 (31, 33, 38). An appropriately sized product was not amplified from DNA of individuals whose lymphocytes do not contain mu class GST by Western blotting (31). When this assay was applied to study human breast carcinomas, DNAs from 7 carcinomas positive for mu class GST on Western blot were all positive on the PCR assay and only one of the 11 DNAs from Western blot-negative tumors produced an appropriately sized DNA fragment ($P < 0.001$, $\chi^2$ test) (Fig. 6).

### DISCUSSION

The clinical course of carcinoma of the breast is quite variable. Patients can develop recurrences either soon after their primary therapy or many years later. The risk of relapse, to some extent, is predictable, most notably by clinical and pathological staging and hormone receptors (24). However, even within a group of patients of the same stage and hormonal receptor status, the course of disease and response to therapy is quite variable. Numerous investigators have sought to resolve the factors involved through the study of additional markers such as assays that estimate the number of cells in S phase (24) or, more recently, the amount of neu gene expression (39).

The glutathione transferases were selected for study because
of evidence from in vitro studies that they might be associated with the development of drug resistance and might be linked to other tumor markers such as estrogen receptor proteins (22). We have found that primary human breast carcinomas are quite heterogeneous with regard to their content of GST activity. This finding is consistent with prior reports, in which smaller numbers of breast carcinoma samples were studied (2–4, 22). However, we observed no significant correlation between the level of GST activity in human breast cancer and factors known to be prognostic in these patients including hormonal receptor status and nodal status. Moscow et al. (22) reported that GST-π mRNA levels in primary breast carcinomas are inversely correlated with estrogen receptor content in the same tumor specimens. Similarly, Howie et al. (23) reported a negative (but quantitatively weaker) correlation between GST-π protein and estrogen receptor content. Our results do not confirm such a correlation and suggest that such a relationship is either substantially weaker than previously suggested or does not exist.

There are several human GSTs of the mu class which cross-react immunologically and are coded for by several mu class genes (33, 34, 38, 41). Therefore, it was not possible to determine which of the mu class GSTs was present in the breast carcinoma extracts by Western blotting alone. Only about 31–66% of normal individuals have detectable GST-μ activity in peripheral mononuclear leukocytes (37) or liver (40). The presence or absence of this enzyme is inherited as an autosomal dominant marker and the absence of this enzyme has recently been shown to be due to a homozygous gene deletion (38). The similar frequency for mu class GST positivity among breast carcinomas (Table 1) and for GST-μ positivity of liver or mononuclear leukocytes for a normal population suggests that the absence of mu class GST in breast carcinomas (and possibly other neoplasms) may be simply determined by the absence of a copy of the GST-μ gene in the germ line of the individual rather than being a characteristic of the carcinoma itself. The results of the studies using the polymerase chain reaction are consistent with this hypothesis. We have found that the target sequence for PCR, a portion of the GST1 gene locus that codes for the GST-μ and GST-π alleles, is present in all breast carcinomas positive for mu class protein on Western blotting and absent in all but one of the carcinomas negative for mu class protein. The single disparate sample (PCR positive but GST-π protein negative on Western blot) may simply be due to GST-π protein degradation during tissue handling and storage. Alternatively, this result may represent a tumor that contains the GST1 gene but is deficient in one or more of the steps required in production of a stable GST-μ protein.

These results demonstrate three different types of variation in GST content for human malignancies. First, the total GST activity and the quantity of GST-π protein present in primary human breast carcinomas vary substantially. The mechanisms by which this variation in GST-π occurs have not yet been determined. Second, breast carcinomas can be classified as either GST-μ positive or GST-μ negative, and this phenotype appears to be determined by the heredity of the individual who develops the carcinoma. Third, breast carcinomas differ from some other forms of human malignancy, such as renal cell carcinomas, by their lack of detectable alpha class GST (3, 41). The difference in GST-α isoenzyme content among neoplasms of different histology is presumably a function of the differentiation of the tissue of origin. However, the molecular mechanism for such tissue-specific regulation has yet to be defined.

The lack of correlation with known prognostic factors does not necessarily rule out a role for GST content as an independent predictor of prognosis or response to therapy. Such a hypothesis, that GST content is predictive of outcomes, can only be tested through studies of a larger group of patients and their tumors where long-term follow-up information concerning relapse rates and response to therapy is obtained.

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