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Expression of glutathione-S-transferases α and π in gastric cancer: a correlation with cisplatin resistance

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Abstract. Glutathione-S-transferase (GST) in one of several factors that are proposed to affect tumor sensitivity to anticancer drugs, including cisplatin (CDDP). Attempts are made herein to evaluate the significance of the enzymes in resistance to CDDP in clinical samples of gastric cancer. A total of 22 gastric cancer specimens, 16 of which were obtained with matching normal mucosae, underwent immunoblotting with polyclonal antibodies against GST-α and GST-π. At the same time, the chemosensitivity of 15 gastric cancer specimens to CDDP was evaluated by the succinic dehydrogenase inhibition (SDI) test. The expression of GST-π was detected in all the specimens, and its content in the neoplasms exhibited a significant positive correlation with that in the matched normal mucosae. The expression of GST-α was detected in 18 of 22 cancer specimens (82%), but its content in the neoplasms did not correlate with that in the matched mucosae. A comparison of the drug-sensitivity findings with the results of immunoblotting revealed a weak but interesting correlation between the protein levels of GST-α and CDDP resistance. The cellular content of GST-α correlated weakly with CDDP resistance in gastric cancer, and its quantification could contribute to prediction of the clinical effects of CDDP in patients with gastric cancer.

Introduction

Gastric cancer remains one of the commonest neoplasms worldwide, with high incidence rates being reported in Eastern Europe, Asia, and South America [17]. The mortality of patients with unresectable disease is extremely high, and the median duration of survival is 4 months [2]. Adequate chemotherapy is required whenever surgical treatment is considered insufficient, and evaluation of the chemosensitivity of cancer cells from gastrectomy specimens to anticancer agents provides useful information for choosing the correct drugs. We have been working on the clinical application of one such evaluation method, the succinic dehydrogenase inhibition (SDI) test [13, 31], and a brief comparison has been made between the results of P-glycoprotein immunostaining and the chemosensitivity to doxorubicin as evaluated by the SDI test [32], indicating that the cell lines and surgical specimens with positive P-glycoprotein staining are doxorubicin-resistant.

Glutathione-related enzymes have also been shown to be associated with resistance to anticancer drugs such as alkylating agents [29, 30] and cisplatin (CDDP) [23] by conjugation reactions [8], peroxidase activity [1], and ligand-binding properties [4]. These diverse biological functions are mediated by multiple glutathione-S-transferase (GST) enzymes. Mammalian cytosolic GSTs are generally grouped into three classes, GST-α, GST-μ, and GST-π, corresponding to three distinct gene families. Of these, GST-π is known to be invariably detected in gastric mucosa and cancer tissues by immunoblot analysis [25], and a 1.3- and 1.4-fold overexpression of the enzyme has been reported in gastric [25] and colon [24] cancer tissues, respectively, as compared with the corresponding normal mucosae. GST-π mRNA has also been invariably detected in human tumors [20] and cancer cell lines [27], and an increase in its expression over that in matched normal tissues has been readily observed [11, 20]. On the other hand, GST-α is not detected in some gastric cancer tissues by immunoblot analysis and GST-μ levels are negligible in gastric cancer specimens [25].

In the present study, the protein contents of GSTs in human gastric mucosae and gastric cancer tissues were quantified by immunoblot analyses using polyclonal antibodies against GST-α and GST-π, and correlation studies were made between these results and the ID_{50} value (50% growth-inhibitory dose) for CDDP as evaluated by the SDI test.

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Materials and methods

Cell line. A gastric cancer cell line, MKN45, was obtained from the Japanese Cancer Research Resources Bank and cultured in RPMI 1640 medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal calf serum.

Tissue procurement. Gastric neoplasms were obtained at surgery from patients who underwent gastrectomy at Nagoya University Hospital, Nagoya, Japan. A total of 15 such gastrectomy specimens were rinsed with sterilized saline, and cancer tissues were resected from the mucosal side and kept in ice-cold Hanks' balanced solution until their use in the chemosensitivity test, which was performed within a few hours of resection. Further cancer sections from the 15 specimens described above, 9 of which were obtained with adjacent normal mucosae, and 7 additional cancer specimens with matched normal mucosae were obtained from the surgically resected specimens, frozen promptly in liquid nitrogen, and kept at –80° C until their use in the protein-extraction procedure. None of the patients had received chemotherapy prior to the surgery.

Chemosensitivity test. CDDP was provided by Nihon Kayaku Pharmaceutical Industries, Tokyo. It was diluted into solutions of 10, 25, and 50 μg/ml with RPMI 1640 medium supplemented with 20% fetal calf serum just before its use in the chemosensitivity test.

The SDI test was performed as previously described [28, 32] using 3-(4,5-dimethylthiazol-2-yl)2,5-d:phenyl-formazan bromide (MTT). Cell suspensions were prepared by mincing of the tumor followed by incubation for 30 min at 37° C with 0.02% (W/V) collagenase type I (Worthington Biochemical Corp., New Jersey, USA), 0.02% DNase I (Boehringer Mannheim GmbH, Germany), and 0.02% Pronase (Boehringer Mannheim GmbH, Germany). Cell numbers were adjusted to 5×10^5 cells/ml of RPMI medium supplemented with 20% fetal calf serum, and 100 μl of the cell suspensions thus prepared was distributed into each well of 96-well microplates containing 100 μl of CDDP solutions of the serial concentrations described above. A set of six wells containing no CDDP served as a control.

The cancer cells were incubated for 48 h at 37° C in an atmosphere containing 5% CO_2. After washing of the cells with phosphate-buffered saline (PBS), 10 μl of 0.1 *M* sodium succinate (Katayama Chemical, Osaka, Japan) and 10 μl of 0.4% MTT (Sigma Chemical, St. Louis, USA) were added and the solution was allowed to react for 3 h at 37° C. After completion of the reaction, 150 μl of dimethylsulfoxide (DMSO; Katayama Chemical, Osaka, Japan) was added to each well to dissolve formazan, a product of the reaction, and the optical density (OD) was measured with a spectrophotometer (Easy Reader EAR-340, SLT Lab Instuments, Salzburg, Austria) at a wavelength of 540 nm. The OD value was found to reflect accurately the proportion of cells surviving at a total cell count of over 10^4/ml (data not shown). The ID_{50} value was worked out from the line plotted with three OD values, which represented the mean values obtained for six wells at each of the CDDP concentrations described above. The SDI test was considered valid only when the coefficient of variation (CV) of the OD values obtained for six control wells containing no CDDP was under 20%. In the present study, ID_{50} was defined as the dose of CDDP required to reduce the OD value in the assay to 50% of the control value.

Protein extraction and immunoblotting. Immunoblotting using anti-GST polyclonal antibodies (Novocastra Laboratories Ltd. Newcastle, UK), each of which is known to be non-cross-reactive with other isoenzymes [9], was performed as previously described [5], with modifications. Briefly, samples of cell sonicate containing 25 μg of protein as quantified according to Lowry et al. [16] were electrophoresed through 15% polyacrylamide gels containing sodium dodecyl sulfate and then electrophoretically transferred to a polyvinylidene difluoride (PVDF) filter (Millipore). The filter was incubated first for 1 h in anti-GST-α or anti-GST-π polyclonal antibodies (1:500) and then for 1 additional h in ^{125}I-labeled protein A. The membrane was then air-dried and a BAS2000 Imaging Analyzer (Fuji Photo Film, Kanagawa, Japan) was used for the densitometric analysis of the blots.

Table 1. GST contents and ID_{50} values in mucosal and cancerous specimens of the stomach

Case	GST-α		GST-π		ID_{50} CDDP
	N	T	N	T	($\times10^{-4}$ *M*)
1	453	103	114	81	1.32
2	100	169	106	73	1.56
3	519	21	108	122	1.55
4	ND	65	ND	108	1.35
5	ND	34	ND	54	1.36
6	96	391	115	100	ND
7	148	42	35	33	ND
8	4	7	12	51	1.01
9	15	0	47	5	0.75
10	4	11	67	130	1.05
11	12	15	217	185	ND
12	40	81	48	123	ND
13	ND	22	ND	96	0.57
14	31	6	26	89	ND
15	169	103	173	136	1.28
16	ND	0	ND	132	1.12
17	ND	0	ND	175	1.00
18	47	100	28	45	ND
19	163	76	28	47	2.17
20	64	79	103	88	0.91
21	34	178	37	53	ND
22	ND	16	ND	62	1.22

The expression of GSTs in the control specimens (cancer tissue of case 18 for GST-α and gastric cancer cell line MKN45 for GST-π) was designated as 100, and the contents in the rest of the specimens were expressed in relation to the controls. N, Normal gastric mucosa; T, gastric cancer tissue; ND, not done

Each filter included control specimens (cancer tissue of case 18 for GST-α and cell line MKN45 for GST-π), and the intensities of the blots of individual specimens quantitated by BS2000 imaging analysis were expressed in relation to the control values, which were designated as 100.

Statistical analysis. Wilcoxon's paired test was performed for comparison of the protein levels between neoplasms and normal mucosae. Pearson's correlation coefficients were calculated both for the evaluation of correlation between ID_{50} values for CDDP and levels of GSTs and for the evaluation of correlation between levels of GSTs in normal mucosae and cancer tissues.

Results

Chemosensitivity test

The ID_{50} values for CDDP were worked out in 15 gastric cancer tissues. They ranged from 5.70×10^{-5} *M* to 2.17×10^{-4} *M* in gastric cancer specimens. The values are summarized in Table 1.

Expression of GSTs in gastric cancer and mucosae

The expression of GST-α and GST-π as qunatified in 16 matched pairs of gastric mucosae and cancer tissues by immunoblot analysis (Fig. 1) are summarized in Table 1. The intensities of the bands corresponding to GST-π in the

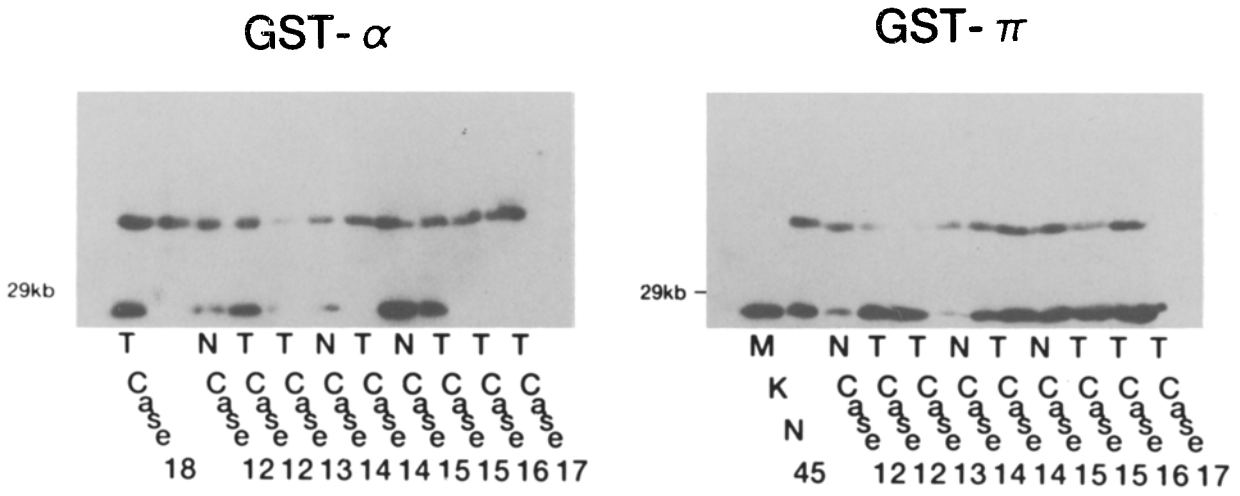
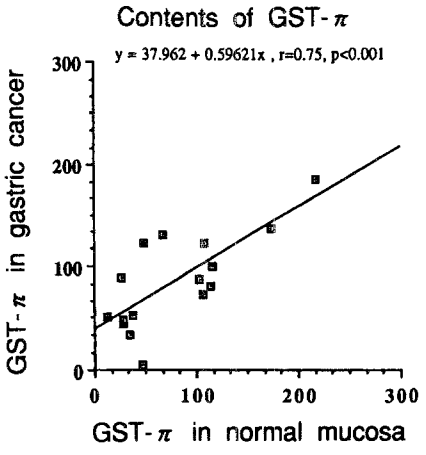


Fig. 1. Immunoblotting of GST-α (*left*) and GST-π (*right*) in protein extracts obtained from normal gastric mucosae (*N*) and gastric cancer (*T*). Case numbers correspond to those listed in Table 1



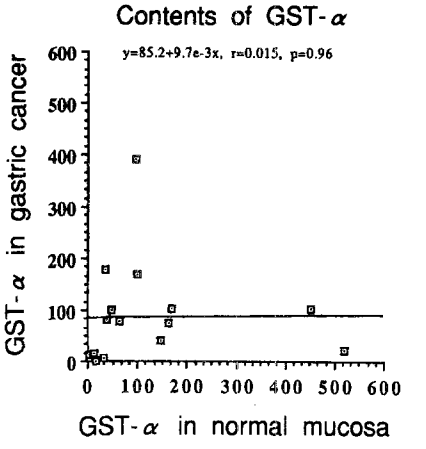


Fig. 2. Correlation of the relative expression of GST-π (*top*) and GST-α (*bottom*) between tumor and normal mucosae. The expression in the positive controls (cancer tissue of case 18 for GST-α and gastric cancer cell line MKN45 for GST-π) was designated as 100. No significant correlation was evident between GST-α contents in cancer tissues and matched mucosae. There was, on the other hand, a significant correlation between GST-π contents in cancer tissues and normal mucosae.

MKN45 cell line and GST-α in the cancer tissue of case 18 (Table 1) were determined to be 100, and the levels of expression in the rest of the specimens were expressed in relation to these values. GST-π was detected in all 16 specimens of gastric mucosae and all 22 specimens of gastric cancer. The GST-π content was greater in cancer tissues (mean, 85.1 ± 46.3) in comparison with matched normal mucosae (mean, 79.0 ± 58.2), but the difference was not statistically significant ($T = 59.0$, $P = 0.67$). The GST-π content in cancer tissues correlated well with that in matched normal mucosae ($r = 0.75$, $P < 0.001$; Fig. 2). GST-α was detected in all 16 specimens of gastric mucosae and in 18 of 22 specimens (82%) of cancer tissues. The content of GST-α was greater in normal mucosae (mean, 118.7 ± 154) than in cancer tissues (mean, 86.4 ± 98.7), but the difference was again not statistically significant ($T = 62.5$, $P = 0.78$). The expression of GST-α in cancer tissues did not correlate with that in matched mucosae ($r = 0.02$, $P = 0.96$; Fig. 2).

Correlation between ID_{50} values and GST contents

There was a lack of correlation between the ID_{50} value for CDDP and the cellular GST-π content in 15 gastric cancer specimens that were evaluated both by SDI test and by immunoblot analysis ($r = 0.09$, $P = 0.74$; Fig. 3). On the other hand, there was a weak correlation between the ID_{50} value for CDDP and the cellular content of GST-α in the same specimens ($r = 0.48$, $P = 0.07$; Fig. 3).

Discussion

Although CDDP has often been used in the treatment of gastric cancer, the response rate for CDDP against gastric cancer is not satisfactorily high even when the drug is given together with 5-fluorouracil and doxorubicin [19]. Moreover, if the administration of CDDP proves to be ineffective, the adverse effects of the drug, including nausea and renal dysfunction, could only ruin the quality of life of

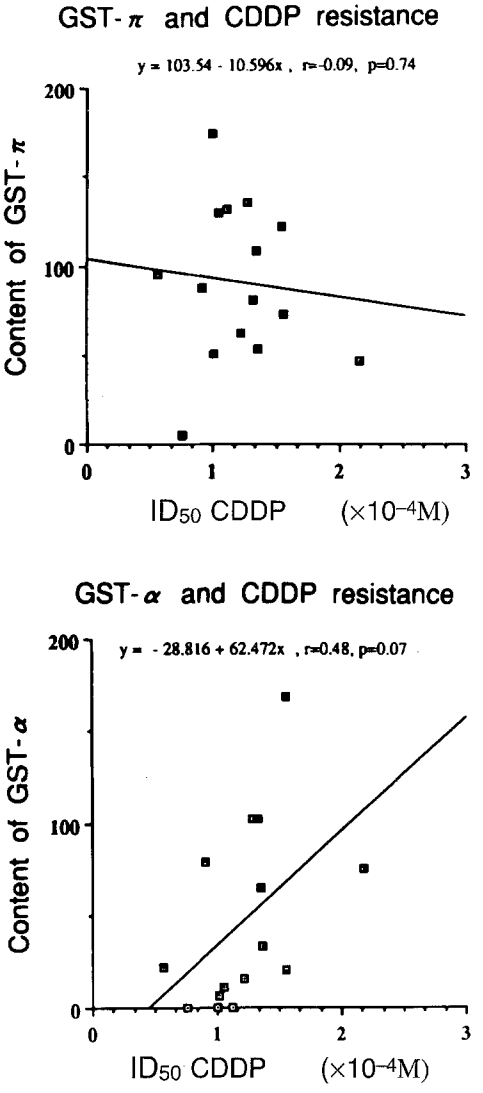


Fig. 3. Correlation of the contents of GST-π (*top*) and GST-α (*bottom*) with ID_{50} values for CDDP. A weak correlation ($r = 0.48$, $P = 0.07$) was observed between the tissue contents of GST-α and the ID_{50} values, whereas no correlation was found between the contents of GST-π and the ID_{50} values.

patients with advanced disease. Prediction of the degree of inherent resistance of individual cancer tissues to antineoplastic agents before the initiation of chemotherapeutic protocols could be immensely helpful from both economical and clinical points of view. We have made one such attempt by immunostaining with the use of a monoclonal antibody against P-glycoprotein [32] to predict the resistance to doxorubicin. According to the results of the present study, quantification of GST-α by immunoblotting may be promising as a predictor of CDDP resistance in gastric cancer specimens.

Several studies on the correlation between the expression of glutathione-related enzymes and drug resistance have been carried out in vitro and in vivo using human cancer cell lines [1, 7, 14, 18, 21, 23, 29] and xenografts [26]. However, similar studies using surgically resected or biopsy specimens have seldom been performed, with the exception of investigations of breast cancer [12] and ovarian cancer [33], in which no significant correlation has been shown between the response to chemotherapeutic agents and the gene or protein expression of GST-π. The present study attempts for the first time to correlate the expression of two predominant forms of GST isozymes in gastric cancer specimens with the results of chemosensitivity tests.

Increased GST-π mRNA levels have been demonstrated in several drug-resistant xenografts established from a human colon-cancer cell line, SW480 [26]. The levels of GST-π mRNA are reported to correlate with resistance to CDDP and carboplatin in human lung-cancer cell lines [23]. However, although similar increases in GST-π enzymatic activities, protein levels, and mRNA levels have been demonstrated in melanoma cell lines resistant to alkylating agents, the lack of cross-resistance observed among drug-resistant cell lines implies that overexpression of GST-π cannot be the predominant mechanism responsible for the drug resistance [29]. Transfection studies with GST-π carried out in MCF7 human breast cancer cells have indicated that increased expression of the enzyme does not confer significant resistance to melphalan or cisplatin [21]. The relevance of GST-π in acquired resistance to anticancer drugs thus remains a matter of controversy. In the present study, the content of GST-π in gastric cancer tissues did not reflect their resistance to CDDP in terms of correlation coefficients, although the constant expression of the isozyme in cancer tissues could contribute to the observation that gastric cancers are generally resistant to CDDP. On the other hand, the content of GST-π in cancer tissue correlated well with that in matched normal mucosa, resulting in a relatively constant cancer/normal ratio, with a slight but statistically nonsignificant up-regulation being noted in the malignancy.

Similar controversies exist over the relevance of GST-α content in drug resistance. GST-α activity is elevated in a colon-cancer cell line resistant to CDDP but is not raised in a cell line resistant to doxorubicin [7]. Amplification and increased expression of GST-α-encoding genes is associated with resistance to nitrogen mustards in Chinese hamster ovary lines [14]. Transfection with a human GST-α gene, on the other hand, has failed to confer resistance to alkylating agents or CDDP [15] in human breast-cancer cell lines MCF7. The results of the present study reveal a varied GST-α expression among the individual cancer tissues that is generally weaker than that in normal mucosa or benign tissues as previously reported in gastric [25] and ovarian [22] cancers. Most important of all, there was a weak but interesting correlation between levels of GST-α and inherent resistance to CDDP. It was also found that unlike the case with GST-π, there was a total lack of correlation between the GST-α contents in cancer and matched normal mucosae, indicating that malignant transformation endows a varied control over the regulation of GST-α.

The SDI test used in the present study to evaluate chemosensitivities was established in the authors' laboratory in 1961 [13], with a few modifications being made thereafter, such as application of the MTT-based colorimetric assay

[6]. In a way, this test was deliberately simplified to enable broad clinical trials. An extensive study to evaluate the extent to which the results of the assay may reflect actual clinical responses to the drugs used in gastric cancer patients is now under way, and promising results have been accumulating for analysis in a study on the selection of optimal anticancer drugs organized by the Ministry of Health and Welfare, Japan. However, one weakness that should be taken into account in any interpretation of the results of the present study involves the inevitable contamination of surgically resected cancer tissues with nontumorous cells [3, 10]. This weakness is common to several other methods of quantification of the chemosensitivity of surgically resected specimens as well as to assays using tumor homogenates, such as immunoblotting, another method that was performed in the present study. Apart from the problem of contamination, these studies using tumor homogenates can give only an average value for the tumor of what is to be quantified. Tumor cells are essentially heterogenous, and there is always a possibility that a small and perhaps undetectable population of cancer cells whose enzymatic profiles are different from those of the vast majority of cells could decide the degree of drug resistance in the whole tumor mass.

From these points of view, our results are not sufficient to rule out the relevance of GST-π in the mechanism of drug resistance. The problems concerning contamination with nontumorous cells could partially have been solved by use of the tumor volume index as described by van der Zee et al. [33], and the qualitative analysis of individual cells could be achieved by immunostaining. Quantitative analysis, namely, immunoblot analysis, might nevertheless be considered of value in the case of GST-α, for which an interesting correlation was obtained, and further studies and analyses of clinical response rates are under way to confirm the relevance of the isozyme in the prediction of tumor resistance to CDDP.

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