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Expression of π-Glutathione S-transferase Gene (GSTP1) in Gastric Cancer: Lack of Correlation With Resistance Against Cis-diamminedichloroplatinum (II)

Y. Kodera, S. Akiyama, K. Isobe, K. Kondo, K. Ito, M. Yamauchi and H. Takagi

Class π-glutathione S-transferase (GSTP-1) is one of several factors proposed to affect drug sensitivity to cis-diamminedichloroplatinum (II) (CDDP). It has also been investigated as a potential marker for the serodiagnosis of various types of cancers. In this study, attempts were made to quantify mRNA levels of the enzyme in healthy and cancerous gastric mucosa specimens, and to evaluate their significance in inherent drug resistance to CDDP. Thirty gastric cancer specimens were analysed by northern blotting with radiolabelled GSTP1 cDNA. Of these, the chemosensitivities of 22 specimens were evaluated by the succinic dehydrogenase inhibition (SDI) test. GSTP-1 mRNA was detected in all the specimens, with slightly increased, but non-significant expression in the neoplasms. Comparison of these drug sensitivities with results of northern blotting analysis showed no inverse correlation, as was expected from the widely investigated role of the enzyme in drug resistance.

Key words: glutathione S-transferase, drug resistance, gastric cancer, cis-diamminedichloroplatinum (II)
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INTRODUCTION

THE EFFECTIVENESS of several clinically useful anticancer drugs can be severely limited by drug resistance, which appears to be intrinsic to some tumours. According to studies carried out using cultured tumour cell models and other systems, a variety of mechanisms can contribute to drug resistance [1–3]. The present study deals with one such mechanism—glutathione S-transferase (GST) which is a group of isozymes that is known to play an important role in the resistance of cells to anticancer agents, including alkylating agents [4, 5] and cis-diamminedich-

loroplatinum (II) (CDDP) [6, 7]. GSTs are widely distributed in the animal and plant kingdoms [8]. Mammalian cells contain both soluble (cytosolic) and membrane-bound isozymes, and Mannervik and associates [9] have recognised that soluble GSTs can be separated into three classes; α, μ, and π, on the basis of structural, immunological and enzymatic properties. Of these, GSTP1 is known to be invariably detected in healthy and cancerous gastric mucosa tissues by immunoblot analysis [10] and 1.3- and 1.4-fold increases in expression of the enzyme have been reported in gastric [10] and colon [11] cancer tissues, respectively. GSTP1-1 mRNA has also been widely detected in human tumours [12] and cancer cell lines [13], and increases in expression compared with matched normal tissues have been readily observed [12, 14], indicating its potential as a tumour marker. Over 80% of patients with stage III or IV gastric cancer, and even 50% of those with stage I and II disease have been reported to have elevated serum GSTP1-1 levels [15].

Gastric cancer remains one of the most common neoplasms worldwide, with high incidence rates in eastern Europe, Asia and South America. Mortality rates of the unresectable disease are extremely high and median survival time is 4 months [16]. Adequate chemotherapy is required whenever surgical treatment is considered insufficient, and evaluation of the chemosensitivity of cancer cells from gastrectomy specimens will provide useful information with which to make an informed choice of drugs. The authors have been working on the clinical application of such an assay system—the succinic dehydrogenase inhibition test (SDI test) [17, 18]. A brief comparison has already been made between the results of immunostaining using the monoclonal antibody C219 raised against p-glycoprotein and the chemosensitivity against doxorubicin evaluated by the SDI test [19], indicating that the cell lines and surgical specimens with positive p-glycoprotein staining are doxorubicin resistant. Others have also shown that protein levels of α-GST correlate with intrinsic resistance to CDDP in gastric cancer specimens [20]. The present study attempts to evaluate to what extent GSTP1-1 mRNA levels are associated with inherent drug resistance to CDDP in gastric cancer specimens.

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% foetal calf serum.

Tissue preparation

Gastric cancer tissue and morphologically normal mucosa were obtained at surgery from patients who underwent gastrectomy at Nagoya University Hospital (Nagoya, Japan). Twenty gastrectomy specimens were rinsed with sterilised saline and cancerous tissue was resected from the mucosal region and kept in ice-cold Hank's balanced solution until use in the chemosensitivity test, performed within a few hours of resection.

Thirty surgically resected specimens of gastric cancer, 16 of which had matched normal mucosa and 22 of which had results of chemosensitivity tests (as mentioned above), were frozen promptly in liquid nitrogen and kept at −80°C until use in total RNA extraction.

None of the patients had received chemotherapy prior to surgery.

Chemosensitivity test

CDDP was provided by Nihon Kayaku Pharmaceutical Industries (Tokyo, Japan). It was diluted to 10, 25 and 50 μg/ml (33, 83 and 160 μM) in RPMI 1640 medium supplemented with 20% foetal calf serum, just before use in the chemosensitivity test. The concentration of 25 μg/ml was selected as adequate to evaluate the effect of CDDP in the fresh specimen [18].

The SDI test was performed as described previously [18–20], using 3-(4,5-dimethylthiazol-2-yl)2,5-D:phenylformazan bromide (MTT). Cell suspensions were prepared by mincing the tumour, followed by incubation for 30 min at 37°C with 0.02% (W/V) collagenase type I (Worthington Biochemical Corp., New Jersey, U.S.A.), 0.02% DNase I (Boehringer Mannheim GmbH, Germany) and 0.02% Pronase (Boehringer Mannheim GmbH). Cell numbers were adjusted to 5×10^5 cells per ml of RPMI medium supplemented with 20% foetal calf serum. Cell suspensions (100 μl/well) were distributed into 96-well microplates containing 100 μl of the serial dilutions of CDDP. A set of six control wells contained no CDDP. The cancer cells were incubated for 48 h at 37°C, 5% CO_2. After washing 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, Missouri, U.S.A.) were added and incubated for 3 h at 37°C. After completion of the reaction, 150 μl/well of dimethylsulphoxide (DMSO, Katayama Chemical) were added to dissolve formazan, a product of the reaction, and the optical density (OD) was measured with a spectrophotometer (Easy Reader EAR-340, SLT Lab Instruments, Salzburg, Austria) at 540 nm. The OD value accurately reflects the number of living cells when the cell count is over 10^4/ml [19]. In 13 of the specimens, ID_{50} was calculated from the line plotted from the three OD values for the three serial concentrations of CDDP. In nine specimens, the single cell suspension obtained was not sufficient for the evaluation of ID_{50} and so the inhibition index was calculated by the formula:

$$\frac{(a-p)}{(a-m)\,100}$$

where p is the OD when the tumour cells are exposed to 25 μg/ml of CDDP, a is the OD determined by the same procedure but without addition of CDDP, and m is the OD when neither the anticancer drug nor MTT is added [18, 19]. The SDI test was considered valid only when the coefficient of variation from the OD values of six control wells containing no CDDP was under 20%.

RNA extraction and northern blotting

Total RNA was extracted from each specimen by the method of Chomczynski and colleagues [21]. Ten micrograms per lane of RNA were electrophoresed on 1% agarose gels containing 1.1 M formaldehyde, and were then transferred to Hybond-N nylon membrane (Amersham, U.K.). Full-length human GSTP1 cDNA, pGPi2, was kindly provided by Professor Muramatsu [22] (Second Department of Biochemistry, Saitama Medical School, Saitama, Japan). Human β-actin cDNA [23] was used as an internal control. These probes were labelled with ^{32}P

Correspondence to Y. Kodera.
Y. Kodera, S. Akiyama, K. Kondo, K. Ito and H. Takagi are at the Department of Surgery II and K. Isobe is at the Department of Immunology, Nagoya University School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466; and M. Yamauchi is at the Department of Surgery, Tokai Chuo Hospital, Kagamihara-shi, Gifu 504, Japan.
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by Multiprime labelling systems (Amersham, Japan) according to the manufacturer's instructions, hybridised for 18 h at 42°C and washed to the final stringency of 0.1 × standard saline citrate (SSC) (1 × SSC consists of 0.15 M sodium chloride and 15 mM sodium citrate) with 0.1% sodium dodecyl sulphate (SDS) at 65°C. The membranes were autoradiographed at −70°C using Fuji RX film (Fuji Photo Film, Kanagawa, Japan), and a BAS2000 Imaging Analyser (Fuji Photo Film) was used for densitometric analysis of the blots. Each filter included a control specimen (cell line MKN45), the intensity of whose blot was quantified by BAS2000 and designated as 100, the intensities of the blots of the rest of the specimens were expressed relative to the control. The values were corrected for the level of β-actin mRNA.

Statistics

Wilcoxon's paired test was performed for comparison of mRNA levels between neoplasms and normal mucosae. Spearman's correlation coefficients were calculated for the evaluation of the correlation between ID_{50} values for CDDP and levels of GSTP1, and of the correlation between ID_{50} values and inhibition indices.

RESULTS

Chemosensitivity test

The values of inhibition indices for CDDP were calculated in 22 gastric cancer tissue samples and ranged from 23.9 to 73.1%. ID_{50} values were obtained in 13 gastric cancer tissues and ranged from 5.70×10^{-5} to 2.17×10^{-4} M. The values of ID_{50} exhibited an inverse correlation with inhibition indices that was statistically significant ($r = -0.93, P < 0.0001$, Figure 1). The values are summarised in Table 1.

Expression of GSTP1 in gastric cancer and mucosae

The levels of expression of GSTP1 quantified in 30 gastric cancer specimens and 16 matched gastric mucosa by northern blot analysis (Figure 2) are summarised in Table 1. GSTP1 mRNA was detected in all 30 specimens of gastric cancer and all 16 specimens of gastric mucosae. There was no significant increased expression of GSTP1 mRNA in gastric cancer specimens (mean 39.2 ± 19.8) over the matched mucosae (mean 29.6 ± 16.2) in the 16 matched pairs in the present series ($T = 35.5, P = 0.09$, Figure 3).

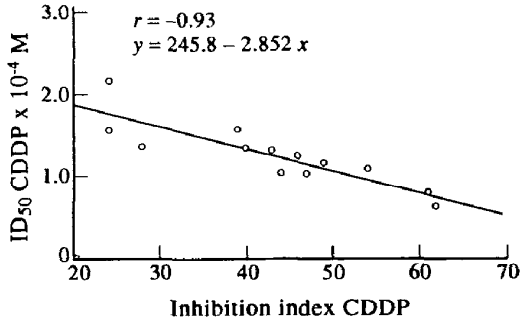

Figure 1. The chemosensitivity of gastric cancer specimen against CDDP evaluated by the SDI test. Both ID_{50} and inhibition index were worked out in 13 of the specimens. The inverse correlation between ID_{50} values and inhibition indices was found to be statistically significant ($r = -0.93, P < 0.0001$).

Table 1. GST contents and chemosensitivities against CDDP in mucosal and cancerous specimens of the stomach

Case no.	GSTP1 N	GSTP1 T	Inhibition index (%)	ID_{50} CDDP ($\times 10^{-4}$M)
1	26	85	38.9	1.32
2	26	9	24.4	1.56
3	30	31	39.0	1.55
4	ND	90	27.7	1.35
5	ND	86	73.1	ND
6	56	70	44.8	ND
7	67	23	43.8	1.01
8	ND	22	56.9	ND
9	ND	42	23.9	2.17
10	ND	51	61.0	0.75
11	ND	8	41.2	ND
12	21	54	ND	ND
13	15	44	54.1	1.05
14	56	32	68.3	ND
15	35	37	ND	ND
16	ND	46	61.5	0.57
17	16	26	ND	ND
18	18	17	43.3	1.28
19	ND	15	49.0	1.12
20	ND	45	47.2	1.00
21	ND	20	46.3	1.22
22	ND	25	61.3	ND
23	ND	60	41.7	ND
24	ND	40	56.2	ND
25	15	22	ND	ND
26	25	52	ND	ND
27	ND	31	45.9	ND
28	17	33	ND	ND
29	31	51	ND	ND
30	20	41	ND	ND

The expression of GSTP1 mRNA in the control specimen (gastric cancer cell line MKN45) was designated as 100 and the contents of the other specimens were expressed relative to the control. N, normal gastric mucosa; T, gastric cancer tissue; ND, not determined.

Correlation between the ID_{50} values and GSTP1 contents

There was a lack of correlation between inhibition index for CDDP and GSTP1 mRNA expression in 22 gastric cancer specimens ($r = 0.13, P = 0.56$, Figure 4).

DISCUSSION

Although CDDP has been used in the treatment of gastric cancer, the response rate of CDDP against gastric cancer is not satisfactorily high even when co-administered with 5-fluorouracil and doxorubicin [24]. The information concerning the extent of inherent drug resistance prior to chemotherapy would be extremely helpful when making choices about drug regimens. The SDI test, a simplified method utilised in the present study for evaluating chemosensitivities, has been established in our laboratory since 1961 [17] and, after a few modifications such as application of the MTT-based colorimetric assay [25], has been applied to clinical practice in the Department of Surgery II, Nagoya University for selection of anticancer drugs in the treatment of gastrointestinal cancer. One major problem encountered in practice is that a clinical sample as large as 1–2 g is needed for the chemosensitivity assay to determine the ID_{50}.

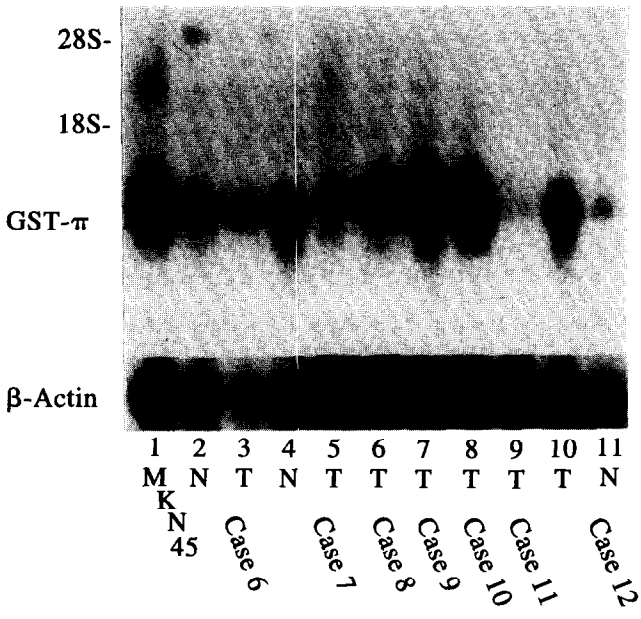

Figure 2. Representative northern blots of gastric cancer specimens (T) and normal mucosae (N). The gastric cancer cell line, MKN45, was used as a positive control and expression of GSTP1 in MKN45 was designated as 100.

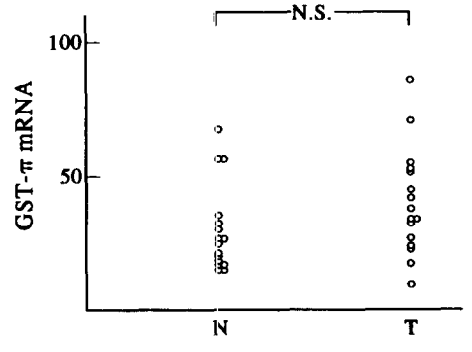

Figure 3. Expression of GSTP1 mRNA in gastric cancer (T) was increased in comparison with corresponding normal mucosa (N), but the difference was not statistically significant ($T = 35.5$, $P = 0.09$).

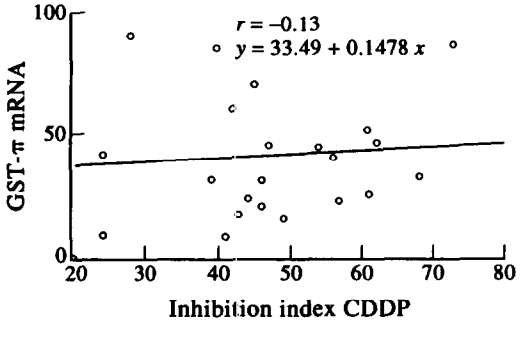

Figure 4. The correlation between expression of π-GST and chemosensitivity against CDDP expressed in terms of inhibition index. No significant correlation was found ($r = 0.13$, $P = 0.56$).

However, the inhibition index that is more easily obtained, as described in the Materials and Methods, exhibited a significant inverse correlation with the ID_{50}. We, therefore, suggest that the inhibition index could substitute for the value of ID_{50} when only small samples are available.

One general weakness that the SDI test and several other chemosensitivity assays have in common is the inevitable mixture of unknown proportions of non-neoplastic cells [26]. Tumour volume index, which represents the percentage of malignant epithelial tissue in a tumour specimen, is measured by a point-counting technique using a 42-point grid placed on a projection microscope at a magnification of 200-fold as described by Baak and associates [27], and is one possible answer to the problem. Another problem is the heterogeneity of the cancer cells found in a single specimen. A value obtained by such a chemosensitivity assay is no more than an average value for the whole cell population included in the specimen. However, the characteristics of a minority of cells, which make little impact on the result of the assay, could in fact be the crucial factor that decides the chemosensitivity of the whole mass. These problems are shared by assays that utilise tissue homogenates such as northern blotting, in which observations, perhaps by *in situ* hybridisation, might supply additional and worthwhile information.

Increases in GSTP1 mRNA levels have been demonstrated in several drug-resistant xenografts established from the human colon cancer cell line, SW480 [28]. The levels of GSTP1 mRNA are reported to correlate with resistance to CDDP and carboplatin in human lung cancer cell lines [7]. However, although similar increases in GSTP1 enzymatic activities, protein levels and mRNA levels have been demonstrated in the melanoma cell lines resistant to the alkylating agents, lack of cross-resistance among the drug-resistant cell lines indicate that the increased expression of GSTP1 cannot be the predominant mechanism for drug resistance [5]. Significance of GSTP1 in acquired resistance to anticancer drugs is thus still controversial. Several studies on the correlation between expression of glutathione-related enzymes and drug resistance have been carried out *in vitro* and *in vivo* using human cancer cell lines [5, 7, 13, 29] and xenografts [28]. However, similar studies using surgically resected or biopsy specimens have seldom been performed, with the exception of Keith and associates [30], who showed a lack of significant correlation between gene expression of GSTP1 and ID_{50} against doxorubicin in biopsy specimens of breast cancer, and van der Zee and colleagues [26] who demonstrated, with specimens of ovarian cancer, that the expression of GSTs was not reflected in the acquired resistance to CDDP.

In the present study, GSTP1 mRNA was consistently detected in all the specimens of gastric cancer and gastric mucosa; a result that concurs with what has been reported at the protein level [10, 20]. However, the mRNA levels of GSTP1, as detected by northern blotting, did not reflect the inherent resistance of cancer cells against CDDP in the clinical samples of gastric cancer. The present study therefore indicates that there is no statistical correlation between drug resistance and gene expression of GSTP1, although the results are not sufficient to reflect that GSTP1 is involved in the detoxification of CDDP. Post-transcriptional control could always cause lack of correlation between the mRNA level and the protein level, even though the latter does correlate with enzymatic activity, which is what really counts in the process of detoxification [10]. The universal expression of GSTP1 in specimens of gastric cancer could simply be indicative of the fact that gastric cancers, in general, do not respond well to chemotherapy with CDDP. It has been reported, however, that gene expression of GSTP1 is even more prominent in cancers of the head and neck and the oesophagus [12], in which CDDP is generally considered more effective, although a simple comparison of the mRNA levels

between adenocarcinomas and squamous cell carcinomas may not be valid.

There might be controversy over the use of ID_{50} values as a parameter for determination of drug resistance. The death of 50% of the cells calculated as ID_{50} might not necessarily mean the eventual death of the remaining 50%, but the response in the clinical setting aims at the death of all viable cancer cells. In the present series, the patients with gastric cancer were not always treated with CDDP alone. Some of the patients did not undergo chemotherapy. It is hoped in the future that a greater number of cancer tissues will be obtained so as to evaluate the correlation between the expression of GSTP1 and actual response rate of the patients undergoing chemotherapy.

Finally, it has already been shown by several investigators [10–12, 14] that expression of GSTP1 is significantly elevated in some cancer tissues over the corresponding normal mucosa. Based on these facts, the role of GSTP1 as a tumour marker has been implicated [14]. The mRNA levels of GSTP1 in gastric cancer were inclined to be elevated over the corresponding mucosa in the present study, but the difference was not significant. Our results, then, do not support the concept of GSTP1 as a tumour marker in the diagnosis of gastric cancer.

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