Expression of Glutathione S-Transferase pi (GST-pi) in Human Malignant Ovarian Tumors

(ヒト卵巣悪性腫瘍における Glutathione S-Transferase pi (GST-pi) の
免疫組織学的研究)

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茨城西南医療センター病院
佐藤 豊実

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Expression of Glutathione S-Transferase pi (GST-pi)  
in Human Malignant Ovarian Tumors

Toyomi Satoh, M.D.,† Masato Nishida, M.D., Ph. D., †  
Hajime Tsunoda, M.D., Ph.D., † and Takeshi Kubo, M.D., Ph.D., †

†Department of Obstetrics and Gynecology, Ibaraki Seinan Central Hospital, Ibaraki 306-0433, Japan,  
and † Department of Obstetrics and Gynecology, University of Tsukuba, Tsukuba, 305-8575, Japan

†To whom reprint requests should be addressed at Department of Obstetrics and Gynecology, Ibaraki  
Seinan Central Hospital, 2190 Sakai-Machi, Ibaraki 306-04, Japan.  
Fax: +81-280-86-7702

A short running head: GST-pi in Human Malignant Ovarian Tumors

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Abstract

Objectives. In recent years, Glutathione S-Transferase pi (GST-pi) has attracted much attention and has been studied as a mechanism of multidrug resistance of tumors to anticancer drugs. In the present study, we immunohistologically measured the expression of GST-pi in tumor tissues using surgical specimens obtained from patients with malignant ovarian tumors.

Methods. Of 137 patients with malignant ovarian tumors treated and managed during a period of 20 years since the establishment of Tsukuba University Hospital, 117 patients were selected as subjects because of the presence of complete data on their clinical courses as well as paraffin blocks preserved in a good condition. GST-pi in these specimens was immunohistochemically stained to determine the correlation between GST-pi stainability and clinical outcomes. Stainability was graded as 0 when GST-pi was completely absent, 1 when less than 20% of tumor cells were stained, 2 when 20-60% were stained, and 3 when more than 60% were stained.

Results. When the correlation between stainability and clinical outcomes was analyzed with Kaplan-Meier method, excluding stage Ia cases that did not receive adjuvant chemotherapy at Tsukuba University Hospital, significantly better clinical outcomes were observed in the low stainable group, compared with the high stainable group (p < 0.01-0.05, Cox-Mantel test, Wilcoxon’s test).

Conclusion. Since the stainability for GST-pi was high in tumors of histological types with strong resistance to anticancer drugs, and better clinical outcomes were observed in cases having a lower stainability score, the expression of GST-pi was thought to play some role in the resistance of malignant ovarian tumors to anticancer drugs.

Key Words : glutathione S-transferase-pi; ovarian malignant tumors; resistance to anticancer drugs; clinical outcomes.

Introduction

Although clinical outcomes of progressive malignant ovarian tumors have markedly improved since the introduction of cisplatin (CDDP) to clinical practice, the increase in the efficacy rate of CDDP has recently remained unchanged [1]. Resistance of tumors to anticancer drugs through multidrug
resistant mechanisms is a possible cause of this phenomenon.

GST-pi, a molecule of the multifunctional enzyme glutathione S-transferase, is thought to serve as a mechanism of multidrug resistance, together with the pumping effect of P-glycoprotein, manifested by the expression of multidrug resistance gene [2], and chelation of heavy metals by metallothionein [3].

The involvement of GST-pi in multidrug resistance is thought to be mediated by glutathione conjugation, extracellular excretion of anticancer drugs through the ATP-dependent GSH conjugate efflux pump, inhibition of actions of anticancer drugs by DNA bridge formation, and resistance to anticancer drugs as the result of elimination of free radicals because those drugs can manifest their cytotoxic effects through radicals [4,5]e. Studies in cell lines derived from various organs have suggested that GST-pi is a factor involving the resistance to cisplatin [6].

Although there have been many reports on the involvement of GST-pi in the acquisition of drug resistance in malignant ovarian tumors [7-9], such drug resistance has been rarely investigated according to four representative histological types of malignant ovarian tumors, serous, mucinous, endometrioid, and clear cell, or in relation to the degree of differentiation.

Thus we carried out the present study to immunohistologically measure the expression of GST-pi in malignant ovarian tumors treated at Tsukuba University Hospital during the past 20 years. Further, we investigated differences in GST-pi expression according to histological types and degrees of differentiation and also the correlation between the intensity of expression of GST-pi and clinical outcomes in patients with primary malignant ovarian tumors treated with anticancer drugs, including cisplatin.

Subjects and Methods

1) Subjects

Of 137 patients with malignant ovarian tumors treated and managed at Tsukuba University Hospital during a period of 20 years from 1976 until 1995, 117 were selected as subjects because these patients had complete data on their medical histories and clinical courses, and their paraffin blocks were
preserved in a good condition.

Surgical specimens from these patients were stained with hematoxylin and eosin to determine their histological types according to the WHO's criteria. The degree of differentiation was defined in serous adenocarcinomas, mucinous adenocarcinomas, and endometrioid adenocarcinomas.

There were 40 cases with serous adenocarcinoma, 30 with mucinous adenocarcinomas, 23 with clear cell carcinoma, 13 with endometrioid adenocarcinoma, and 11 with ovarian cancer of other type.

The degree of differentiation was defined as borderline malignancy (BM) in 16 cases, well differentiated type (GI) in 34, moderately differentiated type (GII) in 27, and poorly differentiated type (GIII) in 6.

Clinical outcomes were analyzed in 87 cases classified as stage II or severer, excluding stage Ia cases because of no implementation of chemotherapy. Of 87 cases, 67 were in stage II or severer, and 56 were in stage III or severer. Any anticancer drug was administered to none of these 87 patients before their surgical specimens were sampled.

The period of observation after surgery was set to be 24 months or longer.

2) Method of Immunostaining of GST-pi and Judgment of Stainability

In immunohistological staining, mouse anti-GST-pi monoclonal antibody (DAKO Laboratories Inc., Carpinteria, CA) was used as the primary antibody. NEVISION+/HRP (DAKO Laboratories Inc.) was used as the secondary antibody. The primary antibody was used at 50-fold dilution and reacted at room temperature for 30 min.

Stainability of each specimen was judged under microscopy in a way of stainability scoring. Stainability was graded as 0 when GST-pi was completely absent, 1 when less than 20% of tumor cells were stained, 2 when 20-60% were stained, and 3 when more than 60% were stained. When a stainability score could not be judged at a glance, positive cells per 100 cells were counted in 5 random regions on one slide, and a percent of positive cells was calculated. When one case had multiple slides, the mean number of positive cells in all slides was calculated. Cells having stained cytoplasm or nucleus were all judged to be positive. When puzzled about judgment, cells were regarded as unstained.

3) Study on the Correlation Between Histological Types and GST-pi Stainability
Differences in stainability were analyzed with Mann-Whitney U-test as to serous adenocarcinomas, mucinous adenocarcinomas, clear cell carcinomas, and endometrioid adenocarcinomas.

4) Study on the Correlation Between Histological Differentiation and GST-pi Stainability

The degree of differentiation was determined in each of serous adenocarcinomas, mucinous adenocarcinomas, and endometrioid adenocarcinomas, and then differences in stainability were analyzed with the Mann-Whitney U-test.

5) Study on the Correlation Between Clinical Outcomes and GST-pi Stainability

Clinical outcomes were analyzed according to stainability scores using Kaplan-Meier method. Significant differences were tested using Wilcoxon’s test and Cox-Mantel test.

From this study, stage Ia cases were excluded because of no implementation of adjuvant chemotherapy on these cases at Tsukuba University Hospital. Early cases that had received chemotherapy, but not CDDP were also excluded from this study. The reasons for this were that we attempted to avoid biases in therapeutic backgrounds as much as possible because clinical outcomes of chemotherapy without CDDP were thought to be poorer, compared with chemotherapy with CDDP, and that we intended to investigate the resistance of GST-pi to CDDP.

As mentioned above, the correlation between the stainability for GST-pi and clinical outcomes was analyzed in limited groups of cases; namely, 87 cases in stage Ib or severer, 67 in stage II or severer, and 56 in stage III or severer.

More, subjects were further divided according to stainability scores into the low stainable group, containing cases with a stainability score of 0 or 1, and the high stainable group, containing cases with a stainability score of 2 or 3. After that, the correlation between the stainability for GST-pi and clinical outcomes was determined in each group in relation to serous adenocarcinoma, mucinous adenocarcinoma, clear cell carcinoma, and endometrioid adenocarcinoma. In addition, analysis only in cases with adenocarcinoma in stage IIIc was also made.

There were no differences in clinical stage, the presence or absence of residual tumor after initial surgical resection, the duration of follow-up, or age at operation among groups of patients divided according to four histological type, serous, mucinous, endometrioid, and clear cell. ($\chi^2$ test, t-test).
Results

Among overall cases, 9.80%, 24.50%, 16.70%, and 49.00% were found to have stainability scores of 0, 1, 2, and 3, respectively. In most of cases with a stainability score of 1, only a very small portion of cells were stained (about 1-10 cells per 500 cells).

1) Stainability Scores by Histological Types

Stainability showed no consistent tendency among 40 cases with serous adenocarcinoma. Stainability scores of 0, 1, 2, and 3 were found in 15.00%, 25.00%, 25.00%, and 35.00% of these cases, respectively.

Thirty cases with mucinous adenocarcinoma tended to have a high stainability score. Stainability scores of 0, 1, 2, and 3 were found in 0%, 13.30%, 10.0%, and 76.70% of them, respectively.

Thirteen cases with endometrioid adenocarcinoma tended to have a relatively low stainability score. Stainability scores of 0, 1, 2, and 3 were found in 7.70%, 46.20%, 23.10%, and 23.10% of them, respectively.

Twenty-three cases with clear cell carcinoma had a high stainability score, showing a similar tendency to that seen in cases with mucinous adenocarcinoma. Stainability scores of 0, 1, 2, and 3 were found in 0%, 21.70%, 17.40%, and 60.90% of them, respectively.

The stainability in cases with mucinous adenocarcinoma was significantly higher than in cases with serous adenocarcinoma and those with endometrioid adenocarcinoma (p < 0.01, Mann-Whitney U-test). The stainability in cases with clear cell carcinoma was also significantly higher than in cases with serous adenocarcinoma and those with endometrioid adenocarcinoma (p < 0.05 and p < 0.01, Mann-Whitney U-test).

A larger number of cases following those of the above 4 histological types were found in mature cystic teratoma (squamous cell carcinoma), occurring in 5 cases. As for stainability in these 5 cases, stainability scores of 0 and 2 were found in 1 case each, and a stainability score of 3 was found in 3 cases, disclosing a similar tendency to that observed in cases with mucinous adenocarcinoma and those with clear cell carcinoma, although the number of cases was small. In addition, both 2 cases with undifferentiated carcinoma had a stainability score of 0; one each of 2 cases with granulosa cell tumor had
stainability scores of 1 and 2; one case with undifferentiated germ cell tumor has a stainability score of 0; one case with embryonal carcinoma had a stainability score of 1.

2) Stainability Scores by Degrees of Histological Differentiation

The degree of histological differentiation was determined in a total of 83 cases, including 40 with serous adenocarcinoma, 30 with mucinous adenocarcinoma, and 13 with endometrioid adenocarcinoma. These cases were classified into borderline malignancy (BM) and grade I to III (GI to III).

High stainability was observed in 16 BM cases, showing stainability scores of 0 to 3 in 0%, 6.3%, 31.3%, and 62.5%, respectively.

Stainability scores of 0 to 3 were observed in 2.9%, 17.6%, 11.8%, and 67.6% of 34 GI cases.

Among 27 GII cases, low stainability was frequently observed, showing stainability scores of 0 to 3 in 29.6%, 33.3%, 14.8%, and 22.2%, respectively.

Six GIII cases tended to have low stainability, showing stainability scores of 0 to 3 in 16.7%, 66.7%, 0%, and 16.7%, respectively.

Stainability was significantly higher in BM and GI cases than in GII and GIII cases (p < 0.01, Mann-Whitney U-test).

As mentioned previously, both 2 cases with poorly-differentiated carcinoma had a stainability score of 0.

3) Correlation with Clinical Outcomes

Comparison of survival curves by stainability scores among cases in stage Ib or severer disclosed no significant differences (Fig. 1).

Among cases in stage II or severer, significantly better clinical outcomes were observed in cases with a stainability score of 0 than in those with a score of 2 (p < 0.05, Wilcoxon’s test; p < 0.05, Cox-Mantel test). Cases with a stainability score of 1 had significantly better clinical outcomes than those with a stainability score of 2 or 3 (N.S., Wilcoxon’s test; p < 0.01, Cox-Mantel test) (Fig. 2).

In comparison of a limited group of cases in stage III or severer, the same tendency was also observed; namely, cases with a stainability score of 1 showed significantly better clinical outcomes than
those with a stainability score of 2 or 3 (N.S., Wilcoxon’s test; p < 0.01, Cox-Mantel test) (Fig. 3).

By histological types, better clinical outcomes were observed in the low stainable group among cases with serous adenocarcinoma in stage II or severer and also in stage III or severer, compared with the high stainable group (N.S., Wilcoxon’s test; p < 0.01 and p < 0.05, respectively, Cox-Mantel test) (Fig. 4). Among cases with serous adenocarcinoma in stage III or severer, significantly better clinical outcomes were observed in the low stainable group (N.S., Wilcoxon’s test; p < 0.05, Cox-Mantel test).

In comparison of a limited group of cases with adenocarcinoma in stage IIIC, the low stainable group had significantly better clinical outcomes (N.S., Wilcoxon’s test; p < 0.01, Cox-Mantel test) (Fig. 5).

Discussion

GST is a multifunctional enzyme participating in a wide range of activities in the body, not only acting as a catalyst in detoxification of various drugs through glutathione conjugation in the liver, but also acting in transport of organic ions, reduction of active oxygen and lipid peroxide, and synthesis and catabolism of prostaglandin and leukotriene[10]. GST-pi, a molecule of this enzyme, has currently been studied with suspicion of its potential involvement in multidrug resistance to anticancer drugs. However, results so far reported have shown great discrepancy. With regard to its involvement in resistance to CDDP, which has attracted keen attention, some investigators agree[11-13], but some others disagree[6,14].

Besides, it has also been reported that adriamycin, cyclophosphamid, and mitomycin C, which are widely used as anticancer drugs in the field of gynecology, are detoxified with reduced glutathione (GSH) [15], indicating the involvement of GST.

In an immunohistological study of GST-pi in lung cancer, it was reported that cases with squamous cell carcinoma were all positive, and some cases with well-differentiated adenocarcinoma were positive while cases with small cell cancer were all negative [16], disclosing the correlation between histological types and stainability. It is said that the sensitivity of lung cancer to anticancer drugs generally increases in the order of squamous cell carcinoma, adenocarcinoma, and small cell carcinoma.
This seems to indicate that the higher the stainability for GST-pi, the higher the resistance.

In the present immunohistological study, we also observed different tendencies of stainability among different histological types. Mucinous adenocarcinoma and clear cell cancer, which were highly stainable, are generally insensitive to anticancer drugs. Thus it is inferred from the stainability according to histological types that GST-pi plays some role in drug resistance of ovarian tumors, as with lung cancer.

However, since immunohistochemical expression of GST-pi may not always correlate with the activity of GST-pi, further studies should be performed on biochemical analysis.

When the stainability was examined in relation to degrees of differentiation, tumors of higher differentiation tended to have a higher stainability score. As for adenocarcinoma of the lung, the expression of GST-pi was more remarkable in more differentiated tumors [16]. Measurement of tissue levels of GST-pi in cases with esophageal cancer also disclosed higher levels in more differentiated tumors [17]. Together with these results, it seems to be rational if one thinks that the degree of differentiation is correlated to the stainability for GST-pi in certain regions of tumor origin.

Meanwhile, the results of present study revealed that the stainability of GST-pi became poorer as ovarian tumors were more poorly differentiated. However, ovarian tumors generally have poorer outcomes as they are more poorly differentiated. This contradictory finding suggests the following possibilities: some mechanisms of drug resistance, other than the GST-pi-involved mechanism, may be present in poorly-differentiated ovarian tumors: tumor growth may exceed the reduction of tumor cells during a non-dosing period although the tumor cells do not have high drug resistance, and they are killed by chemotherapy to a large extent: poorly-differentiated tumor cells may have a higher ability to acquire drug resistance than highly-differentiated tumor cells. However, any definite conclusion cannot be drawn from the results of the present study.

Although no significant correlation between stainability for GST-pi and clinical outcomes was observed among 87 cases in stage Ib or severer, the analysis of 67 cases in stage II or severer and that in 56 cases in stage III or severer revealed that the higher the stainability, the poorer the clinical outcome with significant differences.
In the present study, the stainability for GST-pi was high in cases with clear cell cancer and mucinous adenocarcinoma, which are generally considered to be insensitive to anticancer drugs. Therefore, cases with mucinous adenocarcinoma and clear cell cancer, which usually have poor clinical outcomes, were more often included in the high stainable group than in the low stainable group. Because of these results, it became unclear whether high stainability for GST-pi served as a factor of poor clinical outcome, or cases with high stainability had poorer clinical outcomes because these cases often had mucinous adenocarcinoma or clear cell cancer.

Thus, groups of patients divided according to histological types were further divided into subgroups according to their clinical stages, and then the correlation between the stainability of GST-pi and outcome was investigated in each of these subgroups. Because the number of cases for each histological type was small, we divided the cases into 2 groups, low-stainable and high stainable groups. As a result, cases with serous adenocarcinoma were found to have significantly poorer clinical outcomes in the high stainable group. A similar tendency was also observed in other histological types, but did not reach a different level of significance, probably because of small numbers of cases. Based on these results, it was inferred that the stainability for GST-pi was correlated to clinical outcomes, even within same histological types, strongly suggesting that GST-pi is involved in the resistance of tumors to anticancer drugs, and therefore in clinical outcomes.

When we also compared clinical outcomes in relation to the stainability for GST-pi only using a limited group of cases with adenocarcinoma in stage IIc, in order to compare patients at a similar level of tumor severity as possible, poorer clinical outcomes were also observed as the stainability for GST-pi became higher.

Although the data are not described in “Results”, we then analyzed individual cases in stage III or IV. There were 11 5-year survivors having a stainability score of 0 or 1, whereas only 2 cases with a stainability score of 2 or 3 could survive for 5 years; namely, one progressing to stage IIIc from dermoid cystoma survived for 84 months, and another case had superficial papillary tumor of borderline malignancy in stage IIIb. These results again suggested a very close correlation between the expression of GST-pi and clinical outcomes.
The involvement of GST-pi in multidrug resistance was observed in artificially-induced resistant stains prepared in vitro\cite{18,19}, and in experimental systems of gene transection \cite{6,14}, but the results of these studies were not consistent. Different results according to different experimental systems are thought to indicate the dependency of results of experiments on cell characteristics or other factors.

As so far mentioned, no definite conclusion has been drawn on the involvement of GST-pi in drug resistance. However, the results of the present study on ovarian cancers revealed that GST-pi positive cases often showed histological types that are considered to be insensitive to drugs, and they had well-differentiated cancers, and that positive cases, in fact, had poor clinical outcomes. Thus our results indicate the involvement of this enzyme in drug resistance. Although the involvement of GST-pi in drug resistance is supported by some in vitro reports, but is not by some others, the present study, conducted in relation to clinical cases, agrees on the involvement. In the future, it seems to be necessary not only to clarify the mechanism of drug resistance associated with GST-pi, but also to find a way to overcome such drug resistance.
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Figure 1. Comparison of survival curves in cases Ib or severe according to S:S: survivability score.
Figure 2: Cumulative survival rates for different scores in stage II or severe according to duration of survival.

- (S.S. 3) \( n=23 \)
- (S.S. 2) \( n=12 \)
- (S.S. 1) \( n=24 \)
- (S.S. 0) \( n=8 \)
S.S.: Simulated Score

Comparison of survival curves in cases in stage III or IV according to

Duration of survival

Cumulative survival rate

Years

S.S.3
S.S.2
S.S.1
S.S.0
Comparison of survival curves in cases with serous adenocarcinoma in stage III or IV between low and high stageable groups.

Diagram:

- Duration of survival
- Cumulative survival rate

Legend:
- High stageable group (n=II)
- Low stageable group (n=II)
Comparison of survival curves in stage III C cases between low and high cumulative survival groups.

High survival group (n=21)  
Low survival group (n=16)  

Duration of survival (years)  
Cumulative survival rate (%)
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