γ-Glutamyl Cysteine Synthetase Up-Regulates Glutathione and Multidrug Resistance-associated Protein in Patients with Chemoresistant Epithelial Ovarian Cancer

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ABSTRACT
Cellular detoxification, such as that mediated by the glutathione (GSH) system, is involved in the metabolism of various cytotoxic agents. Little is known, however, about the clinical relevance of cellular detoxification in chemoresistance. To elucidate the relevance of the GSH system to the resistance to chemotherapy observed in patients with ovarian cancer, we assayed the expression of mRNA encoded by the multidrug resistance-associated protein (MRP) and γ-glutamyl cysteine synthetase (γ-GCS) genes, as well as the level of GSH protein in 32 patients with epithelial ovarian cancer after chemotherapy. Tumors of 14 of the 32 patients responded to chemotherapy, whereas 18 did not. The levels of MRP and γ-GCS transcripts in tumors from nonresponders were each about 2-fold higher than in responders. In contrast, the level of GSH did not differ between the two groups. We observed coordinated expression of γ-GCS mRNA and GSH protein levels, and between γ-GCS and MRP in nonresponders, but not in responders. Expression of MRP-encoded mRNA did not correlate to GSH level, however, in either group. These results suggest that γ-GCS may up-regulate GSH and MRP expression in tumors unresponsive to chemotherapeutic agents, and that the GSH system may be involved in the mechanism of chemoresistance in ovarian cancer.

INTRODUCTION
Platinum-based combination chemotherapy is currently the standard treatment for epithelial ovarian cancer, and has achieved a high response rate, but the success of treatment is limited by the development of resistance to chemotherapy (1, 2). Resistance to anticancer drugs presents a major obstacle to the attempt to improve the prognosis of patients with ovarian cancer. Several mechanisms of drug resistance have been proposed, including a decrease in the accumulation of the drug, an increase in cellular detoxification of the drug, and an increase in DNA repair activity (3–7).

Cellular detoxification via the GSH2 system is known to be involved in metabolism of various cytotoxic agents including the platinum agents (5, 8). Elevation of GSH is commonly observed in cellular resistance to a number of anticancer agents (2). In addition, cellular mechanisms for eliminating cytotoxic agents by GSH conjugation are conserved throughout evolution (9). Increased mRNA expression and enzymatic activity of the heavy subunit of γ-GCS, the first enzyme of the GSH biosynthetic pathway, are associated with the elevated GSH levels (8). MRP gene, which encodes a 190-kDa membrane polypeptide, is thought to function as an ATP-dependent pump for cytotoxic drugs (MRP/GX pump; Ref. 10). A recent study by Ishikawa et al. (11) showed the efflux of a GSH-chelate complex by a MRP/GS-X pump. In that study, arsenite efflux from the MRP-transfected cells was accompanied by a significant increase in GSH efflux.

Most studies on the role of the GSH system in drug resistance have been conducted in vitro. Consequently, little is known about the clinical relevance of these findings. To elucidate the relevance of the GSH cellular detoxification system to the chemoresistance in clinic, we assayed levels of MRP and γ-GCS gene transcripts, and of GSH protein in specimens of ovarian tumors obtained from patients after chemotherapy.

MATERIALS AND METHODS
Patients and Materials. A total of 32 Japanese women with epithelial ovarian cancer, who had undergone laparotomy after chemotherapy at Tottori University Hospital between 1990 and 1996, were examined. All patients had a measurable lesion in the abdomen. Patients with borderline malignancy were excluded from this study. Informed consent was obtained from all patients. Chemotherapy consisted of a combination of 50 mg/m2 cisplatin (Nihon Kayaku, Tokyo, Japan), 40 mg/m2 doxorubicin (Adriamycin; Adria Laboratories, Columbus OH), and 400 mg/m2 cyclophosphamide (Shionogi, Osaka, Japan). The interval of each course for chemotherapy was 4 weeks. Chemotherapy was repeated for a maximum of three courses.

The effect of chemotherapy was evaluated 3 weeks after each chemotherapy by ultrasonography, magnetic resonance imaging, and computerized tomography according to the fol-
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Total RNA was isolated from each tissue sample by the acid guanidinium thiocyanate-phenol-chloroform extraction method. The antisense RNA probe was synthesized using SP6 RNA polymerase. To prepare the -γ-GCS probe, a 0.88-kb cDNA for the heavy subunit of the -γ-GCS gene was synthesized by reverse transcription-PCR, using the forward and reversed primers, 5'-GCTGCATCTCCCCCCTTT-TACCAGG and 5'-TGCGACATGTGATTTAGTAGTGTAGT-TCTTGAG, respectively. The PCR product was cloned into pCRII vector. The antisense RNA probe was synthesized using SP6 RNA polymerase.

Expression of MRP and γ-GCS Gene Encoded mRNA. Total RNA was isolated from each tissue sample by the acid guanidinium thiocyanate-phenol-chloroform extraction method (12). mRNAs of MRP and γ-GCS were detected by RNase protection assay (13). Because of vast differences in abundance between test and reference RNA, 20 μg total RNA were hybridized with 32P-labeled MRP and γ-GCS RNA probes, and 1 μg RNA was hybridized with an 18S rRNA probe (2 × 105 cpm each), which serves as a reference. After annealing, the reaction mixtures were combined and digested with RNases A and T1, followed by phenol-chloroform extraction. The nucleic acids were ethanol precipitated and analyzed by PAGE. The expression level was quantitated on a Macintosh Quadra 840AV computer using the public domain NIH image program (written by Wayne Rasband at NIH and available from the Internet by anonymous ftp from zippy.nimh.nih.gov or on floppy disc from NTIS, 5285 Port Royal Rd., Springfield, VA 22161, part number PB93–50464).

MRP and γ-GCS mRNA levels are expressed relative to the 18S RNA used as an internal standard.

Measurement of GSH Concentration. For the extraction of protein, samples were homogenized in PBS on ice and passed through a 40 μm nylon mesh. After centrifugation (25,000 × g for 5 min), the total protein concentration of the supernatant was determined (14). GSH concentration was measured enzymatically, according to the method of Owens and Belcher (15) and in this assay, GSH is oxidized by reduced NADP⁺ to produce oxidized glutathione with the stoichiometric formation of TNB, and oxidized glutathione is subsequently reduced to GSH by GSH reductase mixed plus NADPH. Briefly, 200 μl of sample were incubated with 100 μl of 16 mM DTNB and 700 μl of 0.3 mM NADPH for 5 min at 37°C. GSH reductase was added after the incubation. The rate of TNB formation was determined at 412 nm with a U-2000A Spectrophotometer (Hitachi, Tokyo, Japan).

### Table 1 Patient characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Nonresponders (n = 18)</th>
<th>Responders (n = 14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>41–78</td>
<td>28–69</td>
</tr>
<tr>
<td>Range</td>
<td>62.3</td>
<td>59.8</td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serous cystadenocarcinoma</td>
<td>13</td>
<td>10</td>
</tr>
<tr>
<td>Mucinous cystadenocarcinoma</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Histological grade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>G2</td>
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</tr>
<tr>
<td>IV</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Recurrent disease</td>
<td>5</td>
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</tr>
</tbody>
</table>

*FIGO, International Federation of Gynecology and Obstetrics.

Fig. 1 Expression of mRNA encoded by MRP and γ-GCS genes. RNase protection assay was performed as described in “Materials and Methods,” using 18S rRNA as the internal control. N, nonresponder; R, responder; A, only probes, negative control; B, HeLaR cell which expressed MRP and γ-GCS genes, positive control; nt, nucleotide.
Fig. 2. Expression of MRP and γ-GCS encoded mRNA relative to 18S rRNA in nonresponders and responders. Mean value of MRP was 0.79 for nonresponders and 0.36 for responders, and that of γ-GCS was 0.82 and 0.43, respectively.

Statistical Analysis. All assays were performed in duplicate. Statistical analyses were performed using the Macintosh Excel software. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Response to Chemotherapy. Of the 32 patients with epithelial ovarian cancer, 14 (CR 0, PR 14) responded to chemotherapy, whereas 18 did not (NC 10, PD 8). Nonresponders included five patients with recurrent disease. Five of the responders received two courses of chemotherapy, and the remaining nine responders and all of the nonresponders received three courses (mean, 2.6 for responders and 3.0 for nonresponders). There were no significant differences in age, histology, and histological grade between the responders and nonresponders (Table 1).

Expression of MRP and γ-GCS Gene Encoded mRNA and GSH Protein. Specific transcripts of the MRP and γ-GCS genes were detected in 30 and 31 of the tumors, respectively (Fig. 1). In the one tumor, surgically removed from a responder, the MRP gene was not expressed; in a second tumor, also from a responder, both genes were not expressed. There was no difference in tumor cell versus normal stromal cell ratio between responders and nonresponders. When normalized relative to 18S rRNA, the level of MRP and γ-GCS mRNA was about 2-fold higher in nonresponders than in responders (Fig. 2). The level of GSH protein (μg/mg protein) of responders ranged from 2.1–43.0 (mean and SD, 15.8 and 15.1, respectively) and those in nonresponders ranged from 3.2–52.8 (mean and SD, 19.8 and 15.4, respectively).

Statistical analysis. All assays were performed in duplicate. Statistical analyses were performed using the Macintosh Excel software. A value of $P < 0.05$ was considered statistically significant.

DISCUSSION

It is shown that, in cisplatin-resistant ovarian cancer cell lines, transcriptional activation can lead to increased expression of the γ-GCS gene (16) and that exposure to ethacrynic acid can increase the half-life of γ-GCS (17). These alterations were associated with elevated GSH levels in chemoresistant cells (18). Furthermore, several in vitro studies have shown a correlation between GSH levels in cancer cells and the sensitivity of these cells to anticancer agents (7, 19, 20). However, most clinical studies have failed to demonstrate any role for the GSH system in chemoresistance (21–24). In the present study, GSH concentration did not differ between responder and nonresponder, because it distributed wide range. In our previous study, mean values of GSH concentrations in tumors of ovarian cancer before and after chemotherapy did not differ between responders and nonresponders for the same reason (25). We observed the coordinate expression of γ-GCS mRNA and GSH protein in ovarian cancer from patients unresponsive to chemotherapy, but not in responders. In drug-resistant cell lines, the
coordinate regulation of GST, γ-GCS, and MRP has been found (26). To our knowledge, this is the first clinical report to show the γ-GCS coordinate regulation of GSH in ovarian cancer resistant to chemotherapy. The present result supports our previous finding that GSH concentration and expression of GST-π increased after chemotherapy in the nonresponders but not in the responders (25). These results suggest that the GSH system may be involved in the mechanism of chemoresistance of ovarian cancer, at least in some nonresponding individuals.

Overexpression of MRP occurs in several human cancers such as lung, breast, ovarian, and leukemia (27). A significant association was found in neuroblastoma between a high level of MRP expression and poor clinical outcome (28). Additionally, MRP was shown to be an important predictor for poor prognosis in patients with breast cancer who were treated by chemotherapy (29, 30). However, the association of MRP with chemoresistance has not been elucidated in ovarian cancer yet. In ovarian cancers, positive immunostaining for MRP was not associated with any prognostic factors such as response to treatment and patient survival (31). The study had a serious drawback, however, in that MRP expression was assayed before chemotherapy. It is noteworthy that cellular detoxification enzymes are induced by cytotoxic agents (25, 32, 33). Before chemotherapy, we also found no differences in the level of MRP transcription between responders and nonresponders (Fig. 4). Subsequent to chemotherapy, however, MRP gene expression was significantly higher in nonresponders than in responders. These results suggest that MRP is associated with a chemoresistance mechanism in ovarian cancer.

The recent literature suggested that MRP gene expression was regulated by MYCN oncogene in neuroblastoma (34). There are no reports concerning MYCN oncogene in ovarian cancer. Further study is necessary to know the correlation between the MRP gene and the MYCN oncogene in ovarian cancer.

Cytotoxic agents bind to GSH protein; thus, increases in cellular GSH levels lead to an increased generation of these complexes and their excretion by a MRP/GS-X pump (10, 11). Both the GSH content and GS-X pump activity increased in a γ-GCS gene transfected lung cancer cell line (35). In drug resistant colon tumor cells, Ciaccio et al. (26) found coordinate increase in defense mechanisms that detoxify the drug and remove its conjugate via plasma membrane efflux by MRP. On the other hand, the present study showed that there was no correlation between MRP gene expression and GSH protein levels in either nonresponders or responders. The wide range of GSH levels may be a reason for the result.

We observed the coordinated expression of γ-GCS and MRP in nonresponders. MRP and γ-GCS genes have also been shown to be coordinately overexpressed in cisplatin-resistance cells (9). Therefore, γ-GCS may directly up-regulate MRP in chemoresistant ovarian cancer. Coordinated up-regulation of MRP and γ-GCS was observed in nonresponders but not in responders. In our recent findings, cellular detoxification systems such as GSH and GST-π have been enhanced by chemotherapy only in nonresponders (25, 36). Enhancement of detoxification systems may be a mechanism of chemoresistance.

Mechanisms other than cellular detoxification may also contribute to chemoresistance in ovarian cancers, because the expression of mRNAs of MRP and γ-GCS and the levels of GSH in tumors did not increase in some nonresponding tumors. The results of this investigation suggest that, at least in some cases of ovarian cancer unresponsive to chemotherapy, γ-GCS can up-regulate GSH and MRP. Activation of the GSH system may be involved in the mechanism of chemoresistance in patients with epithelial ovarian cancer.

REFERENCES


Gamma-glutamyl cysteine synthetase up-regulates glutathione and multidrug resistance-associated protein in patients with chemoresistant epithelial ovarian cancer.

J Kigawa, Y Minagawa, X Cheng, et al.