Metallothionein Content Correlates with the Sensitivity of Human Small Lung Cancer Cell Lines to Cisplatin†

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ABSTRACT

We have established cis-diaminedichloroplatinum(II) (cisplatin)-resistant human small cell lung cancer cell lines, H69/CDDP2 and H69/CDDP2, to investigate the mechanism of acquired resistance to cisplatin. H69/CDDP2 and H69/CDDP were 6- and 11-fold resistant to cisplatin compared with the H69 parental cell line. H69/CDDP was also resistant to cadmium chloride (2-fold), cis-diamine(glycolato)platinum (4-fold), 4-hydroperoxycyclophosphamide (3-fold) and 3-(4-amino-2-methyl-5-pyrimidinyl)methyl-1-(2-chloroethyl)-1-nitrosourea (4-fold) if the drug concentrations that inhibit cell growth by 50% from growth inhibition assay were compared. There was no significant difference in the cisplatin accumulation among these cell lines. Although DNA interstrand cross-link formations, determined by filter elution assay in H69/CDDP2 and H69/CDDP, was decreased to 20 to 30% of that in H69 parental cells, the repair capacity of DNA interstrand cross-links was equivalent in all three cell lines. Intracellular glutathione content was equal in all cell lines. H69/CDDP had the highest glutathione S-transferase activity (H69, 11 nmol/min/mg protein, H69/CDDP2, 12 nmol/min/mg protein; H69/CDDP, 74 nmol/min/mg protein, respectively) and an overexpression of glutathione S-transferase α mRNA. The drug concentrations that inhibit cell growth by 50% for cisplatin in all cell lines were decreased by treatment with ethacrynic acid, an inhibitor of glutathione S-transferase α, but this did not alter the relative degree of resistance. Intracellular metallothionein content (H69, 14 pmol/mg protein, H69/CDDP2, 22 pmol/mg protein; H69/CDDP, 33 pmol/mg protein, respectively) and expression of metallothionein mRNA were correlated with the drug concentrations that inhibit cell growth by 50% of the three cell lines for cisplatin and cadmium chloride. The present study suggested the importance of metallothionein in the mechanisms of cisplatin resistance.

INTRODUCTION

Cisplatin is a key anticancer agent for the treatment of human small cell lung cancer (1), although development of resistance to cisplatin limits its efficacy and results in failure of cancer treatment. Thus it is essential to determine the mechanism(s) of cisplatin resistance, not only to improve the present chemotherapy but also to develop new anticancer agents, especially new platinum derivatives. We have already reported characteristics of cisplatin-resistant human non-small cell cancer cell lines (2). A variety of mechanisms of cisplatin resistance have been described (3), including decreased drug accumulation (4–6), increased detoxification by thiol containing scavenger molecules such as GSH4 and MT, and increased repair of DNA damage (7–9). Fujiwara et al. (2) reported that the non-small cell lung cancer cell line, PC-9/CDDP, which showed 28-fold resistance to cisplatin, had higher GSH content than that of parental PC-9. Although dl-buthionine,R,S-sulfoximine depleted the GSH of the resistant line to a level approximately equal to that of the parental line, the IC50 of the resistant line was still 21-fold higher than that of the parental line. This indicates that factors other than increased GSH confer cisplatin resistance. Increased levels of GST isozymes have been correlated with the resistance to alkylating agents (10–12). However, there was no difference in GST activities and GST-α, -μ, and -π contents between in vitro acquired cisplatin-resistant cells and parental cells in our previous studies (2). Transfection of a GST-α expression vector into NIH3T3 cells could not induce cisplatin resistance (13). A potential role for GST-α as a marker of cisplatin resistance remains to be demonstrated (11), although GST-α by itself may not induce cisplatin resistance. MT is the most prominent of the protein sulfhydryl. Andrews et al. (14) reported elevation of the MT level in cadmium chloride-resistant human ovarian carcinoma cells that were cross-resistant to cisplatin. Furthermore, mouse C127 cells transfected with the human MT II gene became resistant to cisplatin and alkylating agents (15). Thus MT may be one possible mechanism of resistance.

In the present study, we have examined the mechanisms of cisplatin resistance in the human small cell lung cancer cell line, H69, and its resistant cell lines with different degrees of resistance to cisplatin.

MATERIALS AND METHODS

Drugs and Chemicals. RPMI 1640 and PBS were purchased from Nissui Pharmaceutical Co., Tokyo, Japan. Cisplatin and etoposide were obtained from Bristol Myers Co., Tokyo, Japan. ACNU was purchased from Sankyo Co., Ltd., Tokyo, Japan. ADM was purchased from Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan. Vincristine, 4-hydroperoxycyclophosphamide, and cis-diamine(glycolato)platinum were obtained from Shionogi Co., Ltd., Osaka, Japan. 7-Ethyl-10-(4-[1-piperidino]-1-piperidino)-carbonyloxycamptothecin was obtained from Daiichi Seiyaku Co., Ltd., Tokyo, Japan. Ethacrynic acid was purchased from Banyu, Tokyo, Japan, and was dissolved in absolute ethanol. [a-32P]CTP and [methyl-3H]thymidine were purchased from Amersham Japan, Tokyo, Japan. [103P]Cisplatin (specific activity, 7.4 × 106 Bq/mg cisplatin; 200 μCi/mg cisplatin) was made available by Dr. M. Akaboshi at Research Reactor Institute of Kyoto University. Other drugs and chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) if not otherwise mentioned.

Cell Lines. H69, which is a human small cell lung cancer cell line, was established at the National Cancer Institute, Bethesda, MD, and was obtained from Dr. Y. Shimosato, National Cancer Center Hospital. H69/CDDP2 was established by exposure of H69 cells to stepwise increasing cisplatin concentrations up to 0.2 μg/ml and the resistant cell line was isolated by limited dilution (16). H69/CDDP2 was further exposed to cisplatin at a concentration of 0.4 μg/ml for 1 year leading to the H69/CDDP cell line. Cells used in all experiments were subcul-
CISPLATIN RESISTANCE MECHANISMS

In order to study cisplatin resistance mechanisms, we performed a series of experiments on two cisplatin-resistant cell lines, H69/CDDP0.2 and H69/CDDP. These cells were derived from the parental cell line H69, which is sensitive to cisplatin.

1. **Drug Sensitivity Test**: The drug sensitivity of each cell line to various antitumor agents was determined by growth inhibition as previously reported (17). In brief, a number of 60-mm dishes were set up containing 2 × 10^5 cells. Drugs were added to each dish at appropriate concentrations. After incubation for 7 days, the cells were counted in a Microcellcounter CC-108. All assays were conducted using continuous drug exposure. Each experiment was performed in triplicate and each experiment was done at least 3 independent times. The degree of drug sensitivity of cells was determined from the graph of cell number versus drug concentration and was expressed as IC50 value, defined as the drug concentration inhibiting cell growth by 50% compared to the control dishes.

2. **Cisplatin Accumulation Study**: For cisplatin accumulation studies, exponentially growing cells of the three cell lines were harvested and seeded into 60-mm culture dishes at a density of 1 × 10^6 cells/ml. The cells were incubated with 5 µg/ml [3H]cisplatin in RPMI 1640 plus fetal bovine serum for 1, 2, or 3 h. In efflux studies, cells treated with 5 µg/ml [3H]cisplatin for 3 h were washed with cold PBS and resuspended in fresh medium and incubated for 1, 2, and 3 h. At the end of each time period, cells were collected by centrifugation and washed twice with cold PBS. The radioactivity of each sample was counted with a Packard Auto-Gamma scintillation counter. All results were normalized to cellular protein as measured by the BCA protein assay kit (Pierce, Rockford, IL).

3. **Filter Elution Assays**: ICL induced by cisplatin were determined by the filter elution method (18–20). In brief, 4 × 10^5 cells were radiolabeled with 1.0 µCi/ml [methyl-3H] thymidine for 60 h, followed by a 3-h chase period. Labeled cells were incubated with cisplatin at various concentrations for 3 h. For γ-irradiation, labeled cells were irradiated on ice with 60Co γ-irradiation at a dose rate of 0.22 Gy/min. In the experiments of repair of ICL, cells treated with cisplatin were incubated in cisplatin-free medium for various periods before irradiation. Cells (1 × 10^6) were layered on a polycarbonate membrane, washed with cold PBS and lysed with a lysis solution containing 2% sodium dodecyl sulfate, 50 mM glycine, 25 mM disodium EDTA, 50 mM Tris (pH 10.0), and 0.5 mg/ml proteinase K. The lysis solution was drawn through the filter elution method (18–20). In brief, 4 × 10^6 cells were radiolabeled with 0.2 mCi/ml [methyl-3H] thymidine for 60 h, followed by a 3-h chase period. Labeled cells were incubated with cisplatin at various concentrations for 3 h. For γ-irradiation, labeled cells were irradiated on ice with 60Co γ-irradiation at a dose rate of 0.22 Gy/min. In the experiments of repair of ICL, cells treated with cisplatin were incubated in cisplatin-free medium for various periods before irradiation. Cells (1 × 10^6) were layered on a polycarbonate membrane, washed with cold PBS and lysed with a lysis solution containing 2% sodium dodecyl sulfate, 50 mM glycine, 25 mM disodium EDTA, 50 mM Tris (pH 10.0), and 0.5 mg/ml proteinase K. The lysis solution was drawn through the filter elution method (18–20).

4. **Statistical Analysis**: The data were analyzed for significance by Student's t test.

#### RESULTS

**Characteristics and Drug Sensitivity of H69 Parental and Cisplatin Resistant Cell Lines.** Two cisplatin-resistant cell lines, H69/CDDP0.2 and H69/CDDP, were derived from H69 parental cells. The doubling times of each cell line were 60, 67 and 67 h, respectively, and the cell sizes were 6.92, 6.59, and 6.96 µm, respectively. There was no significant difference in doubling times and cell sizes in the three cell lines. Table 1 shows the sensitivities for various agents of three cell lines. H69/CDDP0.2 and H69/CDDP were more resistant to cisplatin than H69 with relative resistance (IC50 of resistant cell line/IC50 of parental cell line) of 6.2 and 10.9, respectively. H69/CDDP was cross-resistant to 254-S, a new platinum compound. H69/CDDP0.2 and H69/CDDP were more resistant to ACNU, h-CPA, the active form of cyclophosphamide, and cadmium chloride. Sensitivities to ADM, vincristine, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin, and etoposide were not different between H69 and H69/CDDP.

#### Table 1 Sensitivities for various agents of H69 and its cisplatin-resistant cell lines

<table>
<thead>
<tr>
<th>Agents</th>
<th>H69</th>
<th>H69/CDDP0.2</th>
<th>H69/CDDP</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC50 (µg/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cisplatin</strong></td>
<td>0.076 ± 0.003*</td>
<td>0.47 ± 0.07 (6.2)†</td>
<td>0.83 ± 0.13 (10.9)</td>
</tr>
<tr>
<td>254-S</td>
<td>0.21 ± 0.006</td>
<td>ND</td>
<td>0.86 ± 0.02 (4.1)</td>
</tr>
<tr>
<td>h-CPA</td>
<td>0.26 ± 0.017</td>
<td>0.56 ± 0.06 (2.2)</td>
<td>0.73 ± 0.11 (2.8)</td>
</tr>
<tr>
<td>ACNU</td>
<td>0.52 ± 0.12</td>
<td>1.8 ± 0.2 (3.5)</td>
<td>2.2 ± 0.1 (4.2)</td>
</tr>
<tr>
<td>ADM</td>
<td>0.058 ± 0.007</td>
<td>ND</td>
<td>0.063 ± 0.005</td>
</tr>
<tr>
<td>VCR</td>
<td>0.017 ± 0.008</td>
<td>ND</td>
<td>0.017 ± 0.008</td>
</tr>
<tr>
<td>CPT-11</td>
<td>0.85 ± 0.16</td>
<td>ND</td>
<td>0.79 ± 0.12</td>
</tr>
<tr>
<td>VP-16</td>
<td>0.82 ± 0.11</td>
<td>ND</td>
<td>0.55 ± 0.19</td>
</tr>
<tr>
<td>GC1</td>
<td>9.6 ± 0.7</td>
<td>14.7 ± 1.5 (1.5)</td>
<td>19.8 ± 1.0 (2.1)</td>
</tr>
</tbody>
</table>

* Each value is the mean ± SD of the three independent experiments.
† Relative resistance value: IC50 value of resistant cells/IC50 value of parental cells.
ND, not determined.

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CISPLATIN RESISTANCE MECHANISMS

Fig. 1. Accumulation and efflux of [1⁹⁵⁰Pt]cisplatin in H69 and its cisplatin-resistant cell lines. A, incorporation of radiolabeled cisplatin into H69 parental and drug-resistant cells. All cells were treated with 5 μg/ml of cisplatin for 1, 2, or 3 h. B, efflux of [¹⁹⁵⁰Pt]cisplatin from H69 cells. Cells treated with 5 μg of cisplatin per ml were washed with cold PBS and incubated for 1, 2, or 3 h in drug-free medium. All data were normalized to that of H69 cells with no drug-free incubation period (control). ○, H69; □, H69/CDDP₀₂; ●, H69/CDDP. Bars, SD.

formation of DNA ICL in the 3 cell lines after exposure to various concentrations of cisplatin for 3 h. ICL in each cell line was formed in a concentration-dependent manner. In H69/CDDP₀₂ and H69/CDDP cells, ICL was decreased to 30.0 and 21.1%, respectively, compared with H69. To elucidate any difference in repair of ICL, we washed platinated cells with fresh medium and incubated them for various times. The concentrations of cisplatin to which H69, H69/CDDP₀₂, and H69/CDDP were exposed were 2, 6, and 10 μg/ml, respectively, to induce an equal level of ICL. As shown in Fig. 2B, ICL increased during the first 6 h of the repair time and after 6 h ICL decreased in a time-dependent manner. No significant difference was observed in peak levels and rates of decrease of ICL among the 3 cell lines. No single or double strand breaks induced by cisplatin were found in the filter elution assays (data not shown).

Detoxification Mechanisms. In order to elucidate the role of the potential drug detoxification systems in the acquisition of cisplatin resistance, we measured GSH content, MT content, and GST activity. The results are summarized in Table 2. There was no difference in GSH content among the 3 cell lines. The MT levels in H69/CDDP₀₂ and H69/CDDP were 1.6- and 2.3-fold higher, respectively, than that of H69. The MT levels were positively correlated with IC₅₀ values to cisplatin and cadmium chloride in these cell lines (Fig. 3). GST activity was not different between H69 and H69/CDDP₀₂, but H69/CDDP showed an approximately 6-fold increase in GST activity. To investigate the role of GST in cisplatin resistance, we used ethacrynic acid, an inhibitor of GST-α and GST-π (27), in a growth inhibition assay (Table 3). An ethacrynic acid concentration of 0.1 μg/ml was chosen because this was not cytotoxic in all the cell lines. Ethacrynic acid sensitized each cell line, but the relative resistance of the H69 cell lines was not changed.

Table 2 Detoxification mechanisms of H69 and its cisplatin-resistant cell lines

<table>
<thead>
<tr>
<th></th>
<th>H69</th>
<th>H69/CDDP₀₂</th>
<th>H69/CDDP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative resistance</td>
<td>1.0</td>
<td>6.2</td>
<td>10.9</td>
</tr>
<tr>
<td>GSH content (nmol/mg protein)</td>
<td>16.6 ± 4.7a</td>
<td>14.4 ± 2.9</td>
<td>19.9 ± 3.0</td>
</tr>
<tr>
<td>GST activity (nmol/min/mg protein)</td>
<td>11.5 ± 8.7</td>
<td>12.3 ± 4.5</td>
<td>74.3 ± 16.1b</td>
</tr>
<tr>
<td>MT content (pmol/mg protein)</td>
<td>14.5 ± 1.5</td>
<td>22.3 ± 2.0c</td>
<td>32.6 ± 1.6d</td>
</tr>
</tbody>
</table>

a Mean ± SD.
b P < 0.001 compared to value for H69 and H69/CDDP₀₂ by unpaired Student’s t test.
c P < 0.001 compared to value for H69 by unpaired Student’s t test.
d P < 0.001 compared to value for H69 and H69/CDDP₀₂ by unpaired Student’s t test.
A Northern blot analysis was undertaken to investigate the overexpression of genes in the resistant cell lines. Fig. 4 shows the mRNA levels for GST-π, MT, and β-actin. The β-actin probe demonstrated that an equivalent amount of total RNA from each cell line was loaded in each lane. There was no difference in the expression of GST-π mRNA between H69 and H69/CDDP0.2 but it was overexpressed in H69/CDDP. The expressions of MT mRNA were 2.6- and 4.3-fold higher in H69/CDDP0.2 and H69/CDDP, respectively. The expression of MT mRNA was inversely correlated with the sensitivity to cisplatin. No evidence for MT gene amplification could be seen in H69/CDDP0.2 and H69/CDDP and no evidence of GST-π gene amplification could be seen in H69/CDDP cells (data not shown). No cell lines expressed mdr-1 mRNA, although all cell lines had mdr-1 gene (data not shown).

**DISCUSSION**

The resistant cells used in this study had equal doubling times and cell sizes, making them suitable to analyze mechanisms of acquired cisplatin resistance. We evaluated five factors that are thought to play a role in cisplatin resistance. The analysis using \[^{195m}Pt\]cisplatin revealed equal amounts of cisplatin accumulation and no difference in efflux. Thus we concluded the difference in sensitivity to cisplatin among the 3

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**Table 3. Modification of sensitivity to cisplatin by ethacrynic acid**

<table>
<thead>
<tr>
<th></th>
<th>H69</th>
<th>H69/CDDP0.2</th>
<th>H69/CDDP</th>
</tr>
</thead>
<tbody>
<tr>
<td>GST activity EA(−)</td>
<td>11.3 ± 8.7*</td>
<td>12.3 ± 4.5</td>
<td>74.3 ± 16.1</td>
</tr>
<tr>
<td>EA(+)</td>
<td>3.4 ± 1.9</td>
<td>7.1 ± 0.3</td>
<td>12.7 ± 5.6</td>
</tr>
<tr>
<td>IC₅₀ value to cisplatin</td>
<td>0.076 ± 0.002</td>
<td>0.47 ± 0.07</td>
<td>0.83 ± 0.13</td>
</tr>
<tr>
<td>Relative resistance EA(−)</td>
<td>0.057 ± 0.019</td>
<td>0.25 ± 0.07</td>
<td>0.63 ± 0.02</td>
</tr>
<tr>
<td>EA(+)</td>
<td>1</td>
<td>4.4</td>
<td>11.1</td>
</tr>
</tbody>
</table>

* EA, ethacrynic acid.
* Mean ± SD.
* Cells were treated with 0.1 μg of ethacrynic acid per ml.

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**Fig. 4.** Expression of MT and GST-π in H69 and its cisplatin-resistant cell lines. Samples of total RNA (10 μg each) were electrophoresed in 1% formamide-agarose gel, transferred onto a nylon membrane, and hybridized with each \[^{32}P\]labeled DNA probe. All hybridizations were performed on the same filter and hybridization with β-actin was used to show the equivalent loading of the total RNA.

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**Fig. 3.** Correlation of IC₅₀ values with intracellular MT content. A, cisplatin; B, cadmium chloride. Each IC₅₀ value is the mean of three independent experiments.
CISPLATIN RESISTANCE MECHANISMS

The primary mode of action for cytotoxicity of cisplatin is likely a result of its reaction with DNA. DNA ICL may be one of the important types of cisplatin-DNA adducts. We studied the formation of DNA ICL by the filter elution assay. The formation of DNA ICL significantly decreased in H69/CDDP and H69/CDDP under the same concentration of cisplatin. However, the equivalent amounts of cross-link formation and DNA repair were observed in all three cell lines at approximately equitoxic concentrations of cisplatin. These data suggest that formation of DNA ICL is important for cisplatin cytotoxicity and that repair of DNA ICL did not contribute to the difference in the resistance in H69 cell lines.

Detoxification mechanisms have been thought to play an important role in cisplatin resistance. GSH may be one of the important factors. In our studies, no significant difference was observed in GSH content. GSH-related enzymes, especially GST, have been considered to be important factors for cisplatin resistance. However, Nakagawa et al. (13) could not demonstrate the induction of cisplatin resistance in GST-IR-transfected NIH3T3 cells. In our resistant cell lines, H69/CDDP showed approximately 6-fold higher GST activity and overexpression of GST-IR mRNA, but in a less cisplatin-resistant cell line, H69/CDDP, from which H69/CDDP was derived, there was no significant difference in GST activity and the expression of GST-IR mRNA was similar to the parental H69. As shown in Table 3, the IC50 values to cisplatin were decreased by treatment with ethacrynic acid, an inhibitor of GST-IR and -IR in all cell lines, but the relative resistant values were unchanged. Although the GST activity of H69/CDDP was inhibited by ethacrynic acid, H69/CDDP and H69/CDDP showed 4.2- and 11.0-fold resistance compared with H69 cells. These data suggest that GST may contribute to cisplatin resistance but other factors must be responsible for the difference between these lines.

MTs comprise a class of isoproteins with molecular weights of 6000-7000. They are involved in zinc homeostasis and the detoxification of heavy metal such as cadmium, copper, and mercury (30). Bakka et al. (31) showed that human epithelial cells and mouse fibroblasts, which showed resistance to cadmium chloride and contained a higher MT content, showed cross-resistance to cisplatin. Andrews et al. also reported that the cadmium chloride-resistant cell lines, 2008/MT and COLO/MT, showed cross-resistance to cisplatin and that MT levels were elevated 23-fold in 2008/MT and 9.4-fold in COLO/MT compared to their parental cell lines (14). Although the resistant cells also had increased GSH, depletion of GSH by dibutyryl-R,S-sulfoximine treatment had no effect on the sensitivity to cisplatin and cadmium chloride. They suggested that MT content might be one mechanism of cisplatin resistance (14). C-127 cells transfected with bovine papilloma virus-containing human MT IIA were 4-fold resistant to cisplatin compared to cells transfected with only bovine papilloma virus (15). On the other hand Schilder et al. (32) reported that there was no causal relationship between MT mRNA expression and sensitivities to cisplatin and cadmium chloride in human ovarian cancer cell lines. Thus, the role of MT in cisplatin resistance remains controversial. H69/CDDP and H69/CDDP had higher MT content than H69, and as shown in Fig. 3, MT levels were positively correlated with IC50 values to cisplatin and cadmium chloride. The overexpression of MT mRNA was observed in cisplatin-resistant cells. Because Southern blot analysis showed no DNA amplification, an enhanced rate of gene transcription or increased mRNA stability may be the cause of MT protein overexpression. We speculate that the increased MT in resistant cells may react with more cisplatin than in parental cells resulting in decreased ICL formation in H69/CDDP and H69/CDDP. To our knowledge, this is the first report to show a direct correlation between sensitivities to cisplatin and MT content and MT mRNA in extensively analyzed cell lines. Thus, MT may play an important role in the resistant mechanisms in H69 cells. Andrews et al. showed 23- and 9.4-fold elevation of MT content in only 4.1- and 3.3-fold resistant cell lines selected with cadmium chloride, respectively.

In our study, MT levels were elevated only 1.6- and 2.3-fold in 6.2- and 10.9-fold resistant cell lines selected with cisplatin. MT isoforms may not be equivalent with respect to metal-binding properties (33), MT isoforms may be responsible for the discrepancy between relative resistance and increment of MT contents. H69/CDDP and H69/CDDP showed cross-resistance to 4-hydroperoxycyclophosphamide and ACNU. GST activity cannot be responsible for the cross-resistance to these alkylating agents because GST activities of the 3 cell lines were not correlated with IC50 values. MT has been postulated to be a factor in resistance to alkylating agents (30) and the elevation of MT content that we detected in these cells is consistent with this hypothesis.

In summary, the MT content and MT mRNA levels correlated with the resistance levels to cisplatin in these human small cell lung cancer cell lines. High GST activity and overexpression of GST-IR mRNA were observed only in H69/CDDP but not in H69/CDDP and ethacrynic acid did not overcome the resistance. These data suggest that MT is an important factor in cisplatin resistance and GST may be a minor one in our human small cell lung cancer cell lines.

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