Sensitivity to Anticancer Agents and Resistance Mechanisms in Clear Cell Carcinoma of the Ovary

Hiroaki Itamochi, Junzo Kigawa, Habiba Sultana, Takahiro Iba, Ryoji Akeshima, Shunji Kamazawa, Yasunobu Kanamori and Naoki Terakawa

Department of Obstetrics and Gynecology, Tottori University School of Medicine, 36-1 Nishimachi, Yonago 683-8504

We conducted the present study to determine the chemoresistance mechanisms in clear cell carcinoma of the ovary (CCC). Five human CCC cell lines (HAC-2, RMG-I, RMG-II, KK, and KOC-7c) were used in this study. The sensitivity of the cells to the anticancer agents was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and we assessed drug sensitivity by calculating assay area under the curve (AUC) for each agent. The expression of multi-drug resistance genes (MDR-1, MRP-1, MRP-2) was detected by reverse transcription-polymerase chain reaction (RT-PCR). Glutathione (GSH) concentration was measured by an enzymatic assay. Topoisomerase (topo) I activity was assayed in terms of relaxation of supercoiled plasmid substrate DNA. The IC₅₀ to anticancer agents ranged widely. The assay AUC indicated that 3 of 5 cell lines (RMG-I, RMG-II, and KK) were sensitive to paclitaxel (PTX), 3 (HAC-2, RMG-I, and RMG-II) were sensitive to 7-ethyl-10-hydroxycamptothecin (SN-38), which is an active metabolite of camptothecin (CPT-11), and only one (HAC-2) was sensitive to cisplatin (CDDP). All cell lines were resistant to mitomycin-C (MMC) and etoposide (VP-16). The MRP-1 gene was detected in all cell lines. Only one cell line showed both MRP-2 and MDR-1 gene expression. Except for HAC-2 cells, expression of MRP genes was related to CDDP resistance, and MDR-1 gene expression was associated with PTX resistance. GSH concentrations increased after exposure to CDDP or MMC in all cell lines. There was a significant correlation between topo-I enzymatic activity and the response to SN-38. The present study revealed several resistance mechanisms in CCC and the results suggested that PTX and CPT-11 might be effective agents to treat CCC.

Key words: Ovarian cancer — Clear cell carcinoma — Chemotherapy — Resistance mechanism
The cDNA were amplified individually by PCR, using published primer sequences.23) The PCR primers, annealing temperature, and expected product size were as follows: for MDR-1, a 623-bp product was generated using 5′-ACACCCGACTTACAGATGATGC-3′ (forward primer) and 5′-CGAGATGGTAACTGAAGTGAACAT-3′ (reverse primer) at an annealing temperature of 58°C; for MRP-1, a 657-bp product was generated using 5′-AGTGACCTCTGGTCTTTAACAAGG-3′ (forward primer) and 5′-AGGATGACATCAGAATAAGAGACC-3′ (reverse primer) at an annealing temperature of 56°C; for MRP-2, a 322-bp product was generated using 5′-AAGATGACATCAGAATAAGAGACC-3′ (forward primer) and 5′-CTACTCCATCAATGATAATCTGACC-3′ (reverse primer) at an annealing temperature of 52°C. The primers of GAPDH designed for amplification of the gene as a control were 5′-ACCACCATGCATGCCATAC-3′ (forward primer) and 5′-TCCACACA-CCTGTGAT-TGCT-GTA-3′ (reverse primer). The primers were designed to generate a product of 450 bp. The PCR reaction mixture contained cDNA (0.5 µg), dNTPs (0.2 mM each), primers (0.4 µM each), MgCl2 (1.5 mM), Tris-HCl pH 8.3 (10 mM), KCl (50 mM), and Taq polymerase (2 U; Wako Chemical, Tokyo). Thirty-five cycles of PCR amplification were performed.

PCR-amplified samples were separated by electrophoresis on a 2% agarose gel in 89 mM Tris-borate and 2 mM EDTA (pH 8.3) and stained with 1.0 mg/ml ethidium bromide at 100 V for 30–60 min.

GSH concentration GSH concentration was measured by an enzymatic assay utilizing 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB)-GSSG reductase.24) Briefly, 200 µl of supernatant from the protein extraction was incubated for 5 min at 37°C with 100 µl of 16 mM DTNB and 700 µl of 0.3 mM NADPH. After incubation, glutathione reductase was added. GSH is oxidized by DTNB to give GSSG with stoichiometric formation of 5-thio-2-nitrobenzoic acid (TNB). GSSG is reduced to GSH by the action of NADPH and the highly specific glutathione reductase. The rate of TNB formation, proportional to the sum of GSH and GSSG present, was followed at 412 nm with a spectrophotometer (U-2000A, Hitachi, Tokyo).

Assessment of topo I activity To extract the nuclear protein, cell suspensions were centrifuged (150 g, 10 min) and washed 3 times with ice-cold phosphate buffer solution (PBS). Cell pellets were resuspended in extraction buffer [150 mM NaCl, 1 mM KH2PO4, 5 mM EDTA, 0.2 mM dithiothreitol (DTT), 1 mM PMSF, pH 6.4], mixed gently, and allowed to stand on ice for 15 min. Lysis was achieved by pipetting the suspension 50 times. Nuclear enzymes were extracted by adding an equal volume of 850 mM NaCl nucleus buffer with a final concentration of 500 mM NaCl, then mixed using a rotator at 4°C for 120 min. The mixture was centrifuged at 16 000g for 20 min at 4°C.
Total protein concentration of the supernatant was determined by the method of Bradford.\textsuperscript{25} Topo I activity was assayed in terms of the relaxation of supercoiled plasmid substrate DNA.\textsuperscript{26} The standard reaction mixture for the relaxation assay was assay buffer [100 mM Tris-HCl (pH 7.9), 10 mM EDTA, 1.5 M NaCl, 1% BSA, 1 mM spermidine, 50% glycerol] and 1 \( \mu l \) Form I supercoiled DNA (TopoGEN, Inc., Columbus, OH). Relaxation was carried out by incubating a 1 \( \mu l \) sample for 30 min at 37°C. Reactions were terminated by adding 5 \( \mu l \) of 5% sarkosyl, 0.125% bromophenol blue, and 25% glycerol. Samples were incubated with 50 \( \mu g/ml \) proteinase K for 60 min at 37°C. Samples were separated by electrophoresis on a 1% agarose gel in 89 mM Tris-borate and 2 mM EDTA (pH 8.3) at 50 V for 120 min. Gels were stained with ethidium bromide (0.5 mg/ml) for 30 min and destained for 15 min in H2O. The reaction mixture without nuclear protein was assayed as a negative control. DNA relaxation activity was evaluated in terms of the minimum amount of nuclear extract that generated the relaxed form completely.

**Statistical analysis** All assays were performed in triplicate. Data are expressed as the mean±standard deviation of values. Statistical analyses were performed using the Stat view Version 5.0-J program (Hulinks, Inc., Tokyo). A value of \( P < 0.05 \) was considered statistically significant.

**RESULTS**

The IC\textsubscript{50} values of each cell line for anticancer agents are shown in Table I. The IC\textsubscript{50}s ranged from 1.3 to 18.5 \( \mu M \) for CDDP, from 0.045 to 6.9 \( \mu M \) for PTX, from 0.74 to 10.5 \( \mu M \) for MMC, 4.4 to 93.8 \( \mu M \) for VP-16, and 0.016 to 1.9 \( \mu M \) for SN-38, indicating that these cell lines showed various sensitivities to anticancer agents. When the assay AUC was compared with clinically achievable AUC with a standard dose of each drug, 3 of 5 cell lines (RMG-I, RMG-II, and KK) were defined as sensitive to PTX, 3 (HAC-2, RMG-I, and RMG-II) were sensitive to SN-38, and only one (HAC-2) was sensitive to CDDP. All cell lines were resistant to MMC and VP-16 (Table II).

**Fig. 1** shows mRNA expression of MDR-1, MRP-1, and MRP-2. The MRP-1 gene was detected in all cell lines. Both MRP-2 and MDR-1 genes appeared in only KOC-7c cells, which showed strong resistance to CDDP and PTX. Except for HAC-2 cells, MRP-1 gene expression related to CDDP resistance and MDR-1 gene was also associated with PTX resistance.

Concentration of GSH in cells was widely distributed. The concentrations ranged from 10.0 to 23.9 \( \mu g/mg \) before treatment and increased after exposure to CDDP or MMC in all cell lines (Fig. 2). As a result, the concentration of GSH after exposure to CDDP or MMC was significantly higher than that before treatment (31.0±16.8 \( \mu g/mg \) for CDDP and 26.6±15.7 \( \mu g/mg \) for MMC vs. 14.3±5.7 \( \mu g/mg \) before treatment).

The minimum amount of extracted nuclear protein showing complete DNA relaxation ranged from 15.6 to 65.0 ng. The amount of nuclear protein required for complete DNA relaxation was greater in SN-38-resistant cells (KK and KOC-7c) than in sensitive cells (HAC-2, RMG-I, RMG-II).

### Table I. IC\textsubscript{50} Values to Anticancer Agents

| Cell lines | CDDP (\( \mu M \)) | PTX (\( \mu M \)) | MMC (\( \mu M \)) | VP-16 (\( \mu M \)) | SN-38 (\( \mu M \)) |
|------------|------------------|----------------|----------------|----------------|------------------|
| HAC-2      | 1.3              | 6.9            | 0.74           | 11.2           | 0.016            |
| RMG-I      | 10.1             | 0.045          | 10.5           | 4.4            | 0.037            |
| RMG-II     | 11.1             | 0.078          | 2.1            | 13.4           | 0.034            |
| KK         | 3.5              | 0.15           | 0.86           | 8.2            | 0.26             |
| KOC-7c     | 18.5             | 6.7            | 2.5            | 93.8           | 1.9              |

### Table II. Calculated Assay AUC at IC\textsubscript{50} and Clinically Achievable AUC (CA-AUC) for Each Drug

| Cell lines | Calculated assay AUC (\( \mu M \cdot h \)) | CDDP | PTX | MMC | VP-16 | SN-38 |
|------------|------------------------------------------|------|-----|-----|-------|-------|
| HAC-2      | 31.1\textsuperscript{a}                 | 499.7| 18.5| 548.4| 0.4\textsuperscript{a} |
| RMG-I      | 250.2                                    | 3.3\textsuperscript{a} | 260.6| 214.7| 0.9\textsuperscript{a} |
| RMG-II     | 275.8                                    | 5.7\textsuperscript{a} | 51.7 | 65.4 | 0.8\textsuperscript{a} |
| KK         | 87.4                                     | 11.1\textsuperscript{a} | 21.5 | 40.5 | 6.5 |
| KOC-7c     | 458.9                                    | 478.8| 61.9 | 457.7| 46.9 |
| CA-AUC     | 85.8                                     | 19.3 | 0.5 | 55.6 | 1.5 |

\textsuperscript{a} Sensitive.
and RMG-II) (15.6–23.4 vs. 46.9–62.5 ng). Additionally, IC50 values to SN-38 significantly correlate to topo I activity (Fig. 3).

**DISCUSSION**

Although CCC is known to be resistant to anticancer agents, the underlying mechanism has not been understood.1, 2 We, therefore, conducted the present study to determine CCC’s sensitivity to specific anticancer agents and to understand its chemoresistance mechanism. Determining cellular sensitivity is difficult due to the wide range of IC50 to anticancer agents. Additionally, protein binding of drug and drug stability in the culture medium are important pharmacodynamic factors. Assay AUC is a useful method to predict cellular sensitivity to anticancer agents, that correlates to clinical response.27, 28 In the present study, assay AUC was calculated to assess drug sensitivity.

We found that 4 of 5 cell lines, except for HAC-2 cells, were resistant to CDDP and that all cell lines were resistant to MMC and VP-16, although the IC50 values to the anticancer agents ranged widely. The MRP-1 gene, contributing to resistance to CDDP, MMC and VP-16, was detected in all cell lines. In contrast, 3 of 5 cell lines were sensitive to PTX. The expression of the MDR-1 gene, related to PTX resistance, was observed only in KOC-7c cells, which were resistant to PTX.16, 18, 29 On the other hand, HAC-2, which was resistant to PTX, did not show expression of MDR-1. Mechanisms other than multi-drug resistance genes such as MDR-1 and MRP may also contribute to chemoresistance in CCC cells, because the expression of MDR-1 and MRP-1 did not correlate to chemoresistance in HAC-2 cells.

Cellular detoxification via the GSH system is known to be involved in metabolism of various anticancer agents including CDDP and MMC.13, 17 The present study also showed that GSH concentration increased after exposure to CDDP and MMC. It is noteworthy that cellular detoxification enzymes are induced by anticancer agents in resistance cells.30, 31 Therefore, CCC may be insensitive to CDDP or MMC.

Three cell lines were sensitive to SN-38, an active metabolite of CPT-11. In the literature, the sensitivity of tumor cells to CPT-11 was associated with intracellular topo I level.32 We also found a significant correlation between topo I enzymatic activity and the response to SN-38. Furthermore, the combination treatment of PTX and SN-38 could control 4 of 5 cell lines, indicating that these agents might be an effective treatment for CCC.

Except for one cell line, MRP-1 gene expression was related to CDDP resistance and MDR-1 gene expression was associated with PTX resistance. GSH concentration was associated with resistance to CDDP or MMC. There
was a significant correlation between topo-I enzymatic activity and the response to SN-38. These findings suggested that many resistance mechanisms exist in CCC, although we failed to find a particular chemoresistance system. The present study also showed that PTX or an active metabolite of CPT-11 was effective in 3 of 5 cell lines and only one cell line was sensitive to CDDP. All cell lines were resistant to MMC and VP-16. It is suggested that PTX and CPT-11 may be effective agents against CCC. These data were from an in vitro study, and there is as yet no evidence of efficacy in vivo. Therefore, further studies are necessary to identify effective agents against CCC.

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