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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 IC_{50} 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

Platinum-based combination chemotherapy, a standard treatment for ovarian cancer, has achieved a high response rate. However, several studies have shown that patients with clear cell carcinoma of the ovary (CCC) have a poor prognosis with a low response rate.[1–3] Recently, CCC has increased in prevalence and now accounts for 18.5% of all ovarian carcinomas in Japan.[4] Patients with CCC require a superior chemotherapy, but no effective anticancer agent has yet been established.

Several anticancer agents with no cross-resistance to platinum analogues, such as paclitaxel (PTX), etoposide (VP-16), and camptothecin (CPT-11), have been developed. PTX as a promoter of tubulin polymerization changes the dynamic equilibrium of assembly and disassembly of microtubules, disrupts the formation of the normal spindle at metaphase, and causes the blockade of mitosis at the metaphase-to-anaphase transition.[5] VP-16, which inhibits the activity of DNA topoisomerase (topo) II, is used as a second-line treatment for patients with platinum-resistant ovarian cancer.[6] CPT-11 inhibits topo I by forming stable topo I-DNA cleavable complexes.[7] We previously found a collateral sensitivity between cisplatin (CDDP) and 7-ethyl-10-hydroxycamptothecin (SN-38), which is an active metabolite of CPT-11.[8, 9] Mitomycin-C (MMC), which is not a new agent, has been shown to be an effective agent for CCC in a recent study.[10]

Many mechanisms involved in drug resistance have been proposed, including decreased drug accumulation, increased drug detoxification, and increased DNA repair activity.[11–15] Above all, a multidrug resistance-associated protein (*MRP*) gene, which encodes a 190-kilodalton membrane polypeptide, and glutathione (GSH) contribute to CDDP, VP-16, and MMC resistance.[13, 16, 17] P-Glycoprotein, encoded by a multidrug resistance gene (*MDR-1*), contributes to sensitivity to PTX.[18] Topo I activity relates to sensitivity to CPT-11.[8, 9]

We conducted the present study to determine the chemosensitivity and to examine the resistance mechanisms in CCC.

MATERIALS AND METHODS

Cell lines and culture Five human CCC cell lines (HAC-2, RMG-I, RMG-II, KK, and KOC-7c) were used in this study. Those cell lines were kindly provided as follows: HAC-2 from Dr. M. Nishida, Tsukuba University, RMG-I

E-mail: itamochi@hotmail.com

and RMG-II from Prof. S. Nozawa, Keio University, KOC-7c from Dr. T. Sugiyama, Kurume University, and KK from Prof. Y. Kikuchi, National Defense Medical College. HAC-2, KK, and KOC-7c cells were maintained in RPMI 1640 medium (Nissui, Tokyo) with 10% fetal calf serum (FCS) in a humidified atmosphere containing 5% CO_2 at 37°C. RMG-I and RMG-II cells were maintained in Ham's F12 medium (Nissui) with 10% FCS also under the same conditions.

Sensitivity to anticancer agents The sensitivity of the cells to the anticancer agents was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[19] Briefly, cells were diluted with culture medium to the seeding density (10^5 cells/ml), suspended in 96-well tissue culture plates (200 μl/well) (Sumitomo Bakelite, Tokyo), and preincubated at 37°C for 4 h. Cells were then treated continuously with 20 μl of various concentrations of the anticancer agents to obtain a dose-response curve for each agent. Each drug concentration was as follows: 0.1–30 μM CDDP, 0.001–20 μM PTX, 0.01–30 μM MMC, 0.1–200 μM VP-16, and 0.001–5 μM SN-38.

After incubation for 72 h, 20 μl of MTT solution (2.5 mg/ml) was added to each well and the plates were further incubated for 4 h. One hundred microliters of dimethylsulfoxide was added to solubilize the MTT-formazan product. Absorbance at 570 nm was measured with a microplate reader Model 550 (BIO-RAD, Hercules, CA). Dose-response curves were plotted on a semi-log scale as a percentage of the control cell number, which was obtained from the no-drug-exposure sample.

To assess drug sensitivity, the assay area under the curve (AUC) was calculated by means of the following formula: initial concentration$\times t^{1/2}\times 1.44[1-\exp\{-(0.693)(72)\times t^{1/2}\}]$. Initial concentration was determined based on the IC_{50} of each agent in the present study. $t^{1/2}$ is the *in vitro* half-life of the drug at 37°C.[20] The half-lives of CDDP and VP-16 are 18.5 and 60 h, respectively.[21] PTX, MMC, and SN-38 were reported as stable in serum-containing medium at 37°C in the presence of 5% CO_2; therefore, the assay AUC of those drugs was calculated as the drug concentration $\times$72 h.[22] The assay AUC was then compared with clinically achievable AUC with a standard dose of each drug. If the calculated assay AUC was less than the clinically achievable AUC, the drug was defined as sensitive.

mRNA expression of MDR-1, MRP-1, and MRP-2 mRNA expression of MDR-1, MRP-1, and MRP-2 was determined by means of reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from each sample by the guanidinium thiocyanate method according to the manufacturer's instructions (Isogen, Nippon Gene, Tokyo). cDNA was synthesized from the isolated RNA by RT with the Gene Amp RNA PCR Core Kit (Perkin-Elmer Corp., Norwalk, CT).

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′-ACACCCGACTTACAGATGATGTCTC-3′ (forward primer) and 5′-CGAGATGGGTAACTGAAGTGAACAT-3′ (reverse primer) at an annealing temperature of 58°C; for MRP-1, a 657-bp product was generated using 5′-AGTGACCTCTGGTCCTTAAACAAGG-3′ (forward primer) and 5′-GAGGTAGAGAGCAAGGATGACTTGC-3′ (reverse primer) at an annealing temperature of 56°C; for MRP-2, a 322-bp product was generated using 5′-AGGATGACATCAGAAATAGAGACC-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′-ACCACAGTCCATGCCATCAC-3′ (forward primer) and 5′-TCCACCA-CCCTGT-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), $MgCl_2$ (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 KH_2PO_4, 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 16 000*g* for 20 min at 4°C.

Total protein concentration of the supernatant was determined by the method of Bradford.[25]

Topo I activity was assayed in terms of the relaxation of supercoiled plasmid substrate DNA.[26] The standard reaction mixture for the relaxation assay was assay buffer [100 m*M* Tris-HCl (pH 7.9), 10 m*M* EDTA, 1.5 *M* NaCl, 1% BSA, 1 m*M* spermidine, 50% glycerol] and 1 μl Form I supercoiled DNA (TopoGEN, Inc., Columbus, OH). Relaxation was carried out by incubating a 1 μl sample for 30 min at 37°C. Reactions were terminated by adding 5 μl of 5% sarkosyl, 0.125% bromophenol blue, and 25% glycerol. Samples were incubated with 50 μg/ml proteinase K for 60 min at 37°C. Samples were separated by electrophoresis on a 1% agarose gel in 89 m*M* Tris-borate and 2 m*M* 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 H_2O. 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_{50} values of each cell line for anticancer agents are shown in Table I. The IC_{50}s ranged from 1.3 to 18.5 μM for CDDP, from 0.045 to 6.9 μM for PTX, from 0.74 to 10.5 μM for MMC, 4.4 to 93.8 μM for VP-16, and 0.016 to 1.9 μM 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).

Table I. IC_{50} Values to Anticancer Agents

Cell lines	IC_{50} (μM)				
	CDDP	PTX	MMC	VP-16	SN-38
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

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 μg/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 μg/mg for CDDP and 26.6±15.7 μg/mg for MMC vs. 14.3±5.7 μg/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,

Table II. Calculated Assay AUC at IC_{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[a)]	499.7	18.5	548.4	0.4[a)]
RMG-I	250.2	3.3[a)]	260.6	214.7	0.9[a)]
RMG-II	275.8	5.7[a)]	51.7	654.7	0.8[a)]
KK	87.4	11.1[a)]	21.5	401.5	6.5
KOC-7c	458.9	478.8	61.9	4577.1	46.9
CA-AUC	85.8	19.3	0.5	55.6	1.5

a) Sensitive.

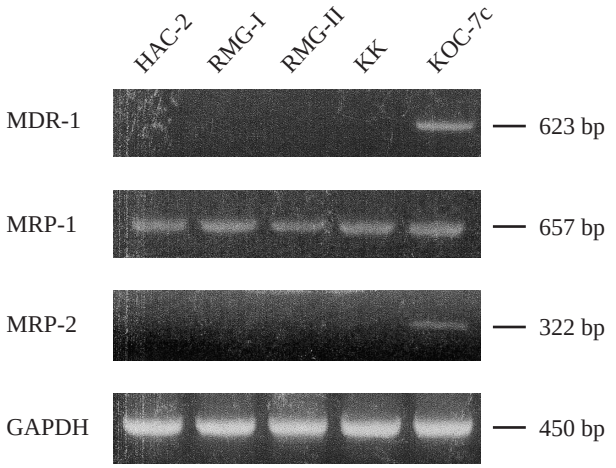

Fig. 1. The gene expression determined by RT-PCR. *MRP-1* gene was detected in all cell lines. Only KOC-7c cells showed both *MRP-2* and *MDR-1* genes.

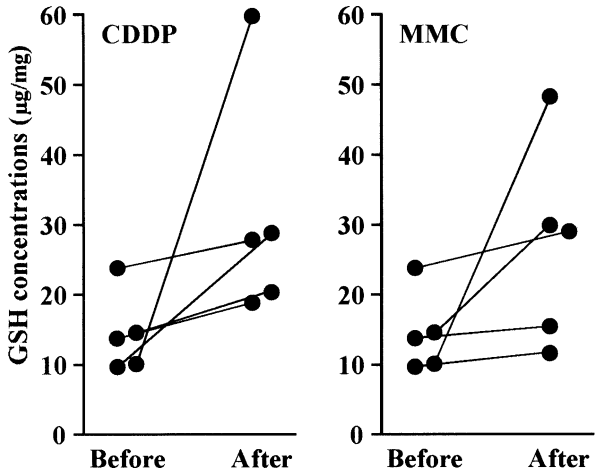

Fig. 2. Distribution of GSH concentrations is shown for each cell line. GSH concentrations of all cell lines increased after exposure to CDDP or MMC. The concentration of GSH after exposure to CDDP or MMC was significantly higher than that before treatment (Wilcoxon test $P<0.05$).

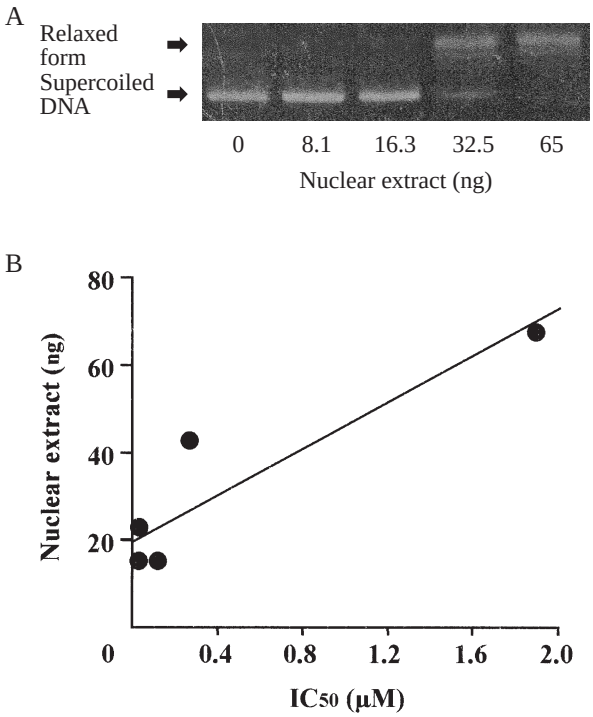

Fig. 3. (A) Topo I catalytic activity in KOC-7c cells. Sixty-five nanograms of extracted nuclear protein showing complete DNA relaxation. (B) Correlation between IC_{50} to SN-38 and topo I activity. IC_{50} to SN-38 showed a significant correlation to topo I activity. $r=0.900$, $P<0.05$.

and RMG-II) (15.6–23.4 vs. 46.9–62.5 ng). Additionally, IC_{50} 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 IC_{50} 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 IC_{50} 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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REFERENCES

1) Sugiyama, T., Kamura, T., Kigawa, J., Terakawa, N., Kikuchi, Y., Kita, T., Suzuki, M., Sato, I. and Taguchi, K. Clinical characteristics of clear cell carcinoma of the ovary: a distinct histologic type with poor prognosis and resistance to platinum-based chemotherapy. *Cancer*, **88**, 2584–2589 (2000).
2) Goff, B. A., Sainz de la Cuesta, R., Muntz, H. G., Fleischhacker, D., Ek, M., Rice, L. W., Nikrui, N., Tamimi, H. K., Cain, J. M., Greer, B. E. and Fuller, A. F., Jr. Clear cell carcinoma of the ovary: a distinct histologic type with poor prognosis and resistance to platinum-based chemotherapy in stage III disease. *Gynecol. Oncol.*, **60**, 412–417 (1996).
3) Tammela, J., Geisler, J. P., Eskew, P. N., Jr. and Geisler, H. E. Clear cell carcinoma of the ovary: poor prognosis compared to serous carcinoma. *Eur. J. Gynaecol. Oncol.*, **19**, 438–440 (1998).
4) Ueki, M. Report of gynecologic cancer in Japan. *Acta Obstet. Gynecol. Jpn.*, **53**, 1038–1048 (2001).
5) Horwitz, S. B. Mechanism of action of taxol. *Trends Pharmacol. Sci.*, **13**, 134–136 (1992).
6) Rose, P. G., Blessing, J. A., Mayer, A. R. and Homesley, H. D. Prolonged oral etoposide as second-line therapy for platinum-resistant and platinum-sensitive ovarian carcinoma: a Gynecologic Oncology Group study. *J. Clin. Oncol.*, **16**, 405–410 (1998).
7) Tsuruo, T., Matsuzaki, T., Matsushita, M., Saito, H. and Yokokura, T. Antitumor effect of CPT-11, a new derivative of camptothecin, against pleiotropic drug-resistant tumors *in vitro* and *in vivo*. *Cancer Chemother. Pharmacol.*, **21**, 71–74 (1988).
8) Minagawa, Y., Kigawa, J., Irie, T., Kanamori, Y., Itamochi, H., Cheng, X. and Terakawa, N. Enhanced topoisomerase I activity and increased topoisomerase IIα content in cisplatin-resistant cancer cell lines. *Jpn. J. Cancer Res.*, **88**, 1218–1223 (1997).
9) Minagawa, Y., Kigawa, J., Ishihara, H., Itamochi, H. and Terakawa, N. Synergistic enhancement of cisplatin cytotoxicity by SN-38, an active metabolite of CPT-11, for cisplatin-resistant HeLa cells. *Jpn. J. Cancer Res.*, **85**, 966–971 (1994).
10) Shimizu, Y., Umezawa, S. and Hasumi, K. A phase II study of combined CPT-11 and mitomycin-C in platinum refractory clear cell and mucinous ovarian carcinoma. *Ann. Acad. Med. Singapore*, **27**, 650–656 (1998).
11) Andrews, P. A., Velury, S., Mann, S. C. and Howell, S. B. *cis*-Diamminedichloroplatinum (II) accumulation in sensitive and resistant human ovarian carcinoma cells. *Cancer Res.*, **48**, 68–73 (1988).
12) Andrews, P. A. and Howell, S. B. Cellular pharmacology of cisplatin: perspectives on mechanisms of acquired resistance. *Cancer Cells*, **2**, 35–43 (1990).
13) Godwin, A. K., Meister, A., O'Dwyer, P. J., Huang, C. S., Hamilton, T. C. and Anderson, M. E. High resistance to cisplatin in human ovarian cancer cell lines is associated with marked increase of glutathione synthesis. *Proc. Natl. Acad. Sci. USA*, **89**, 3070–3074 (1992).
14) Fujiwara, Y., Sugimoto, Y., Kasahara, K., Bungo, M., Yamakido, M., Tew, K. D. and Saijo, N. Determinants of drug response in a cisplatin-resistant human lung cancer cell line. *Jpn. J. Cancer Res.*, **81**, 527–535 (1990).
15) Kasahara, K., Fujiwara, Y., Nishio, K., Ohmori, T., Sugimoto, Y., Komiya, K., Matsuda, T. and Saijo, N. Metallothionein content correlates with the sensitivity of human small cell lung cancer cell lines to cisplatin. *Cancer Res.*, **51**, 3237–3242 (1991).
16) Kuwano, M., Toh, S., Uchiumi, T., Takano, H., Kohno, K. and Wada, M. Multidrug resistance-associated protein subfamily transporters and drug resistance. *Anticancer Drug Des.*, **14**, 123–131 (1999).
17) Sharma, M., He, Q. Y. and Tomasz, M. Effects of glutathione on alkylation and cross-linking of DNA by mitomycin C. Isolation of a ternary glutathione-mitomycin-DNA adduct. *Chem. Res. Toxicol.*, **7**, 401–407 (1994).
18) Horwitz, S. B., Cohen, D., Rao, S., Ringel, I., Shen, H. J. and Yang, C. P. Taxol: mechanisms of action and resistance. *J. Natl. Cancer Inst. Monogr.*, **15**, 55–61 (1993).
19) Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods*, **65**, 55–63 (1983).
20) Park, J. G., Kramer, B. S., Steinberg, S. M., Carmichael, J., Collins, J. M., Minna, J. D. and Gazdar, A. F. Chemosensitivity testing of human colorectal carcinoma cell lines using a tetrazolium-based colorimetric assay. *Cancer Res.*, **47**, 5875–5879 (1987).
21) Gorai, I., Nakazawa, T., Miyagi, E., Hirahara, F., Nagashima, Y. and Minaguchi, H. Establishment and characterization of two human ovarian clear cell adenocarcinoma lines from metastatic lesions with different properties. *Gynecol. Oncol.*, **57**, 33–46 (1995).

22) Ohta, I., Gorai, I., Miyamoto, Y., Yang, J., Zheng, J. H., Kawata, N., Hirahara, F. and Shirotake, S. Cyclophosphamide and 5-fluorouracil act synergistically in ovarian clear cell adenocarcinoma cells. *Cancer Lett.*, **162**, 39–48 (2001).
23) Young, L. C., Campling, B. G., Voskoglou-Nomikos, T., Cole, S. P., Deeley, R. G. and Gerlach, J. H. Expression of multidrug resistance protein-related genes in lung cancer: correlation with drug response. *Clin. Cancer Res.*, **5**, 673–680 (1999).
24) Owens, C. W. I. and Belcher, R. V. A colorimetric micro-method for the determination of glutathione. *Biochem. J.*, **94**, 705–711 (1965).
25) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–254 (1976).
26) Liu, L. F. and Miller, K. G. Eukaryotic DNA topoisomerases: two forms of type I DNA topoisomerases from HeLa cell nuclei. *Proc. Natl. Acad. Sci. USA*, **78**, 3487–3491 (1981).
27) Ozawa, S., Sugiyama, Y., Mitsuhashi, Y., Kobayashi, T. and Inaba, M. Cell killing action of cell cycle phase-non-specific antitumor agents is dependent on concentration—time product. *Cancer Chemother. Pharmacol.*, **21**, 185–190 (1988).
28) Mitsuhashi, Y., Inaba, M., Sugiyama, Y. and Kobayashi, T. *In vitro* measurement of chemosensitivity of human small cell lung and gastric cancer cell lines toward cell cycle phase-nonspecific agents under the clinically equivalent area under the curve. *Cancer*, **70**, 2540–2546 (1992).
29) Kamazawa, S., Kigawa, J., Minagawa, Y., Itamochi, H., Shimada, M., Takahashi, M., Sato, S., Akeshima, R. and Terakawa, N. Cellular efflux pump and interaction between cisplatin and paclitaxel in ovarian cancer cells. *Oncology*, **59**, 329–335 (2000).
30) Cheng, X., Kigawa, J., Minagawa, Y., Kanamori, Y., Itamochi, H., Okada, M. and Terakawa, N. Glutathione S-transferase-π expression and glutathione concentration in ovarian carcinoma before and after chemotherapy. *Cancer*, **79**, 521–527 (1997).
31) Kigawa, J., Minagawa, Y., Cheng, X. and Terakawa, N. γ-Glutamylcysteine synthetase up-regulates glutathione and multidrug resistance-associated protein in patients with chemoresistant epithelial ovarian cancer. *Clin. Cancer Res.*, **4**, 1737–1741 (1998).
32) Bjornsti, M. A., Benedetti, P., Viglianti, G. A. and Wang, J. C. Expression of human DNA topoisomerase I in yeast cells lacking yeast DNA topoisomerase I: restoration of sensitivity of the cells to the antitumor drug camptothecin. *Cancer Res.*, **49**, 6318–6323 (1989).