Anticancer drugs induce *mdr1* gene expression in recurrent ovarian cancer

Stephanie Hille[a], Daniel T. Rein[a], Marion Riffelmann[b], Rainer Neumann[c], Judith Sartorius[d], Andreas Pfützner[e], Christian M. Kurbacher[f], Thomas Schöndorf[e,*] and Martina Breidenbach[g,*]

Ovarian cancer is currently the most lethal gynecologic malignancy in Europe and the US. Platin analogues and paclitaxel demonstrate high remission rates, but unfortunately the efficacy of cytostatic agents is limited by the development of multidrug resistance (*mdr*). Clinical paclitaxel resistance is often associated with *mdr1* overexpression. In a recent study, we introduced a highly specific quantitative real-time reverse transcriptase polymerase chain reaction for the quantification of *mdr1* transcripts. In the present study, we demonstrate that primary tumor cells from patients with recurrent ovarian cancer overexpress *mdr1*. To evaluate *mdr1* expression, we collected tumor cells from 77 ovarian cancer patients (13 chemotherapy-naive ovarian cancer, 64 recurrent ovarian cancer). Cancer cells were aspirated from 49 solid specimens (63%) and 28 ascitic fluids (37%). Subsequently, cancer cells were exposed in 221 short-term cultures either to blank medium (control) or to a single anticancer drug, cisplatin, doxorubicin or paclitaxel. The drug concentrations applied referred to clinical relevant doses. *mdr1* mRNA expression was significantly higher in specimens from recurrent ovarian cancer incubated in paclitaxel than in specimens from chemotherapy-naive ovarian cancer. No significant differences were detectable between the mean value of *mdr1* mRNA expression in tumor specimens from recurrent ovarian cancer incubated in cisplatin or doxorubicin. Differences within the untreated patients group were also not statistically significant. The result of this study confirms clinical observations, as well as in-vitro studies based on tumor cell lines, that paclitaxel resistance is correlated with *mdr1* overexpression. *Anti-Cancer Drugs* 17:1041–1044 © 2006 Lippincott Williams & Wilkins.

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[a]Department of Gynecology and Obstetrics, University of Düsseldorf, Düsseldorf, [b]Klinikum Krefeld, Krefeld, [c]Bayer Vital, Leverkusen, [d]Department of Gynecology and Obstetrics, University of Cologne, Cologne, [e]Institut of Clinical Research and Development (IKFE), Mainz, [f]Oncology Center, Bonn and [g]Department of Gynecology and Obstetrics, University of Aachen Medical Center, Aachen, Germany.

Correspondence to S. Hille, Department of Gynecology and Obstetrics, University of Düsseldorf, Moorenstrasse 5–8, 0225 Düsseldorf, Germany. Tel: +49 211 817501; fax: +49 211 81 8483; e-mail: stephanie_hille@hotmail.com

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Introduction

Ovarian cancer is the most fatal gynecologic malignancy in the US and the incidence is reported to be increasing [1]. The majority of patients present with advanced stage 3 or stage 4 disease, which is associated with a poor prognosis [2,3]. Response rates of chemotherapy-naive ovarian cancer (CNOC) to platinum analogues and paclitaxel (PTX) exceed 75%. The majority of patients with advanced ovarian cancer, however, will have a relapse and finally die from their disease. In this regard, resistance to chemotherapeutic agents is a major obstacle for successful chemotherapy of recurrent ovarian cancer (ROC) and mechanisms to overcome resistance to chemotherapy are highly warranted [2,4,5]. Clinical trials are underway to modulate expression of the multidrug resistance 1 (*mdr1*) gene, one of the contributors to the *mdr* phenotype [6,7]. Chemoresistance, whether intrinsic or acquired, is attributable to the genetic process taking place in neoplastic cells [8]. On the molecular level, overexpression of the ATP-binding cassette transporters such as ABCB1 (*mdr1*) has been implicated in resistance to various chemotherapeutic agents *in vitro*. The *mdr1* gene encodes a membrane-bound P-glycoprotein that has been shown to function as a transmembrane drug-efflux pump [9]. The results from in-vitro studies indicate that *mdr1* overexpression may be the predominant factor in limiting the efficacy of anticancer drugs [10]. In ovarian cancer, as well as in other tumor entities, *mdr1* overexpression has been shown to be correlated with a worse prognosis [5,11].

In a previous study, we were able to introduce a quantitative real-time polymerase chain reaction (PCR) applicable to determine the *mdr1*-inducing potency of commonly used anticancer drugs [12]. The results of this study, which was performed with established ovarian

*Authors contributed equally.

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cancer cell lines, indicate that *mdr1* induction by antineoplastic agents might be responsible for treatment failure in patients suffering from ovarian cancer. The value of experiments based on established tumor cell lines, however, is limited as they reflect the biological situation only to a certain extent. Although the relationship between *mdr1* overexpression and chemoresistance has been well studied in cell lines *in vitro*, little is known regarding the regulation of the *mdr1* gene in chemotherapy-naive or pretreated primary tumor cells from patients.

Evidence shows that antineoplastic agents may induce *mdr1* gene expression, thereby triggering the development of secondary chemoresistance by accelerated extrusion. The aim of the present study was to determine differences between *mdr1*-mRNA expression in primary tumor cells from chemotherapy-naive and pretreated patients following incubation in *cis*-diaminodichloroplatinum(II) [cisplatin (CDDP)], doxorubicin (DOX) or PTX.

Materials and methods

Patients

Ovarian carcinoma specimens were collected from 77 patients who underwent treatment in our institution during the years 1996–2001 after informed consent and approval of the study by the Ethical Board of the University of Cologne Medical Center. Thirteen patients had advanced CNOC (FIGO III or IV). Histological subtyping revealed 10 serous papillary carcinomas, one clear cell adenocarcinoma and two endometrial adenocarcinomas. Patient characteristics are shown in Table 1. All of the 64 patients with ROC had received one previous adjuvant chemotherapy comprising either PTX/carboplatin (24 patients) or cyclophosphamide/carboplatin (40 patients).

Tumor cell culture

Primary tumor specimens were obtained surgically from the ovary at the time of initial diagnosis. Recurrent specimens were collected by a secondary surgery (42 patients, 64%) or aspiration of ascitic effusions (22 patients, 36%). All specimens were snap frozen in liquid nitrogen immediately after acquisition and stored in liquid nitrogen until further investigation. Tumor cells were prepared as described previously [13]. The cells were grown in CAM medium, supplemented with 10% fetal calf serum, 2 mmol/l L-glutamine, 100 IE/ml penicillin and 100 µg/ml streptomycin. Cell cultures were maintained at 10^5 cells/ml and 37°C in a humidified 95% air/5% CO_2 atmosphere. Cells were exposed for 72 h to either blank medium (control) or the different antineoplastic agents (DOX, CDDP and PTX). In order to study the cells after exposure to therapeutically relevant drug dosages, the final concentrations of the cytostatics assayed were adjusted to either the clinical peak plasma concentration after administration of an intravenous standard dose (DOX, CDDP) or the equivalent of the area under the plasma elimination curve [14,15]: DOX, 0.5 µg/ml; PTX, 13.6 µg/ml; CDDP, 3.8 µg/ml. These concentrations have already been shown to produce preclinical activity comparable to the reported clinical response rates when testing naive ovarian cancers *in vitro* [14,15]. Each assay was performed in quadruplicate.

Table 1 Patient characteristics

	Chemotherapy-naive ovarian cancer ($n=13$)	Recurrent ovarian cancer ($n=64$)
Age [years (mean ± SD)]	63.25 ± 10.2	61 ± 12.9
Histology		
serous adenocarcinoma	10 (77%)	58 (90.6%)
clear cell adenocarcinoma	1 (7.7%)	4 (6.3%)
endometrial adenocarcinoma	2 (15.3%)	2 (3.1%)
FIGO		
I	2 (15.4%)	2 (3.1%)
II	3 (23.1%)	19 (29.7%)
III	7 (53.8%)	40 (62.5%)
IV	1 (7.7%)	3 (4.7%)

RNA extraction

Cells were harvested by centrifugation (5 min, 8000 *g*), washed in phosphate-buffered saline and resuspended in lysis buffer. Cells were frozen in liquid nitrogen and stored at −80°C until RNA extraction. Total RNA was extracted using the RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was quantified using the RiboGreen RNA quantification kit (MoBiTec, Göttingen, Germany). Five nanograms of total RNA was applied to each quantitative reverse transcriptase (RT)-PCR assay (quRT-PCR).

Quantitative reverse transcriptase polymerase chain reaction

The quantitative real-time PCR was performed as described earlier [12]. In brief, both the target *mdr1*-mRNA and the internal GAPDH mRNA control were amplified in a 50 µl biplex-quRT-PCR. Each assay was performed in triplicate. For template quantification, resulting fluorescence was detected at each PCR cycle by the ABI 7700 Sequence Detection System (Applied Biosystems, Darmstadt, Germany). For each microplate, threshold was automatically calculated in terms of the reporter dye. Each *mdr1* or GAPDH signal was quantified by the threshold cycle number (C_t).

Statistics

For statistical analysis, we used SPSS 9.0 for Windows (Statistical Package for the Social Sciences, Munich, Germany). Statistical differences among groups were assessed with a two-tailed Student's *t*-test. $^*P < 0.05$ was considered significant.

Results

Of the tumor specimens from 77 patients included in this study, 221 measurements were evaluable. Each

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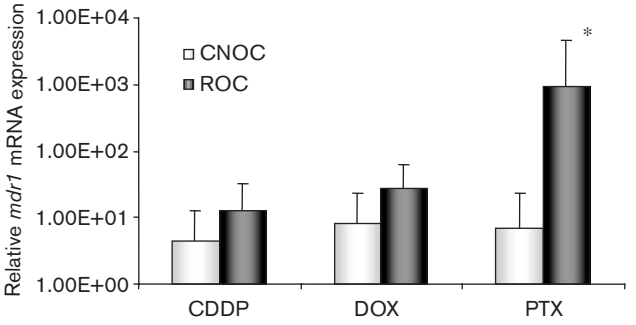
Fig. 1



Relative *mdr1* mRNA expression levels in chemotherapy-naive (CNOC) and pretreated recurrent ovarian cancer (ROC) cells from patients. Quantitative real-time polymerase chain reaction was performed following 72 h of incubation in cisplatin (CDDP), doxorubicin (DOX) or paclitaxel (PTX). Error bars indicate standard variation. $*P<0.05$.

quRT-PCR was performed in triplicate and produced comparable results with acceptable variation (SD). Expression of the *mdr1* gene was indicated as ΔC_t values ($\Delta C_t = C_{t,\ mdr1\ gene} - C_{t,\ GAPDH}$). Specifically, the quantity of *mdr1* mRNA in chemotherapy-naive cells relative to *mdr1* mRNA in pretreated patients was expressed as $2^{\Delta C_t}$.The mean value of the expression of *mdr1* mRNA in tumor specimens from ROC incubated in PTX was significantly ($P < 0.032$) 1.3 log higher than in specimens from chemotherapy-naive tumor specimens (Fig. 1). Differences between the mean value of the *mdr1* mRNA expression in tumor specimens from ROC incubated in CDDP or DOX were not statistically significant ($P = 0.12$ and 0.056, respectively). Differences within the untreated patients group were also not statistically significant (CDDP versus PTX: $P = 0.27$; CDDP versus DOX: $P = 0.117$; DOX versus PTX: $P = 0.28$). Within the ROC patients group, only differences between CDDP and PTX were statistically significant (CDDP versus PTX: $P = 0.002$; CDDP versus DOX: $P = 0.44$; DOX versus PTX: $P = 0.49$). Within the PTX/carboplatin (24 patients) or cyclophosphamid/carboplatin (40 patients) group, no statistically significant differences were detectable.

Discussion

The response to anticancer drugs in patients with ROC remains disappointing. Several studies have demonstrated that various anticancer drugs induce chemoresistance [16,17] and *mdr1*, which encodes P-glycoprotein, has been associated with multidrug resistance *in vitro* [12]. Other studies have demonstrated that *mdr1* expression might be of value in the prediction of therapy response in patients suffering from ovarian cancer [10,18–21]. In particular, PTX resistance has been associated with P-glycoprotein encoded by the *mdr1* gene and with alterations involving tubulin [22,23]. A retrospective survival analysis of ovarian cancer patients based on paired CNOC and ROC samples has demonstrated an inverse correlation between *mdr1*-encoded P-glycoprotein and treatment response to PTX [24]. In a multivariate analysis, the outcome following secondary surgery, P-glycoprotein expression and response to PTX were significant predictors of survival. The prognostic significance of P-glycoprotein expression in advanced ovarian carcinoma and a correlation to tumor chemosensitivity and overall survival has been confirmed by others [25]. It has been speculated that changes in gene expression patterns in tumor cells are specifically influenced by treatment with anticancer drugs [26]. Different studies have been performed to determine the relationship between multidrug resistance and sensitivity to antineoplastic agents *in vitro*. In a recently published study [27], expression of 557 human cancer-related cDNA transcripts was compared in a number of PTX-resistant and PTX-sensitive cell lines. In this study, *mdr1* was one out of eight overexpressed genes within the panel examined. Another study [21] demonstrated that the expression of the *mdr1* gene was overexpressed in a panel of PTX-resistant ovarian cancer cell lines. Other studies have shown that suppression of the *mdr1* gene enhances the anticancer activity of DOX [28] and reverses multidrug resistance [29]. To model more closely the clinical situation with the most stringent substrate, we compared *mdr1* expression in CNOC patient samples and tissue samples from patients with ROC. Quantitative real-time PCR was performed 72 h after incubation of tumor cells in CDDP, DOX or PTX. Our study clearly demonstrated *mdr1* mRNA overexpression in ROC samples. These results are in line with the observation that *mdr1* is overexpressed in PTX-resistant cancer cell lines. In a recent study, the sensitivity of tumor cells to CDDP was restored after a drug-free interval [30]. It has been speculated that this effect is, at least in part, based on the suppression of the *mdr1* gene. In our study, however, no significant differences were detected between chemotherapy naive and pretreated ovarian cancer cells after incubation in CDDP with regard to *mdr1* mRNA. Strategies to prevent or reverse multidrug resistance have focused on agents that are competitive inhibitors of P-glycoprotein. Unfortunately, the use of these agents for clinical purpose is limited by toxicity and limited efficacy [7]. A novel approach is the use of small interfering RNAs that can be used to target *mdr1* in multidrug-resistant tumor cells. A recent study [31] demonstrated the effectiveness of this approach in inhibiting ABCB1 (*mdr1*) expression and the subsequent reversal of resistance to PTX.

In conclusion, our data demonstrate that PTX induces *mdr1* overexpression in pretreated ovarian cancer cells from patients. The results are in line with in-vitro studies based on ovarian cancer cell lines and clinical observations indicating that *mdr1* overexpression may be the predominant factor in limiting the efficacy of chemother-

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apeutic agents. Novel treatment approaches that include the suppression of *mdr1* might be of use in the treatment of ROC.

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