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cDNA Microarray Analysis of Gene Expression in Ovarian Cancer Cells After Treatment with Carboplatin and Paclitaxel

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Abstract. *Background: Tumor resistance to chemotherapy results in ovarian cancer treatment failures. To understand the role of DNA damage, expression profiling was performed in ovarian cancer cells following suboptimal carboplatin (Carbo) and paclitaxel (Tax) treatments. Materials and Methods: Cell lines isolated from one patient were used. UL-3A was isolated at initial diagnosis, UL-3B after failure to cisplatin and Tax, and UL-3C at the final stages of the disease. Five clones isolated from UL-3A were also included. Sulforhodamine B assay was used to determine drug sensitivities. Gene expression was studied by DNA repair pathway specific microarray. Results: The cytotoxic phase was induced in all cells with Carbo and/or Tax treatments. Some cells recovered after individual drug treatments with the UL-3B cells surviving all modalities. Significant changes in the sensitivity of some surviving cells to chemotherapeutic agents were demonstrated. Carbo or Tax treatments resulted in significantly increased MDM2 and MSH6 and decreased 53BP1, EXT1, H2AFL and UNG expression. Combined Carbo and Tax treatment resulted in decreased ATR, CHEK1, PPM1D and PRKDC and increased RAD1, CDS1 and ATRX expression. Conclusion: Differential gene expression patterns were identified in cells that survived Carbo and Tax treatment that may be involved in ovarian cancer drug resistance.*

Ovarian cancer remains the most lethal gynecologic cancer in industrialized countries (1). Currently, standard treatment for Stage IIIC or greater disease includes initial surgical staging and optimal debulking followed by adjuvant chemotherapy with a platinum compound and a taxane. Despite an 80% complete response rate with this treatment approach (2) most patients will experience disease recurrence within 5 years and succumb to their disease (1). The disease-free interval (DFI), has been shown to be a prognostic indicator of response rate with repeated treatments. A DFI between 6 and 24 months is associated with a 30% overall response rate to repeated platinum treatment and DFI >24 months shows a 60% response (3). Because this phenomenon of initial sensitivity to platinum therapy is most often followed by later resistance, therapy itself appears to either allow expression of suppressed inherent proliferative mechanisms or induce resistance mechanisms in tumor cells.

Drug sensitivity can be categorized as physiological or pathological (4). Physiological resistance is predominant in early neoplastic transformation and precedes the acquisition of drug sensitivity. Pathological resistance appears either later in malignant transformation or during chemotherapy as a result of the classic mechanisms of increased detoxification, upregulation of cellular and nucleotide repair pathways, or defective apoptosis. Ovarian cancer is typically diagnosed and treated in the "pathological" drug resistance phase.

Platinum-containing compounds, cisplatin and carboplatin (carbo), induce cytotoxicity by binding to DNA and nuclear proteins, leading to the formation of both inter-strand and intra-strand DNA cross-links that cannot undergo replication or transcription (5). Mechanisms elucidated *in vitro* to explain the treated cells' ability to overcome this DNA damage have included decreased net drug uptake by the cell *via* the multidrug resistance (MDR) pathway, increased DNA repair *via* both nucleotide excision repair (NER) and mismatch repair (MMR) pathways (6) and decreased ability to undergo apoptosis (7). Paclitaxel (Tax) induces cytotoxicity by binding to polymerized tubulin, promoting constitutive microtubule assembly. This action disables the mitotic spindle preventing further cell replication and interferes with cell shape and membrane function. Proposed mechanisms of resistance to Tax have included decreased total intracellular tubulin and variations in the isotypes of tubulin subtypes (8). The significance of these mechanisms *in vivo* is largely unknown.

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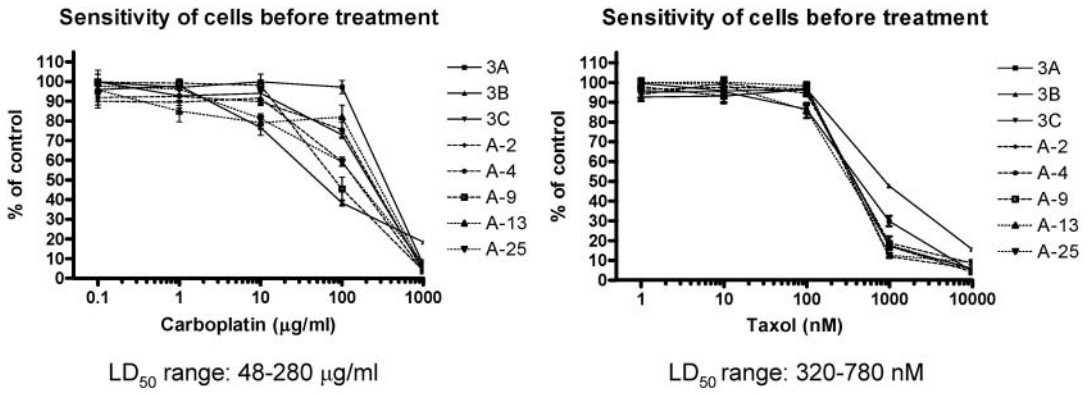


Figure 1. *Sensitivity of ovarian cancer cell lines before in vitro treatment with carboplatin or paclitaxel. Sulforhodamine B assay was used to determine LD_{50} values.*

The effect of each pathway on the others is also unclear, and more functional studies are needed to define their roles in multi-factorial chemoresistant ovarian cancer.

Microarray gene expression profiling has been used in the investigation of ovarian cancer to compare the gene expression profile of ovarian cancer with that of the normal ovary (9, 10). This has subsequently been used to identify biomarkers that have significantly higher expression in benign *versus* malignant disease, and to elucidate the differences between early- and late-stage ovarian disease (11-14). Subsequent studies have described differential expression profiles of cells with evolving Tax resistance and cells with increasing levels of cisplatin resistance (15).

A variety of factors such as accessibility of tumor tissue, maximum achievable plasma concentration of chemotherapeutic agents and inherent drug resistance could result in suboptimal chemotherapy in ovarian cancer. Recurrent disease can be a result of cells that survive the chemotherapy attempts. Our purpose was to study ovarian cancer cells that survive chemotherapy *in vitro* with respect to genetic changes and drug sensitivity to Carboplatin and Paclitaxel.

Materials and Methods

Cell lines and treatment with chemotherapeutic agents. The cell lines were obtained from the ascitic fluid of a patient with Stage IIIC Grade 1 papillary serous adenocarcinoma of the ovary under a University Human Studies Committee approved protocol, UL-3A, at presentation, UL-3B at recurrence six months post-treatment, and UL-3C proximate to the time of death. The cells were cultured in RPMI media supplemented with 10% fetal calf serum. Five different single cell clones previously derived from the heterogenous UL-3A cell line were also included. To study cell recovery, all cell lines were treated with the respective LD-30 and -70 of Carbo, Tax, and a combination of both for two hours. Recovery was defined as formation of a monolayer during which cell proliferation was observed (maximum six weeks). The recovered cells were cultured for an additional 3-4 weeks without the presence of any drugs and drug sensitivity assays were performed.

Cytotoxicity assays. Cytotoxicity assays were performed in triplicate. Carboplatin was made as a stock solution (12.5 mg/mL) in water, while Tax was made 10 mM in DMSO. The cells were added to 96-well plates at $5x10^3$/well input density. After overnight incubation, Carbo and Tax were added for two hours and cultures were incubated for 96 hours. Sulforhodamine B was used for color reaction, and absorbance was detected at 540 nM. A prism software program was used to generate the dose-response curves. The mean EC_{30}, EC_{50} and EC_{70} values were compared with ANOVA to determine significance.

DNA microarray. RNA isolation was performed with Trizol reagent. cDNA was synthesized following the protocol recommended by GEArray Series kit (SuperArray Bioscience Corp., Frederick, MD, USA). The biotin labeled cDNA probe was used for hybridization. cDNAs from UL-3B (untreated), UL-$3B_{C30}$, UL-$3B_{T30}$, and UL-$3B_{C/T30/30}$ were hybridized to respective human DNA damage signaling pathway gene array membranes. Each array included 96 key genes involved in either the ATR/ATM signaling network, the cell cycle arrest pathway, the apoptosis pathway, or the genome stability/repair pathway. CDP-Star chemiluminescent substrate was added to the hybridization tubes. The image was acquired on X-ray film. ScanAlyze software was utilized to convert the image into numerical data. The relative transcript abundances were estimated by examining corrected, normalized signals. All raw signal intensities were corrected for background by subtracting the signal intensity of pUC18 DNA, β-actin, GAPDH and blank spaces.

Results

Sensitivity of cell lines to carboplatin and paclitaxel. The sensitivity of UL-3A, UL-3B and UL-3C cells, and the five UL-3A clones was determined to Carbo and Tax (Figure 1). LD_{50} values for Carbo ranged from 48-280 μg/ml, and for Tax the range was from 320-780 nm. The most sensitive cell to carboplatin was UL-3B, and the most resistant was the

Table I. *Change in sensitivity of ovarian cancer cells to Carboplatin after sublethal treatment. Results are expressed as fold change compared to values obtained before treatment of cells with carboplatin or paclitaxel.*

Cells	LD_{30}	LD_{50}	LD_{70}
UL3-A-C30	1.00	1.32	0.91
UL3-A13-C30	0.74	0.72	0.90
UL3-A2-T30	2.41	1.46	1.28
UL3-A25-T30	0.15	0.44	0.75
UL3-B-C30	2.00	1.30	1.31
UL3-B-T30	3.03	2.56	1.49
UL3-B-C/T30/30	6.04	8.32	3.61
UL3-C-C30	0.20	0.21	0.20

Table II. *Change in sensitivity of ovarian cancer cells to Paclitaxel after sublethal treatment. Results are expressed as fold change compared to values obtained before treatment of cells with carboplatin or paclitaxel.*

Cells	LD_{30}	LD_{50}	LD_{70}
UL3-A-C30	0.01	0.03	0.06
UL3-A13-C30	0.01	0.03	0.08
UL3-A2-T30	0.16	0.59	1.86
UL3-A25-T30	0.19	0.25	0.58
UL3-B-C30	1.50	1.22	1.50
UL3-B-T30	0.25	0.53	3.00
UL3-B-C/T30/30	1.00	2.56	4.00
UL3-C-C30	0.06	0.13	0.27

UL-A13 clone. With respect to Tax, the most sensitive was UL-A25, and the most resistant was UL-3B.

Sensitivity of cell lines to carboplatin and paclitaxel after treatment. All cells were treated with LD_{30} Carbo, LD_{30} Tax, $LD_{30/30}$ Carbo/Tax, $LD_{30/70}$ Carbo/Tax, or $LD_{70/30}$ Carbo/Tax. The cells that recovered from the various treatments were tested for their chemosensitivity to Carbo (Table I). All parent cell lines UL-3A, 3B, 3C and the clone UL-3A-13 recovered from LD_{30} treatments of Carbo. UL-3A-2, UL-3B and UL-3A- 25 were able to survive LD_{30} treatment of Tax. Only the UL-3B cells recovered from both Carbo and Tax treatments at the LD_{30} level. There were no survivors from LD_{70} treatments.

The sensitivities of UL-3A-C30 and UL-3A-13-C30 to Carbo were not significantly different than the untreated cells. UL-3A-A2-T30 and UL-3B-T30 were more resistant to Carbo than the untreated counterparts. UL-3A-A25-T30 and UL-3C-C30 were more sensitive to Carbo after recovery from Tax or Carbo. UL-3B-C/T30/30 cells were more resistant to Carbo after treatment with both agents.

Changes in sensitivity to Tax after recovery from cytotoxic treatment were observed (Table II). UL-3A-C30, UL-3A13-C30, UL-3A25-T30, UL-3C-C30 were all significantly more sensitive to Tax. UL-3A2-T30 and UL3B-T30 were more sensitive at the LD_{30} and LD_{50} levels, but more resistant at the LD_{70} level. UL-3B-C/T30/30 and UL-3B-C30 cells were more resistant to Tax.

DNA microarray analysis. UL-3B cells that recovered from Carbo, Tax and combined treatments were further studied by DNA microarray to determine if heritable changes in DNA damage genes had occurred in comparison with untreated UL-3B cells. The gene expression patterns obtained from the microarrays were analyzed such that a change from the normal two-fold was considered significant (Figure 2). The numbers of genes showing differential expression in each cell group are listed in Table III. MDM2 and MSH6 expression was increased with Carbo or Tax treatments while 53BP1, EXT1, H2AFL and UNG expression was decreased. The combined Carbo and Tax treatment resulted in decreased expression of ATR, CHEK1, PPM1D, PRKDC and an increase in RAD1, CDS1 and ATRX among others. Several of the differentially expressed genes were associated with more than one treatment group, and the direction of change from control was always conserved. RT-PCR was performed to confirm the data obtained with the DNA microarrays (Figure 3). There was general agreement obtained with microarray results and RT-PCR.

Table III. *Summary of gene expression data following suboptimal treatment with Carboplatin, Paclitaxel and a combination of the two agents.*

	Carboplatin	Paclitaxel	Carboplatin and Paclitaxel
Increased expression	ATM, DDB2, MDM2, MSH6, RAD1, RRM2B, XRCC5	ATRX, CDS1, MDM2, MSH2, MSH6, NBS1	ATRX, BAK1, BAX, BCL6, BRCA2, BTG2, CDC25A, CDC25B, CDS1, CENPE, DDIT3, E2-EPF, ERCC6, LRDD, MPG, PA26, PCNA, IN1, PMS1, PMS2, PURA, RAD1, RAD23A, RAD9
Decreased expression	53BP1, CDC25A, CDC25B, CENPE, CHEK1, ERCC2, EXT1, H2AFL, p53AIP1, PPMID, RAD50, UNG	53BP1, APEX, ATR, DDB2, DDIT3, EXT1, H2AFL, PRKDC, UNG	ATR, CHEK1, PPM1D, PRKDC

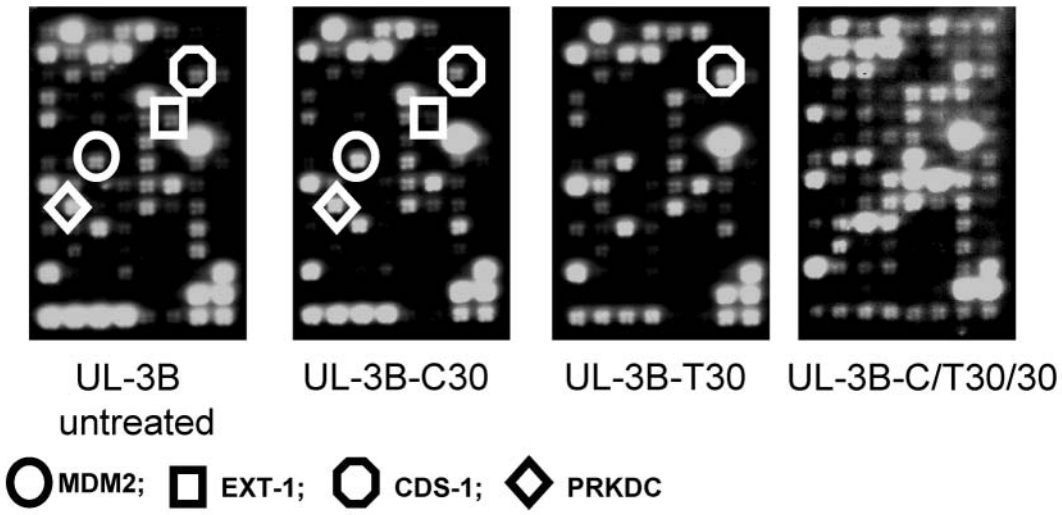
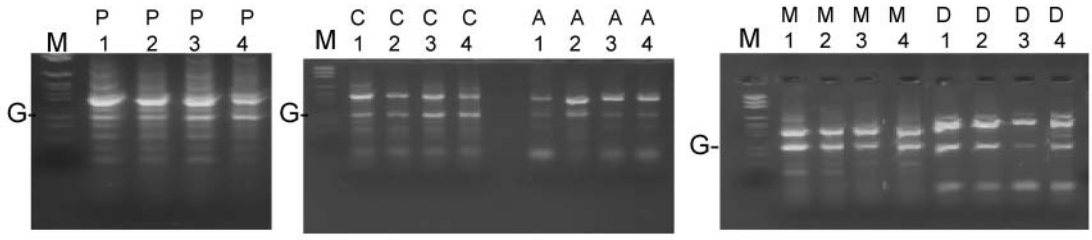


Figure 2. *DNA microarray of UL-3B, UL-3B-C30, UL-3B-T30 and UL-3B-C/T30/30. Representative genes (MDM-2, EXT-1, CDS-1, PRKDC) with significant change in expression are highlighted.*



Genes	Carbo Array[1]/RT-PCR[2]	Tax Array/RT-PCR	Carbo/Tax Array/RT-PCR
ATM	↑ / 135.2	N.S[3]./113.1	N.S./ 107.5
Chk1	↓ / 89.4	N.S./ 95.4	↓/ 84.7
mdm-2	↑ / 119.0	↑ / 147.0	N.S./ 103.0
PCNA	N.S./ 91.2	N.S./ 93.0	↑/ 105.8
PRKDC	N.S./107.0	↓ / 79.0	↓ / 88.1

Figure 3. *RT-PCR of selected genes. M: Molecular weight markers, P: PCNA; C: CHEK1; A: ATM; M: mdm-2; D: DNA-PK. Lanes1: Control; 2: UL-3B C-30; 3: UL-3B T-30; 4: UL-3B C/T30/30. G: GAPD internal normalizer. [1]Significant change of expression, [2]% of control, [3]Not significant.*

Discussion

The characteristics of ovarian cancer with respect to drug sensitivity and gene expression after treatment with carboplatin and/or paclitaxel were studied. Due to the nature of ovarian cancer, suboptimal chemotherapy is likely to occur during clinical treatment of the disease. Thus, these surviving cells may contribute to subsequent recurrences and therapy failures. Changes in drug sensitivity after treatment with Carbo and Tax were demonstrated. Significant resistance to chemotherapy was seen with the UL-3B-C30, UL-3B-T30, and UL-3BC/ T30/30 cells. The early disease group (UL-3A) and the peri-mortem group (UL-3C) had either the same or enhanced sensitivity after treatment.

From the microarray analysis heritable change in gene expression was demonstrated in the recovered cells. The most intriguing aspect of our data is that not only were genes differentially expressed, but distinct patterns of change among treatment groups were observed. Certain genes such as MDM2 and MSH6 were up-regulated with Carbo and Tax, 53BP1, H2AFL and UNG were down-regulated. The cells that recovered from the combination treatment with Carbo and Tax had a large number of genes up-regulated, and four genes down-regulated that were a cross-section of the two cytotoxic agents individually. These patterns suggest that temporal and perhaps coordinated changes are taking place in the cell and producing the phenotypic differences from the control. Because this microarray focused on a manageable number of genes related to DNA damage, it has yielded a number of gene targets for further study. Other genes may also be involved in the ovarian cancer progression to non-responsiveness that require additional studies. Up-regulation of DNA repair mechanisms is a major limitation to the prolonged use of cisplatin in all tumors because of the development of drug resistance (16). Cisplatin induces mostly intrastrand DNA cross-links which resemble UV-induced pyrimidine dimmers. In mammalian cells, NER is essential for the removal of a variety of helix distorting DNA lesions, including those induced by cisplatin. NER deficiencies render cells more sensitive to cisplatin and elevated NER capacity is associated with platinum resistance (17, 18).

Recent studies with human tumors have shown that the loss of 53BP1 expression correlates with cancer progression. 53BP1 participates in the cellular response and associates with various DNA repair/cell cycle factors and is thought to synergize with p53 to suppress tumorigenesis (19).

The MDM2 oncogene was first cloned as an amplified gene on a murine double-minute chromosome in the 3T3DM cell line (20). The MDM2 gene is amplified in a number of human tumors, including breast cancer. The expression of MDM2 is induced by p53, and mdm2 oncoprotein binds to p53 with high affinity, inhibiting its ability to act as a transcription factor (21). Studies have suggested that overexpression of MDM2 is associated with inactivation of wild-type p53, and inhibiting MDM2 expression in these tumors may lead to reactivation of p53 and induction of cell growth arrest or apoptosis. Significantly poorer survival has been seen for those ovarian cancer patients with p53 ($p<0.05$) or MDM2 ($p<0.01$) positive tumors than those with negative p53 or MDM2 staining (22).

Increased expression of DNA repair genes like ERCC-1 and XPAC and augmented intracellular platinum detoxification has been demonstrated. The inactivation of the DNA mismatch repair system has been linked to drug resistance. The presence of even small numbers of DNA mismatch repair-deficient cells in a tumor may adversely affect therapeutic outcome. Not only are such cells intrinsically resistant to cisplatin, but they also have an increased rate of spontaneous mutation to resistance to other chemotherapeutic agents (23).

To our knowledge, this is the first *in vitro* study of gene expression patterns in combination treatment resistance in ovarian cancer cells. Other reports have compared sensitive and resistant cells derived *in vitro* or in patient samples (24-28).

Our approach provides data on the effect of cytotoxic insult with carboplatin and paclitaxel on the DNA repair pathway. Because the gene expression changes that give rise to chemoresistance are multi-factorial in nature, expression modification of interactive gene transcripts may prove to be a more suitable target for molecular therapies.

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