Genome-wide transcriptional analysis of carboplatin response in chemosensitive and chemoresistant ovarian cancer cells

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Abstract

We have recently described an *ex vivo* chemoresponse assay for determining chemosensitivity in primary cultures of human tumors. In this study, we have extended these experiments in an effort to correlate chemoresponse data with gene expression patterns at the level of transcription. Primary cultures of cells derived from ovarian carcinomas of individual patients ($n = 6$) were characterized using the ChemoFx assay and classified as either carboplatin sensitive ($n = 3$) or resistant ($n = 3$). Three representative cultures of cells from each individual tumor were then subjected to Affymetrix gene chip analysis ($n = 18$) using U95A human gene chip arrays. Data were analyzed using the dCHIP software package. We identified a significant number of genes whose expression patterns were altered between carboplatin chemosensitive and chemoresistant cells, in normal culture conditions and in the presence of carboplatin for either 2 or 72 hours. Among these differentially expressed genes, we found a significant proportion to be associated with apoptosis, cell-cell communication, cell adhesion, DNA repair, and cell proliferation. In general, the molecular phenotype displayed by chemoresistant cells was reflective of an extended life span in culture in the presence of carboplatin and the genes that define this phenotype are potential biomarkers for the prognostic management of ovarian cancer patients. [Mol Cancer Ther 2005;4(10):1605–16]

Introduction

Gene expression profiling at the level of transcription by oligonucleotide microarray analysis is a powerful molecular tool for the discovery of genes that are involved in a variety of disease processes, including cancer. Patterns of global gene expression can reveal the molecular pathways relevant to the disease process and identify potential new therapeutic targets. The use of this technology for the molecular classification of cancer was recently shown with the identification of an expression profile that was predictive of patient outcome for B-cell lymphoma (1). In addition, this study showed that histologically similar tumors can be differentiated based on their gene expression profiles. Ultimately, these unique patterns of gene expression may be used as guidelines to direct different modes of therapy.

Although it is widely recognized that patients with the same histologic stage and grade of cancer respond to therapies differently, few clinical tests can predict individual patient responses. The next great challenge will be to use the power of post-genomic technology, including microarray analyses, to correlate gene expression patterns with individual patient responses to clinical therapies. The major objective of the current study was to determine whether predictive gene expression patterns can be identified that correlate with the results of an *ex vivo* chemoresponse assay done on primary cultures of cells derived from ovarian carcinomas of individual patients. Specifically, we used Affymetrix gene chip microarrays to analyze gene expression patterns in 18 cell cultures that were functionally characterized as either chemoresponsive or chemosensitive to carboplatin *in vitro*.

Materials and Methods

Patients

Tumors were submitted to Precision Therapeutics, Inc. (Pittsburgh, PA) for assessment of chemoresponsiveness to oncologist-selected chemotherapeutic agents. They were tested using the ChemoFx phenotypic chemoresponse assay that assesses tumor cell viability in the presence of a range of drug concentrations (see below; Table 1).

Primary Cell Culture and the ChemoFx Assay

Precision Therapeutics' ChemoFx assay involves the isolation, short-term growth, and drug dosage treatment of epithelial cells derived from solid tumors (2, 3). At the time of surgical "debulking," pieces of solid tumor are obtained by the surgeon or pathologist and placed in tissue culture transport medium for overnight shipment to the laboratories of Precision Therapeutics. Upon arrival, the tumor is minced into small pieces and placed with cell culture medium into multiple small flasks for cell outgrowth. Over time, cells move out of the tumor pieces and form a monolayer on the bottom of the flask. Once enough cells have migrated out of the *ex vivo* explant pieces, they are then trypsinized and reseeded into

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Table 1. Patient profiles

Patient no.	Tumor type	Tumor site	Cell kill (%)*	ChemoFx response †
1	PSA	Liver	66	S
2	PSA	Ovary	37	S
3	PSA	Ovary	53	S
4	PSA	Ovary	8	R
5	PSA	Ovary	3	R
6	PSA	Omentum	9	R

Abbreviations: PSA, papillary serous adenocarcinoma; S, sensitive; R, resistant.
*% Cell kill at the highest drug dose (1,000 mmol/L).
†*In vitro* response to carboplatin.

microtiter plates for either the ChemoFx assay (described below) or for immunohistochemistry analysis for the identification of malignant epithelial cells, as previously described (2).

For the ChemoFx assay, ~350 cells in 10 μL of medium are seeded into 60-well microtiter plates and allowed to attach and grow for 24 hours. After 24 hours in culture, 10 μL of six doses of 2× drug are dissolved in HBSS and added to each well for 2 hours at 37°C. For the present study, carboplatin was added in concentrations of 20, 50, 100, 200, 500, and 1,000 mmol/L. Plates are then rinsed rapidly with HBSS four times to remove any drug residue, and then fresh medium is added for 72 hours. After the 72 hours of recovery period, the medium and any nonadherent dead cells are rinsed off with HBSS, and the remaining cells are fixed for 5 minutes in 95% ethanol containing the DNA intercalating blue fluorescent dye, 4′,6-diamidino-2-phenylindole. The number of cells per well was then quantitated by counting fluorescent nuclei using an operator-controlled, computer-assisted image analysis system (Zeiss, Hertfordshire, United Kingdom) customized for Precision Therapeutics. A complete dose-response curve was generated for each drug evaluated. The data are presented graphically as the cytotoxic index. The cytotoxic index (% kill) is defined as 1 − [no. of cells in treated wells / no. of cells in control wells] × 100. For each drug dose, the cytotoxic index was calculated using two columns of untreated cells in the plate as the negative control. Drug responses were scored from 0 to 5, with the score determined by the number of drug doses where the cytotoxic index was ≥35%. For the purposes of the present study, a drug response score of 0 to 1 was considered resistant and a score of 3 to 5 was considered sensitive.

Affymetrix Gene Expression Analysis

For oligonucleotide analysis using the Affymetrix platform, RNA extraction, reverse transcription, cRNA preparation, and chip hybridization were done according to the manufacturer's instructions (Affymetrix, Santa Clara, CA). The full protocol can be found at http://www.affymetrix.com/support/technical/manual/expression_manual.affx. In brief, total RNA was extracted from cultured cells using the Trizol (Invitrogen, Carlsbad, CA) and RNeasy (Qiagen, Valencia, CA) protocols. Twenty micrograms of purified RNA were then used as a template for double-stranded cDNA synthesis primed using a T7-$(dT)_{24}$ oligonucleotide. Double-stranded cDNA was then used as a template for biotin-labeled cRNA preparation using T7 RNA polymerase. Resulting cRNA was fragmented and hybridized to Affymetrix GeneChip Human Genome U95 version 2 (HG-U95A) oligonucleotide microarrays.

Affymetrix data were analyzed using the dCHIP software package (4). The 18 DAT files generated by the Affymetrix Microarray Suite version 5.0 were converted into DCP files using dCHIP,[3] as described previously by Li and Wong (4). The DCP files were normalized, and raw gene expression data generated. Normalization was carried out according to the Invariant Set Normalization method as described by Li and Wong (5). The resulting gene list was then filtered such that only genes whose expression levels satisfied the following criteria were included: 0.5 < SD/mean >10, gene called P (present) in 20% of samples, and expression level >20 in >50% of samples. Global comparisons of data derived from resistant and sensitive cells was done as follows (where E = experimental samples and B = baseline samples): $E_{mean}/B_{mean} > 2$ or $B_{mean}/E_{mean} > 2$; $E_{mean} - B_{mean} > 100$ or $B_{mean} - E_{mean} > 100$; and P for testing $E_{mean} = B_{mean}$ < 0.03. The t statistic was computed as $(\text{mean}_1 - \text{mean}_2) / \sqrt{[\text{SE}(\text{mean}_1)_2 + \text{SE}(\text{mean}_2)_2]}$ and its P computed based on the t distribution, and the degree of freedom is set according to the Welch-modified two-sample t test. The clustering algorithm used is as follows: the distance between two genes is defined as $1 - r$, where r is the Pearson correlation coefficient between the standardized expression values (make mean = 0 and SD = 1) of the two genes across the samples used.[4] Full data sets are available [accession no. GSE1926 (Gene Expression Omnibus series)].[5]

Results

The experiments done in this study were designed to (a) identify genes whose expressions were altered in primary cultures of carboplatin-sensitive versus carboplatin-resistant ovarian carcinoma cells; (b) to determine the differences in genomic response of chemosensitive versus resistant cells to 2 or 72 hours of carboplatin exposure *in vitro*; and (c) to analyze the temporal genomic response to carboplatin in either chemosensitive or resistant cells independently of each other. Affymetrix data sets were obtained for three representative cultures of cells from each of six individual tumors (18 arrays), of which, based on data from the ChemoFx assay (Table 1), three were resistant and three were sensitive to carboplatin. Data were analyzed using the dCHIP package (4) as described in Material and Methods.

[3] http://www.dCHIP.org
[4] http://biosun1.harvard.edu/complab/dchip/clustering.htm
[5] http://www.ncbi.nlm.nih.gov/projects/geo/

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Carboplatin-Sensitive Ovarian Tumor Cells Display a Distinct Molecular Phenotype at the Level of Transcription Relative to Resistant Cells

We first sought to identify genes whose altered expressions correlated with chemosensitivity or resistance. We therefore compared expression profiles of sensitive and resistant cells in culture without *in vitro* exposure to carboplatin. The underlying hypothesis for this experiment was that fundamental differences in molecular phenotype exist at the mRNA level between ovarian tumor cells before

Table 2. Genes whose expressions (>2-fold) are altered between carboplatin-sensitive and carboplatin-resistant cells (*n* = 3)

Gene	Symbol	Accession no.	Function	Fold change (R/S)	*P*
Proteoglycan 1, secretory granule	*PRG1*	X17042	Apoptosis	>−20	0.02932
Armadillo repeat protein	*ALEX2*	AB011084		>−20	0.012058
C-type (calcium-dependent, carbohydrate recognition domain) lectin, superfamily member 2 (activation induced)	*CLECSF2*	X96719	Cell-cell adhesion	>−20	0.014593
MD-2 protein	*MD-2 (Ly96)*	AB018549	Inflammation	>−20	0.001373
Dihydropyrimidinase like 3	*DPYSL3*	D78014	Signal transduction	>−20	0.029675
Cadherin 2, type 1, N-cadherin (neuronal)	*CDH2*	M34064	Cell-cell adhesion	−14.08	0.013468
Fc fragment of IgG, receptor, transporter, α	*FCGRT*	U12255	Immune response	−13.22	0.010178
Zinc finger protein 238	*ZNF238*	AJ223321	DNA binding	−9.37	0.009983
Neuron navigator 3	*NAV1*	AB023155	Nucleotide binding	−7.62	0.02058
Hypothetical protein BC001096	—	W87466	—	−7.58	0.002457
KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 3	*KDELR3*	AL035081	Protein transport	−7.23	0.007759
ALL1-fused gene from chromosome 1q	*AF1Q*	U16954	Unknown	−6.34	0.012183
Small nuclear ribonucleoprotein polypeptide N	*SNRPN*	U41303	RNA metabolism	−6.01	0.009959
γ-Aminobutyric acid(A) receptor-associated protein like 1	*GABARAPL1*	W28281	Unknown	−5.9	0.015469
Expressed sequence tags, weakly similar to hypothetical protein FLJ22184 (*H. sapiens*)	—	AL046940	—	−5.5	0.028947
Fc fragment of IgG, receptor, transporter, α	*FCGRT*	U12255	Immune response	−5.23	0.001166
Serine (or cysteine) proteinase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2	*SERPINE2*	AI743134	Heparin binding	−5.07	0.019903
Adipose differentiation–related protein	*ADFP*	X97324	Lipid metabolism	−4.89	0.002709
Melanoma antigen, family D, 1	*MAGED1*	W26633	Apoptosis	−4.87	0.00796
Major vault protein	*MVP*	X79882	Protein transport	−4.36	0.000862
RecQ protein like (DNA helicase Q1 like)	*RECQL*	D37984	DNA repair	−4.27	0.017178
Retinitis pigmentosa 2 (X-linked recessive)	*RP2*	AJ007590	Protein folding	−4.23	0.005434
SEC24-related gene family, member D (*Saccharomyces cerevisiae*)	*SEC24D*	AB018298	Protein transport	−3.95	0.020784
Lectin, galactoside-binding, soluble, 1 (galectin 1)	*LGALS1*	AI535946	Cell-cell adhesion	−3.8	0.018688
Myosin X	*MYO10*	AB018342	Cytoskelatal	−3.45	0.013408
KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 2	*KDELR3*	M88458	Protein transport	−3.09	0.015031
Nucleobindin 2	*NUCB2*	X76732	DNA binding	−3.08	0.017437
Activin A receptor, type I	*ACVR1*	Z22534	Transforming growth factor-B1 signaling	−2.81	0.004938
Small nuclear ribonucleoprotein polypeptide F	*SNRPF*	AI032612	RNA metabolism	2.59	0.017056
Fatty acid synthase	*FASN*	U29344	Lipid metabolism	2.74	0.006118
KIAA0116 protein	—	D29958	—	3.15	0.014451
D00591/FEATURE = exons 7-14/DEFINITION = HUMRCC1 *H. sapiens* RCC1 gene, exons 1-14, complete cds	—	D00591	—	3.16	0.00635
Hypothetical protein BC008326	—	AI057614	—	3.2	0.003631
Hypothetical protein FLJ11220	—	AL050064	—	3.26	0.001465
BCL2-associated athanogene	*BAG1*	Z35491	Antiapoptosis	4.55	0.003866
DC12 protein	*DC12*	AF035296	Unknown	4.77	0.021593
Peroxisome proliferator-activated receptor γ	*PPARG*	L40904	Signal transduction	6.79	0.02744
Ephrin A-1	*EFNA1*	M57730	Cell-cell signaling	>20	0.067722*

Abbreviations: R, resistant; S, sensitive.

*Differentially expressed genes that did not reach significance but which were clearly altered.

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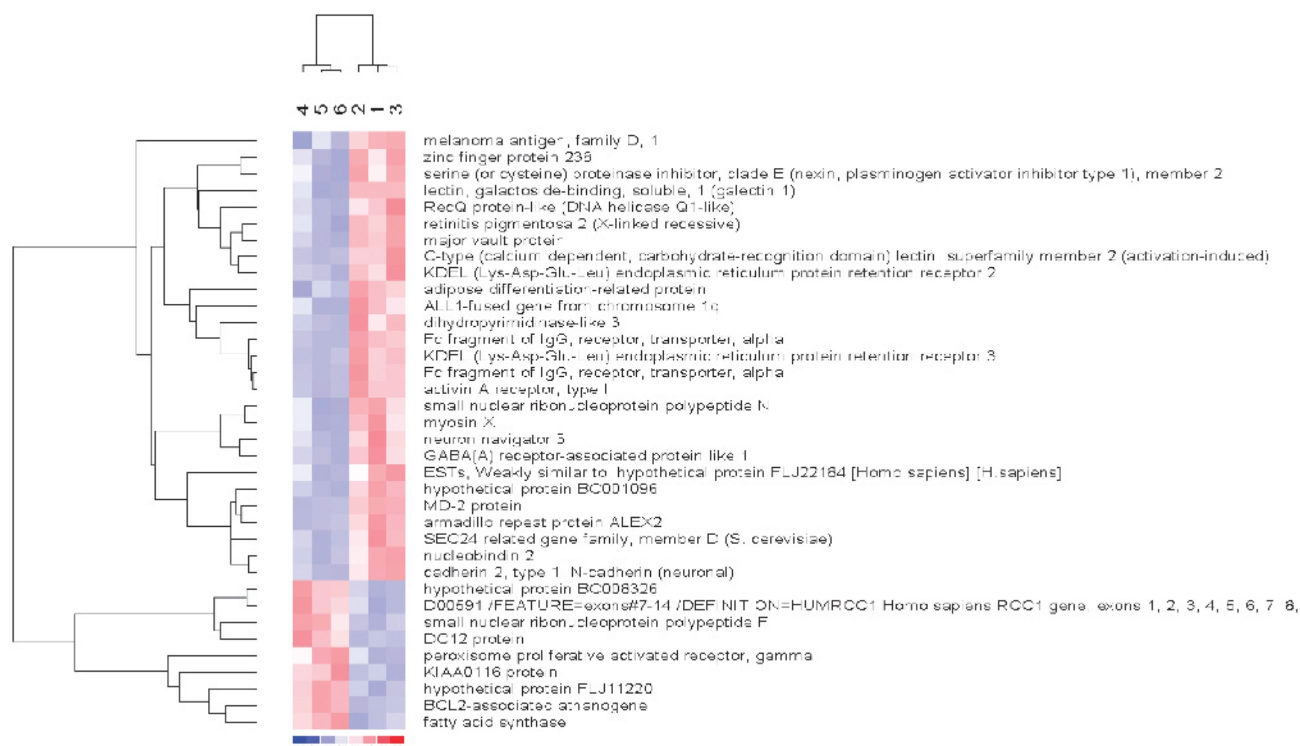

Figure 1. Two-fold expression changes at $t = 0$, sensitive versus resistant (see Table 2). Carboplatin sensitive and carboplatin resistant as determined by ChemoFx assay. Numbers refer to patient ID as in Table 1. Red and blue boxes indicate up-regulation and down-regulation, respectively.

treatment *ex vivo* with carboplatin and are potential prognostic markers for the clinical identification of drug-sensitive and drug-resistant tumors. Genes consistently altered by ≥2-fold differences between chemosensitive and resistant cells are shown in Table 2 and Fig. 1.

Interestingly, a number of the genes whose expressions are altered between sensitive and resistant cells play a role in apoptosis. For example, the expression of BCL2-associated athanogene (*BAG1*) is elevated in the chemoresistant cells relative to the chemosensitive cells. *BAG1* encodes a protein with antiapoptotic function. In keeping with this antiapoptotic phenotype, the proapoptotic factors adipose differentiation–related protein (*ADFP*) and melanoma antigen family D1 (*MAGED1*) are elevated in chemosensitive versus chemoresistant cells. Similarly, proteoglycan 1 (*PRG1*), which has been shown to trigger apoptosis in HeLa cells (6), is very highly expressed 'in chemosensitive cells relative to resistant cells. Chemosensitive cells also expressed higher levels of galectin 1 (*LGALS1*), c-type lectin superfamily member 2 (*CLECSF2*), RecQ protein like (*RECQL*), activin A receptor (*ACVR1*), and armadillo repeat protein (*ALEX2*). The possible significance of these observations is discussed below.

A number of uncharacterized genes are differentially expressed between chemosensitive and resistant cells without *in vitro* exposure to carboplatin. These include expressed sequence tags, similar to hypothetical protein FLJ22184, hypothetical protein BC001096, hypothetical protein FLJ11220, and KIAA0116 protein and expressed sequence tags similar to T00636 hypothetical protein F21856. These may represent entirely novel markers for chemoresistance in ovarian cancer.

Furthermore, there are a number of characterized genes whose altered expressions are difficult to interpret in the context of chemoresistance. These include MD-2 protein (*LY96*), *deafness autosomal dominant 5*, endoplasmic reticulum protein retention receptors 2 and 3 (*KDEL2* and *KDEL3*), γ-aminobutyric acid(A) receptor–associated protein like 1 (*GABARAPL1*), small nuclear ribonucleoprotein polypeptide N (*SNRPN*), SEC24-related gene family member D (*SEC24D*), retinitis pigmentosa 2 (*RP2*), and nucleobindin 2 (*NUCB2*).

Transcriptional Response to Carboplatin Is Altered in Chemoresistant versus Chemosensitive Ovarian Tumor Cells

We next sought to identify genes whose expression levels were altered by exposure to carboplatin *ex vivo* and specifically to define the differences in this response, if any, between chemoresistant and chemosensitive cultured ovarian tumor cells. We hypothesized that the molecular response to drug exposure would be specific to each cell type and that the differences between chemosensitive and resistant cells might define potential markers for use in predictive assays for disease prognosis. Chemoresistant and sensitive cells, as defined by the ChemoFx assay (Table 1), were exposed in culture to carboplatin for 2 or

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Table 3. Genes whose expressions (>2-fold) are altered between carboplatin-sensitive and carboplatin-resistant cells following either 2 h (A) or 72 h (B) exposure to carboplatin *in vitro* (n = 3)

Gene	Symbol	Accession no.	Function	Fold change (R/S)	*P*
A. Two-hour exposure to carboplatin					
Proteoglycan 1, secretory granule	*PRG1*	X17042	Apoptosis	>−20	0.02932
Armadillo repeat protein	*ALEX2*	AB011084		>−20	0.046459*
C-type (calcium-dependent, carbohydrate recognition domain) lectin, superfamily member 2 (activation induced)	*CLECSF2*	X96719	Cell-cell adhesion	>−20	0.03044*
Dihydropyrimidinase-like 3	*DPYSL3*	D78014	Signal transduction	>−20	0.023379
MD-2 protein	*MD-2 (Ly96)*	AB018549	Inflammation	−17.97	0.011945
Zinc finger protein 238	*ZNF238*	AJ223321	DNA binding	−12.25	0.004093
IFNγ-inducible protein 16	*IFI16*	M63838	DNA binding	−11.11	0.012357
Fc fragment of IgG, receptor, transporter, α	*FCGRT*	U12255	Immune response	−10.45	0.009317
KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 3	*KDELR3*	AL035081	Protein transport	−7.78	0.028435
Neuron navigator 3	*NAV1*	AB023155	Nucleotide binding	−6.93	0.004931
Small nuclear ribonucleoprotein polypeptide N	*SNRPN*	U41303	RNA Metabolism	−6.91	0.007716
Hypothetical protein BC001096	—	W87466	—	−6.77	0.005009
ALL1-fused gene from chromosome 1q	*AF1Q*	U16954	Unknown	−6.66	0.019783
Expressed sequence tags, weakly similar to hypothetical protein FLJ22184 (*H. sapiens*)	—	AL046940	—	−5.74	0.027368
Serine (or cysteine) proteinase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2	*SERPINE2*	AI743134	Heparin binding	−5.65	0.019291
RecQ protein like (DNA helicase Q1-like)	*RECQL1*	D37984	DNA repair	−4.74	0.005347
Major vault protein	*MVP*	X79882	Protein transport	−4.72	0.012515
Adipose differentiation–related protein	*ADFP*	X97324	Lipid metabolism	−4.71	0.005218
Fc fragment of IgG, receptor, transporter, α	*FCGRT*	U12255	Immune response	−4.61	0.002221
Melanoma antigen, family D, 1	*MAGED1*	W26633	Apoptosis	−4.53	0.010493
SEC24-related gene family, member D (*S. cerevisiae*)	*SEC24D*	AB018298	Protein transport	−4.3	0.006707
Lectin, galactoside-binding, soluble, 1 (galectin 1)	*LGALS1*	AI535946	Cell-cell adhesion	−3.93	0.012395
Retinitis pigmentosa 2 (X-linked recessive)	*RP2*	AJ007590	Protein folding	−3.4	0.015383
HLA class II region–expressed gene KE4	*SLC39A7*	AL031228	Ion transport	−3.3	0.005645
Myosin X	*MYO10*	AB018342	Cytoskeletal	−3.13	0.019424
Leukotriene A4 hydrolase	*LTA4H*	J03459	Inflammation	2.63	0.029435
Regulator of chromosome condensation 1	*RCC1*	X12654	Cell cycle	2.69	0.003837
Small nuclear ribonucleoprotein polypeptide F	*SNRPF*	AI032612	RNA metabolism	2.8	0.025743
Chloride channel, nucleotide-sensitive, 1A	*CLNS1A*	X91788	Ion transport	2.8	0.01432
REST corepressor	*RCOR1*	D31888	DNA binding	2.81	0.001903
Programmed cell death 2	*PDCD2*	S78085	Apoptosis	2.89	0.004035
Metastasis-associated 1	*MTA1*	U35113	DNA binding	3.22	0.021499
D00591/FEATURE = exons 7-14/DEFINITION = HUMRCC1 *H. sapiens* RCC1 gene, exons 1-14, complete cds	—	D00591	—	3.26	0.010929
DKFZP564C186 protein	—	AL050019	—	3.37	0.003486
Hypothetical protein BC008326	—	AI057614	—	3.37	0.007721
MAX-interacting protein 1	*MX1*	L07648	DNA binding	4.19	0.021369
BCL2-associated athanogene	*BAG1*	Z35491	Antiapoptosis	4.81	0.028184
DC12 protein	*DC12*	AF035296	Unknown	4.91	0.015992
KIAA0601 protein	—	AB011173	—	5	0.018079
Ephrin A-1	*EFNA1*	M57730	Cell-cell signaling	>20	0.06189*
B. Seventy-two-hour exposure to carboplatin					
Armadillo repeat protein	*ALEX2*	AB011084	Unknown	>−20	0.03187*
Dihydropyrimidinase-like 3	*DPYSL3*	D78014	Signal transduction	>−20	0.003027*
Proteoglycan 1, secretory granule	*PRG1*	X17042	Apoptosis	>−20	0.068708*

Abbreviations: R, resistant; S, sensitive.

*Differentially expressed genes that did not reach significance but which were clearly altered.

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Table 3. Genes whose expressions (>2-fold) are altered between carboplatin-sensitive and carboplatin-resistant cells following either 2 h (A) or 72 h (B) exposure to carboplatin *in vitro* (n = 3) (Cont'd)

Gene	Symbol	Accession no.	Function	Fold change (R/S)	*P*
C-type (calcium-dependent, carbohydrate recognition domain) lectin, superfamily member 2 (activation induced)	*CLECSF2*	X96719	Cell-cell adhesion	>−20	0.017664
MD-2 protein	*MD-2 (Ly96)*	AB018549	Inflammation	>−20	0.005349
Fc fragment of IgG, receptor, transporter, α	*FCGRT*	U12255	Immune response	−14.94	0.025556
Sarcospan (Kras oncogene–associated gene)	*SSPN*	N21470	Cell-cell adhesion	−12.65	0.004181
IFNγ-inducible protein 16	*IFI16*	M63838	DNA binding	−7.69	0.006012
Fc fragment of IgG, receptor, transporter, α	*FCGRT*	U12255	Immune response	−6.61	0.02581
Adipose differentiation–related protein	*ADFP*	X97324	Lipid metabolism	−5.65	0.002593
Glutathione peroxidase 1	*GPX1*	X13710	Stress response	−5.16	0.027208
ALL1-fused gene from chromosome 1q	*AF1Q*	U16954	Unknown	−4.84	0.019144
H. sapiens mRNA; cDNA DKFZp564A026 (from clone DKFZp564A026)	—	AL050367	—	−4.57	0.015384
RecQ protein like (DNA helicase Q1-like)	*RECQL1*	L36140	DNA repair	−4.42	0.018595
CLIP-170-related protein	*CLIPR-59*	N99340	Unknown	−4.29	0.003918
Aldehyde dehydrogenase 1 family, member A3	*ALDH1A3*	U07919	Lipid metabolism	−4.24	0.013915
H. sapiens unknown mRNA	—	AI674208	—	−4.12	0.022238
Hypothetical protein BC001096	—	W87466	—	−4.05	0.017738
Serine/threonine kinase 17a (apoptosis inducing)	*STK17A*	AI961743	Apoptosis	−3.6	0.004848
CGI-65 protein	*NDUFAF1*	AI566877	Mitochondrial electron transport	−3.34	0.014449
BET1 homologue (*S. cerevisiae*)	*BET1*	AF007551	Protein transport	−3	0.003188
CGI-100 protein	*TMED5*	AL080084	Protein transport	−2.7	0.003929
H. sapiens clone 24416 mRNA sequence	—	AF052159	—	2.67	0.001003
KIAA1014 protein	—	AB023231	—	2.74	0.022371
CCCTC-binding factor (zinc finger protein)	*CTCF*	U25435	DNA binding	2.77	0.009499
Hypothetical protein MGC5576	—	AI525633	—	2.83	0.004847
L-3-hydroxyacyl-coenzyme A dehydrogenase, short chain	*HADHSC*	X96752	Lipid metabolism	2.83	0.003823
REST corepressor	*RCOR1*	D31888	DNA binding	2.99	0.001522
Chromobox homologue 1 (HP1β homologue *Drosophila*)	*CBX1*	U35451	Chromatin assembly	3.11	0.029683
Espressed sequence tags	—	AI827895	—	3.27	0.014036
Fatty acid synthase	*FASN*	U29344	Lipid metabolism	3.34	0.001912
Uracil-DNA glycosylase	*UNG*	Y09008	DNA repair	3.4	0.00155
Cyclin F	*CCNF*	Z36714	Cell cycle	3.42	0.024405
MCM4 minichromosome maintenance-deficient 4 (*S. cerevisiae*)	*MCM4*	X74794	DNA repair	3.46	0.003692
Vaccinia-related kinase 1	*VRK1*	AB000449	Protein Modification	3.68	0.00879
KIAA0101 gene product	—	D14657	—	3.68	0.009866
Rad2	*RAD2*	HG4074-HT4344	DNA repair	3.69	0.015096
Replication factor C (activator 1) 3, 38 kDa	*RFC4*	L07541	DNA replication	3.73	0.004902
KIAA0186 gene product	—	D80008	—	3.8	0.010346
MCM2 minichromosome maintenance-deficient 2, mitotin (*S. cerevisiae*)	*MCM2*	D21063	DNA replication	3.87	0.015244
H2A histone family, member X	*H2AX*	X14850	Chromosome organization	3.89	0.000189
Paired basic amino acid cleaving system 4	*PCSK6*	M80482	Cell-cell signaling	4.1	0.003952
Flap structure–specific endonuclease 1	*FEN1*	AC004770	DNA /Repair	4.13	0.017241
Hypothetical protein FLJ10719	—	W74442	—	4.21	0.023813
Desmoplakin (DPI and DPII)	*DSP*	AL031058	Cell-cell adhesion	4.28	0.011781
Adenosine deaminase, RNA-specific, B1 (RED1 homologue rat)	*ADARB1*	U76421	RNA metabolism	4.4	0.008376
CDC45 cell division cycle 45 like (*S. cerevisiae*)	*CDC45L*	AJ223728	DNA replication	4.75	0.02302
DC12 protein	*DC12*	AF035296	Unknown	5.22	0.005536

(*Continued on the following page*)

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Table 3. Genes whose expressions (>2-fold) are altered between carboplatin-sensitive and carboplatin-resistant cells following either 2 h (A) or 72 h (B) exposure to carboplatin *in vitro* (n = 3) (Cont'd)

Gene	Symbol	Accession no.	Function	Fold change (R/S)	*P*
v-myb myeloblastosis viral oncogene homologue (avian) like 2	*MYBL2*	X13293	Antiapoptosis	7.26	0.028861
KIAA1028 protein	—	AB028951	—	7.43	0.026059
KIAA0906 protein	—	AB020713	—	8.08	0.006995
Ephrin-A1	*EFNA1*	M57730	Cell-cell signaling	>20	0.000447

72 hours. Genes whose expression levels were altered following 2 hours of exposure to carboplatin are shown in Table 3A and Fig. 2A. These data are strikingly similar to those obtained when chemoresistant and chemosensitive cells were compared in the absence of carboplatin exposure (Table 2). Specifically, of the genes identified as being differentially expressed between sensitive and resistant cells in the absence of carboplatin (Table 2), 79% were also altered after 2 hours of exposure to carboplatin. This likely indicates that relatively few alterations in gene expression occur within the first 2 hours following the onset of carboplatin exposure and therefore provides a convenient external control for the experimental data summarized in Table 2. Notably, a number of genes that were altered between chemoresistant and chemosensitive cells in the absence of carboplatin (Table 2) were also altered after 2 hours (and 72 hours) of carboplatin exposure. These include *PRG1, ALEX2, MD2*, ephrin A-1 (*EFNA1*), and *RECQL1*. Those genes whose altered expression was not common to the unexposed and 2-hour carboplatin-exposed cells are generally poorly characterized, and/or information relating to their potential involvement in chemosensitivity/resistance is limited. They included REST corepressor (*RCOR1*); leukotrine A4 hydrolase (*LTA4H*); HLA class II region–expressed gene KE4 (*KE4*); chloride channel, nucleotide-sensitive 1A (*CLNS1A*); programmed cell death 2 (*PCD2*); MAX-interacting protein (*MX1*); and IFNγ-inducible protein 16 (*IFI16*). One exception is metastasis-associated 1 (*MTA1*), which is discussed below.

In contrast, we found that following 72 hours of exposure to carboplatin (Table 3B; Fig. 2B), differentially expressed genes had only moderate overlap with those identified after 2 hours of exposure or no exposure, indicating a marked response at the level of transcription to 72 hours of carboplatin exposure. Specifically, of the 37 genes whose expressions were altered between chemosensitive and resistant cells in the absence of carboplatin, only 15 (28%) overlapped with the 53 differentially expressed genes identified after 72 hours of carboplatin exposure. This suggests that the transcriptional response to carboplatin overrides many of the transcriptional differences that define the cells in their carboplatin-free baseline state.

We found marked differences in gene expression patterns between the chemosensitive and resistant cells as shown in Table 3B and Fig. 2B. For example, a number of genes of functional significance in the context of drug response were elevated in resistant versus sensitive cells. These genes were either associated with cell proliferation, including CDC45 cell division cycle 45 like (*CDC45L*), cyclin F (*CCNF*), or DNA replication and repair, including replication factor C 4 (*activator 1*) 38-kDa (*RFC4*), minichromosome maintenance-deficient 2 (*MCM2*), *MCM4*, uracil-DNA glycosylase (*UNG*), flap structure–specific endonuclease 1 (*FEN1*; also *RAD2*]. Also elevated in resistant cells was v-myb myeloblastosis viral oncogene homologue (avian) like 2 (*MYBL2*), which encodes a protein with antiapoptotic activity. In keeping with the apoptosis-resistant phenotype of chemoresistant cells, we also found that serine/threonine kinase 17a (*STK17A*) was reduced in chemoresistant cells. STK17A encodes a protein with proapoptotic function (7).

We also identified H2A histone family member X (*H2AX*), which is involved in the maintenance of genome integrity (see below), as being elevated in resistant cells, as well as chromobox homologue 1 (*HP1*). The elevated expression of HP1 in chemoresistant cells is notable, because loss of function of HP1 has been shown to cause cell death (8), and the down-regulation of HP1α expression is associated with the metastatic phenotype in breast cancer (9).

Notable genes whose expressions were elevated in chemosensitive cells included glutathione peroxidase 1 (*GPX1*) and *IFI16*, both of which have been previously implicated in chemoresistance and apoptosis, respectively. The significance of these observations is discussed below.

Finally, a number of uncharacterized genes were found to be differentially expressed by 72 hours of carboplatin exposure. These included *KIAA0101* gene product, hypothetical protein *BC001096, Homo sapiens* clone *24416, CGI-100* protein, and *CGI65* protein.

Characterization of the Temporal Response to Carboplatin in either Chemosensitive or Resistant Cells

We also searched our data for genes whose expressions were independently altered in a temporal fashion in either chemosensitive or resistant cells by exposure to carboplatin. Using this approach with a 2-fold expression change cutoff, we found that in the chemosensitive cells, only 19 and 11 genes were altered when (a) t = 0 hour was compared with t = 72 hours and (b) t = 2 hours was compared with t = 72 hours, respectively, for the sensitive cells (Table 4A). Furthermore, only two and three genes

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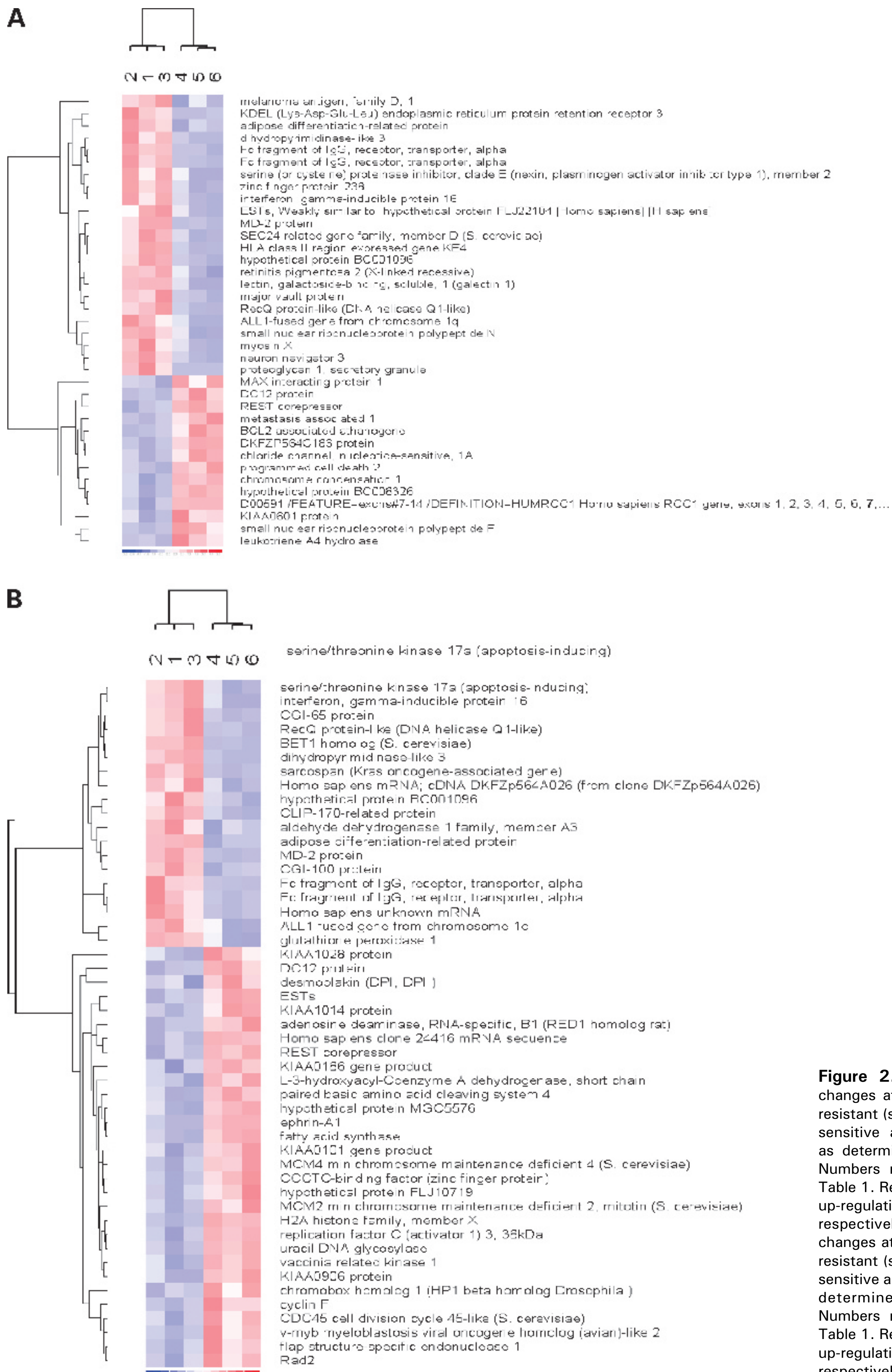

Figure 2. **A,** 2-fold expression changes at $t = 2$, sensitive versus resistant (see Table 3A). Carboplatin sensitive and carboplatin resistant as determined by ChemoFx assay. Numbers refer to patient ID as in Table 1. Red and blue boxes indicate up-regulation and down-regulation, respectively. **B,** 2-fold expression changes at $t = 72$, sensitive versus resistant (see Table 3B). Carboplatin sensitive and carboplatin resistant as determined by ChemoFx assay. Numbers refer to patient ID as in Table 1. Red and blue boxes indicate up-regulation and down-regulation, respectively.

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were altered when (a) t = 0 hour was compared with t = 72 hours and (b) t = 2 hours was compared with t = 72 hours, respectively, for the resistant cells (Table 4B). Not surprisingly, we found considerable overlap between the t = 0 hour versus t = 72 hours and t = 2 hours and t = 72 hours comparisons, which is consistent with the previous finding that few expression changes are evident when chemosensitive and resistant cells were directly compared after 2 hours of carboplatin exposure (Table 3A). Interestingly, we found very few statistically significant gene expression

Table 4. Genes whose expressions are temporally altered in (A) chemosensitive and (B) chemoresistant cells following carboplatin exposure *in vitro*

0 h versus 72 h	*P*	2 h versus 72 h	*P*	Gene	Symbol	Function	Accession no.	Locus link
A. Chemosensitive cells								
↓72 h	0.022345	↓72 h	0.018408	CDC6 cell division cycle 6 homologue (*S. cerevisiae*)	*CDC6*	Cell cycle	U77949	990
↓72 h	0.025498	—	—	Inhibin, β A (activin A, activin AB α polypeptide)	*INHBB*	Cell cycle	J03634	3624
↓72 h	0.01845	↓72 h	0.033219	Kinetochore associated 2	*KNTC1*	Cell cycle/ DNA repair	AF017790	10403
↓72 h	0.019354	↓72 h	0.024426	Flap structure–specific endonuclease 1	*FEN1*	DNA repair	AC004770	2237
↓72 h	0.032797	—	—	Ribonucleotide reductase M2 polypeptide	*RRM2*	DNA replication	X59618	6241
↓72 h	0.032727	↓72 h	0.033662	MCM7 minichromosome maintenance-deficient 7 (*S. cerevisiae*)	*MCM7*	DNA repair	D55716	4176
↓72 h	0.021449	↓72 h	0.02774	Rad2	*RAD2*	DNA repair		
↓72 h	0.016836	↓72 h	0.032576	Ubiquitin-specific protease 1	*USP1*	Ubiquination cycle	AB014458	7398
↓72 h	0.016794	↓72 h	0.027252	H4 histone family, member G	*HIST1H4C*	Chromatin Organization	AA255502	8364
↓72 h	0.039039	↓72 h	0.040177	Replication factor C (activator 1) 4, 37 kDa	*RFC4*	DNA replication	M87339	5984
↓72 h	0.035747	—	—	MCM4 minichromosome maintenance-deficient 4 (*S. cerevisiae*)	*MCM4*	DNA repair	X74794	4173
↓72 h	0.02134	—	—	Sprouty homologue 2 (*Drosophila*)	*SPRY2*	Cell-cell signaling	AF039843	10253
↓72 h	0.024581	—	—	Fusion, derived from t(12;16) malignant liposarcoma	*FUS*	DNA binding/ unknown	S62140	2521
↓72 h	0.033626	—	—	MAD, mothers against decapentaplegic homologue 7 (*Drosophila*)	*SMAD7*	DNA binding transforming growth factor B1 signaling	AF010193	4092
↑72 h	0.021064	—	—	Gelsolin (amyloidosis, Finnish type)	*GSN*	Cytoskeletal	X04412	2934
↑72 h	0.03123	—	—	H2A histone family, member L	*HIST1HH2AH*	Chromatin organization	U90551	8334
↑72 h	0.015597	↑72 h	0.02281	Chemokine (C-C motif) ligand 2	*CCL2*	Inflammation/ cell-cell adhesion	M28225	6347
↑72 h	0.022596	↑72 h	0.022538	p8 protein (candidate of metastasis 1)	*P8*	Apoptosis	AI557295	26471
↑72 h	0.009538	—	—	Chemokine (C-C motif) ligand 2	*CCL2*	Inflammation/ cell-cell adhesion	M26683	6347
B. Chemoresistant cells								
↓72 h	0.037631	↓72 h	0.041482	Inhibitor of DNA binding 2, dominant-negative helix-loop-helix protein	*ID1*	DNA binding/ development	D13891	3398
—	—	↓72 h	0.029638	HIF-1 responsive RTP801 (DNA damage–inducible transcript 4)	*DDIT4*	DNA damage response	AA522530	54541
↓72 h	0.028745	↓72 h	0.028122	Cyclin G2	*CCNG2*	Cell cycle	U47414	901

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changes following 2 or 72 hours of exposure to carboplatin in chemoresistant cells. This suggests that the transcriptional response to carboplatin is subtle in these cells and this may reflect the chemoresistant nature of their behavior in culture in the presence of the drug.

Strikingly, a large number of the genes altered by exposure to carboplatin in chemosensitive cells are involved in DNA replication and maintenance, and these are reduced by carboplatin exposure at the mRNA level. These genes include, for example, *MCM7, MCM4, RFC4* (37 kDa), kinetochore-associated 2 (*KNTC2*), *FEN1*, and ribonucleotide reductase M2 polypeptide (*RRM2*). Genes whose expressions were elevated in chemosensitive cells in response to carboplatin have a variety of functions but include H2A histone family member L (*HIST1H2AC*), chemokine (C-C motif) ligand 2 (*CCL2*), Sprouty 2 (*SPRY2*), Gelsolin (*GSN*), and p8 protein (*P8*).

Discussion

The data presented here are the result of a preliminary investigation into the molecular phenotype of patient-derived ovarian tumor cells in the context of sensitivity or resistance to carboplatin. We have identified a number of differentially expressed genes that define transcriptional differences between chemosensitive and resistant cells and temporal responses to carboplatin expressed in an *ex vivo* setting. These genes represent a variety of functional classes but are biased towards those that encode proteins involved in apoptosis, cell-cell communication, cell anchorage, DNA repair, and chromosome stability. These findings are consistent with increased survival of resistant cells in the presence of carboplatin in culture as defined by the ChemoFx assay.

One feature of our data was the finding that chemosensitive cells in the absence of carboplatin have a transcriptional profile that can be associated with a proapoptotic phenotype compared with chemoresistant cells. BAG-1, for example, was elevated in chemoresistant relative to chemosensitive cells. BAG-1 has been shown to inhibit apoptosis (10, 11), and its overexpression in resistant cells is consistent with the ChemoFx data (Table 1). Significantly, it has recently been suggested that BAG-1 up-regulation is associated with the acquisition of paclitaxel resistance (12).

PRG1, which was highly expressed in the chemosensitive cells at all time points, has been shown to trigger apoptosis in HeLa cells (6) and to mediate the clearance of apoptotic cells by dendritic cells *in vivo*. Similarly, *ADFP*, which is a peroxisome proliferator–activated receptor γ target gene (13), has been shown to be involved in apoptotic processes in epithelial cells derived from cervical carcinomas (14), whereas MAGED1 is involved in the p75 neurotrophin receptor–mediated programmed cell death pathway (15). The increased expression in chemosensitive cells of LGALS1 is perhaps not surprising, given that it encodes a protein with antiproliferative function that may be associated with apoptosis in cancer cells (16). ALEX2, which is also elevated in chemosensitive cells, may play a role in tumor suppression (17). In general, these markers, which were elevated in chemosensitive cells, confer proapoptotic traits that contribute to defining the molecular phenotype underlying the ChemoFx data presented in Table 1.

Our data also highlight a number of other genes that are involved in DNA repair and maintenance of chromosome stability. For example, RECQL is a DNA helicase that acts to restore productive DNA replication following S-phase arrest and hence prevent subsequent genomic instability. RECQL expression was found to be elevated in chemosensitive cells, which is consistent with the previously documented observation that loss of DNA repair function has been correlated with resistance to a variety of DNA-damaging agents, including many anticancer drugs (18). Interestingly, chemosensitive cells were also found to overexpress ACVR1. Activin signaling may play a significant role in cell proliferation and ovarian tumor development. Furthermore, several ovarian cancer cell lines produce activin *in vitro*, and exogenous activin induced their proliferation (19–22). Paradoxically, the gene encoding the major vault protein is overexpressed in chemosensitive versus resistant cells. The major vault protein has been associated with drug resistance in cancer, and its overexpression has been suggested as a marker for chemoresistance in ovarian cancer (23). However, its use in this context has recently been called into question (24).

Our analysis of the transcriptional response to carboplatin at 2 hours revealed that very few robust alterations in gene expression occur during the short duration of this exposure. This likely reflects the fact that the temporal response to drug stimulus requires >2 hours to be manifested at the transcriptional level. Those genes whose expressions are altered following 2 hours of carboplatin exposure are generally poorly characterized. Exception include MTA1, overexpression of which has been associated with metastasis in ovarian cancer (25), and PCD2, which is involved in apoptosis (26).

The effects of carboplatin were much more pronounced after 72 hours and revealed alterations in the expression of a variety of genes. These include those encoding either proteins involved in cell proliferation (*CDC45L* and *CCNF*) or DNA replication and repair (*RFC4, MCM2, MCM4, UNG*, and *FEN1/RAD2*), which not surprisingly were coordinately elevated in chemoresistant cells. Also altered was the gene (*DSP*) that encodes the cell-cell adhesion protein desmoplakin. Desmoplakin is an intercellular junction protein in vertebrate epithelial cells that is able to form both membrane anchorage sites for intermediate-size filaments and interact with the plasma membrane of adjacent cells to mediate stable intercellular adhesion. Interestingly, a reduction in the DSP-encoded protein has been observed in drug-resistant breast cancer cell lines (27), and a reduction in expression of the related protein γ-catenin has been observed in cisplatin-resistant adenocarcinoma cell lines (28), although this was not apparent in our data.

The glycosylphosphatidylinositol-anchored ligand EFNA1 preferentially binds to the receptor tyrosine kinase

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EFNA2, and EFNA1 expression was elevated in chemoresistant versus chemosensitive cells in the absence of carboplatin and after 2 and 72 hours of carboplatin exposure. It has recently been shown, in a colon carcinoma cell line, that activation of EFNA2 by EFNA1 may trigger an indirect growth signal via phosphorylation of E-cadherin and β-catenin by overcoming contact inhibition (29). Similarly elevated in chemoresistant cells is FASN, which is almost universally up-regulated in human cancers and is thought to be involved in the development, maintenance, and enhancement of the malignant phenotype. FASN protein inhibition induces programmed cell death in human breast cancer cells (30). These observations are consistent with our own data and suggest that a relative reduction in FASN expression in chemosensitive cells may be involved in their apoptosis. Paradoxically, however, FASN expression has also been shown to induce apoptosis in a prostate carcinoma cell line (31).

GPX1 is one of the most important antioxidant enzymes in humans. It has been reported that the protein encoded by GPX1 is protective against CD95-induced apoptosis in cultured breast cancer cells, and that GPX1 overexpression increases resistance to toxic challenges. Furthermore, GPX1 executes intracellular inactivation of the carboplatin relative cisplatin, and detoxification of reactive oxygen species is a mechanism for cisplatin resistance (32). Paradoxically, GPX1 is elevated in carboplatin-sensitive cells in our data, suggesting that GPX1 activity is not an essential component of carboplatin resistance in ovarian cancer cells. Clearly, our observation that GPX1 expression is increased in chemosensitive versus chemoresistant ovarian cancer cells requires further investigation in the context of the previous data.

One gene of particular interest that we found to be up-regulated in chemoresistant versus chemosensitive cells is *H2AX*. This encodes a core component of chromatin that is phosphorylated in chromatin-flanking DNA double-strand breaks. It has recently been shown in mice that H2AX helps prevent aberrant repair of both programmed and general DNA breakage and thereby functions as a dosage-dependent suppressor of genomic instability and tumor formation (33). Similarly, Celeste et al. (34) recently showed that loss of a single *H2AX* allele compromises genomic integrity and enhances the susceptibility to cancer in the absence of p53. They report that H2AX acts as a ''genomic caretaker'' in protection against tumorigenesis. Although this evidence is not directly related to chemoresistance, it can plausibly be interpreted to suggest that H2AX overexpression leads to improved maintenance of genomic integrity in the face of carboplatin exposure.

The observation that IFI16 is overexpressed in chemosensitive cells is significant, because the *IFI16* gene product has recently been shown to negatively regulate p21/waf1, resulting in p53-dependent cell cycle arrest (35). Our finding that IFI16 mRNA is elevated in carboplatin-sensitive ovarian tumor cells suggests a possible mechanism whereby drug resistance is conferred, in part, via a reduction in IFI16 expression. For example, cisplatin increases p21/waf1 protein levels (36). If carboplatin has the same effect, elevated IFI16 could potentially counteract p21/waf1 induction, resulting in reduced apoptosis. Interestingly, low p21/waf1 protein levels have recently been shown to sensitize tumor cells, albeit not ovarian tumor cells, to apoptosis (37). However, p21/waf1 overexpression has been found to result in apoptosis in ovarian cancer cell lines (38), which would not support this hypothesis.

In keeping with the above results, and consistent with an association between cellular survival and chemoresistance, analysis of the temporal response to carboplatin in only the chemosensitive cells revealed a coordinated alteration in the expression levels of genes that are involved in DNA replication and/or maintenance. For example, FEN1, whose expression is reduced by carboplatin, is a member of the XPG/RAD2 endonuclease family and is one of 10 proteins essential for cell-free DNA replication. It processes the 5′ ends of Okazaki fragments in lagging strand DNA synthesis and is involved in DNA repair (39). Similarly down-regulated is RFC4, which is involved in the elongation of primed DNA templates by DNA polymerase δ and DNA polymerase epsilon (39) and KNTC2, which is one of several proteins involved in spindle checkpoint signaling. This surveillance mechanism assures correct segregation of chromosomes during cell division by detecting unaligned chromosomes and causing prometaphase arrest until the proper bipolar attachment of chromosomes is achieved. Depletion of KNTC2 has been shown to result in catastrophic mitotic exit (40), which is consistent with the expected cellular response to a cytotoxic compound such as carboplatin. Also down-regulated by carboplatin in sensitive cells was CDC6, which is essential for the initiation of DNA replication. The protein encoded by CDC6 functions as a regulator at the early stages of DNA replication and is rapidly destroyed in cells undergoing apoptosis (41). Finally, RRM2 overexpression is associated with gemcitabine chemoresistance in pancreatic adenocarcinoma cells; in addition, that suppression of RRM2 expression by small interfering RNA enhanced gemcitabine-induced cytotoxicity *in vitro* has been shown to result in markedly suppressed tumor growth, increased tumor apoptosis, and inhibition of metastasis (42). These observations are consistent with our finding that RRM2 is down-regulated by carboplatin in chemosensitive cells.

In summary, we present preliminary data that defines the transcriptome of cultured ovarian tumor cells that display either carboplatin sensitivity or resistance in an *ex vivo* assay of cell survival. Our data suggest that there is a comprehensive and coordinated alteration in gene expression that defines an apoptosis-resistant phenotype in chemoresistant cells. The genes identified in the present study have potential as markers of ovarian tumor chemosensitivity, both in the presence (and absence) of *ex vivo* carboplatin treatment. Although this is a preliminary investigation into the molecular phenotype of carboplatin resistance in ovarian cancer, it provides a platform of comprehensive and robust gene expression data that will form the basis of future investigation by ourselves and others.

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