# Remodeling of the extracellular matrix through overexpression of collagen VI contributes to cisplatin resistance in ovarian cancer cells

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## Summary

**The mechanisms of drug resistance in cancer are poorly understood. Serial analysis of gene expression (SAGE) profiling of cisplatin-resistant and sensitive cells revealed many differentially expressed genes. Remarkably, many ECM genes were elevated in cisplatin-resistant cells. *COL6A3* was one of the most highly upregulated genes, and cultivation of cisplatin-sensitive cells in the presence of collagen VI protein promoted resistance in vitro. Staining of ovarian tumors with collagen VI antibodies confirmed collagen VI expression in vivo and suggested reorganization of the extracellular matrix in the vicinity of the tumor. Furthermore, the presence of collagen VI correlated with tumor grade, an ovarian cancer prognostic factor. These results suggest that tumor cells may directly remodel their microenvironment to increase their survival in the presence of chemotherapeutic drugs.**

## Introduction

Resistance to anti-tumor drugs is common and represents a major cause of cancer death. This problem is particularly apparent in the treatment of ovarian cancer patients with cisplatin-based regimens where the majority of the patients eventually die of their disease with cisplatin-resistant tumors. Cisplatin forms intrastrand and interstrand crosslinks in DNA, which are believed to trigger cell cycle arrest and apoptosis in cancer cells (Pinto and Lippard, 1985; Kartalou and Essigmann, 2001). The molecular mechanisms underlying cisplatin resistance are only partially understood and are thought to be multifactorial (Kartalou and Essigmann, 2001). The most important molecular mechanisms believed to contribute to an increase in cisplatin resistance include decrease in intracellular concentration of cisplatin (Gately and Howell, 1993), interactions with inactivating molecules (Godwin et al., 1992; Kasahara et al., 1991), increased repair or tolerance to DNA adducts (Jones et al., 1994; Mamenta et al., 1994; Fink et al., 1998), and altered expression of modulating genes such as p53 (Fan et al., 1994) and c-*jun* (Potapova et al., 1997).

Recent technological developments such as cDNA arrays and serial analysis of gene expression (SAGE) have allowed large-scale gene expression analysis. These techniques have provided a wealth of information on various aspects of tumorigenesis, including cisplatin-induced changes in gene expression. A number of studies have been published that identify potentially novel targets and pathways important in cisplatin resistance of various cancers (Deng et al., 2002; Sakamoto et al., 2001; Anderson et al., 2002; Zembutsu et al., 2002).

Cisplatin is used for the treatment of a number of malignancies, including ovarian, lung, head and neck, bladder, and testicular cancers (Cohen and Lippard, 2001). It is the most efficacious agent against ovarian cancer with initial response rates varying from 40%–80%. Platinum-based combination therapy, especially cisplatin/paclitaxel, offers a modest but significant improvement over cisplatin alone, and this regimen is now standard for women with advanced ovarian cancer (Omura et al., 1986; McGuire et al., 1996). Unfortunately, a significant fraction of women with tumors that initially respond to chemotherapy eventually relapse with drug-resistant disease (Ozols and Young, 1984). Overall, fewer than 25% of the women diagnosed

SIGNIFICANCE

**Resistance to anti-tumor drugs is common and represents a major cause of cancer death. Although many mechanisms have been shown to be involved in resistance, the role of the microenvironment, and more specifically of the extracellular matrix, has received less attention. Here, we show that tumor cells can remodel their microenvironment through production of collagen VI and that the presence of collagen VI increases resistance of ovarian cells to chemotherapeutic agents. A better understanding of the tumor-ECM interactions and signaling may reveal novel avenues for treatment of drug-resistant tumors.**

with advanced ovarian cancer will show progression-free survival after 4 years, in spite of treatment (McGuire et al., 1996). In this context, a better understanding of cisplatin resistance may lead to the development of novel approaches for the treatment of ovarian and other cancers treated with cisplatin.

Interestingly, ECM has been suggested to provide protection against chemotherapy-induced apoptosis in small cell lung cancer and other cancers (Sethi et al., 1999; Hazlehurst and Dalton, 2001). It was suggested that upon activation by an ECM ligand, integrin-mediated signal transduction, including PTK-dependent mechanisms, can provide protection against apoptosis. In the microenvironment of the tumor, cancer cells and stroma cells interact to create favorable ECM interactions. The key components of the ECM in chemotherapy resistance and the mechanisms of ECM reorganization are not understood (DeClerck, 2000). In addition, it is currently unclear whether the stroma or the cancer cells themselves can produce the ECM components necessary for remodeling.

It is clear that a better understanding of the molecular mechanisms leading to cisplatin resistance may provide targets for agents that may modulate cisplatin resistance and increase the effectiveness of cisplatin chemotherapy. In order to address this question, we have generated ovarian cancer cell lines resistant to cisplatin through intermittent exposure to cisplatin, conditions similar to the development of cisplatin resistance in patients. Using SAGE, we report the identification of many genes differentially expressed as a result of cisplatin resistance. Genes encoding ECM proteins were particularly striking, and we show that collagen VI, an ECM protein that has been ascribed growth factor-like properties, is highly upregulated in cisplatin-resistant cells and can significantly increase resistance to cisplatin in ovarian cancer cells in vitro. Importantly, staining of ovarian tumors with a collagen VI antibody reveals areas of high collagen VI concentration in the microenvironment of the tumor cells and shows that collagen VI is expressed by the tumor cells. Collagen VI RNA levels in tumors were associated with tumor grade, a prognosis factor in ovarian cancer. These findings suggest that tumor cells can modulate their microenvironment to favor survival in the presence of chemotherapeutic drugs.

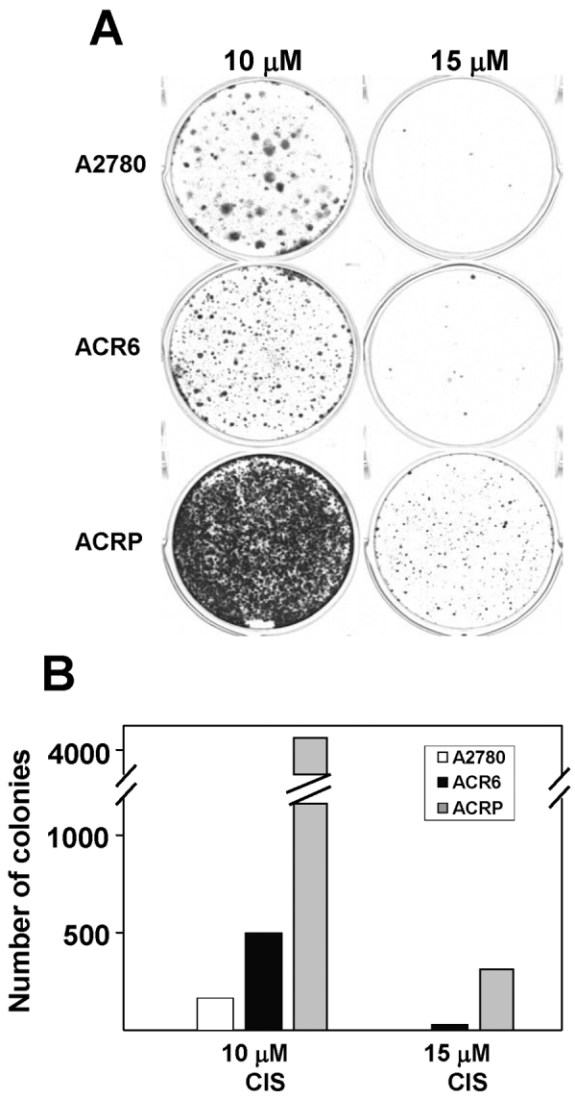


**Figure 1.** Characterization of cisplatin-resistant clones ACRP and ACR6 by clonogenic assays

**A:** Surviving colonies of a typical experiment are shown for the cell lines A2780, ACR6, and ACRP following treatment with 10 or 15 μM cisplatin.
**B:** Colonies from (**A**) were counted and plotted for the various lines at 10 uM and 15 uM cisplatin.

## Results

### Generation of cisplatin-resistant lines

In order to obtain perfectly matched cisplatin-sensitive and -resistant ovarian cancer cells, we generated these lines in our laboratory. In addition, rather than passaging the lines continually in high concentrations of cisplatin, the parental A2780 ovarian cancer line was treated intermittently with cisplatin for 24 hr and then expanded in the absence of the drug. We reasoned that this more closely resembles the treatment leading to the formation of cisplatin-resistant tumors in patients. Of the ten clones obtained after three rounds of cisplatin selection, two of the clones, ACR6 and ACRP, exhibited significant increase in cisplatin survival compared to the A2780 parental line. Indeed, after treatment with 10 μM cisplatin, the ACRP line yielded approximately 30 times more colonies compared to A2780 (Figures 1A and 1B). ACR6 had a more modest resistance level with approximately 3-fold increase in surviving colonies. Although the number of colonies was lower overall, this trend was similar following 15 μM cisplatin treatment, where ACR6 and ACRP exhibited 3-fold and 60-fold increases in colony formation, respectively. Through a series of clonogenic assays with a wide range of drug concentration, it was determined that the cisplatin $IC_{50}$ for A2780 and ACRP were 0.75 μM and 3 μM, respectively (data not shown). Finally, we tested whether cisplatin resistance was associated with crossresistance to other chemotherapeutic agents. Similar experiments were thus repeated in order to evaluate the resistance of A2780 and ACRP to doxorubicin and taxol. We found that ACRP cells were more resistant to both these agents, although the resistance to taxol was less pronounced (see Supplemental Data at http://www.cancercell.org/cgi/content/full/3/4/377/DC1).

We believed the ACRP cell line to be particularly interesting for many reasons. First, it was selected from a pool of clones and subsequently reselected with cisplatin, in a fashion similar

to the development of resistance in vivo. Secondly, ACRP exhibited a much higher level of cisplatin resistance than ACR6. Finally, ACRP acquired co-resistance to other agents, as is often observed in tumors from patients undergoing chemotherapy.

### Identification of genes differentially expressed using SAGE

In an attempt to identify molecular determinants underlying cisplatin resistance, we used SAGE to obtain gene expression profiles of A2780 and ACRP. SAGE is a powerful quantitative technique that allows determination of gene expression profiles in tissues of interest (Saha et al., 2002; Velculescu et al., 1995). The SAGE libraries were constructed and over 30,000 tags were obtained for each of the two libraries. After correction for sequencing errors (Zhang et al., 1997), a total of approximately 15,000 unique genes were identified in each of the libraries. The libraries were clearly similar: a scatter plot of the tag frequency in the libraries demonstrated a high degree of similarity with a Pearson correlation coefficient of 0.75 (Figure 2A). However, in spite of the high degree of similarity, many genes were significantly ($p < 0.05$) differentially expressed between these lines and are indicated as solid diamonds in the scatter plot (Figure 2A).

Genes differentially expressed between A2780 and ACRP ($p < 0.05$) are shown in Table 1 and Table 2. Genes with a high level confidence differential expression ($p < 0.001$) are indicated in italics in these tables. All the genes listed were divided into functional categories in an attempt to clarify the possible mechanisms behind ACRP's drug resistance (Figure 2B). A large number of both the down and upregulated genes fell into three main functional categories. These are cell cycle control/apoptosis (cyclin I, growth arrest-specific 2 like 1), metabolism/energy homeostasis (NADH dehydrogenase subunit 1, creatine kinase), and DNA repair/modification enzymes (RAD23 homolog B, DNA polymerase 1). Interestingly, the SAGE data exhibited evidence of known mechanisms of chemotherapeutic resistance such as cell cycle control and DNA repair, but five genes involved in ECM structure and/or function were also found upregulated in ACRP. The involvement of ECM proteins in anticancer drug resistance has not been extensively studied. Four of the genes encode protein products that are secreted into the ECM (collagen XI, collagen VI, cartilage linking protein 1, decorin) and PAPSS functions in posttranslational modification (sulfate esterification of polysaccharides) of proteoglycans of the ECM. The downregulated genes included some genes encoding proteins involved in chromatin structure/remodeling (chromodomain helicase DNA binding protein and H1 histone), as well as products involved in protein synthesis (Figure 2B, Table 2).

### Validation of differentially expressed genes

Many genes identified by SAGE as differentially expressed were selected for validation by semi-quantitative real-time RT-PCR using the SYBR green I assay. The validation process was also an opportunity to extend the SAGE finding to ACR6, a cell line also resistant to cisplatin but to a much lesser degree. Because many genes of the ECM appeared upregulated in cisplatin-resistant ACRP and because of the lack of precedent for the involvement of these genes in cisplatin resistance, they were chosen for validation by semi-quantitative real-time RT-PCR. Primers were designed for RT-PCR of *COL6A3*, *COL11A1*, *DCN*, *CRTL1,* and *PAPSS*, and the expression levels of these genes were measured in A2780, ACR6, and ACRP. RT-PCR confirmed the levels of upregulation obtained by SAGE (Figure 3A). *DCN* and *PAPSS* were also found upregulated in ACR6. Among the genes downregulated in cisplatin-resistant cells, *FADS*, *FLNA*, and *CRABP* were selected for validation. These genes were indeed found downregulated in ACRP and ACR6 compared to A2780 (Figure 3B).

### Collagen VI increases cisplatin resistance

Because two different types of collagen were found upregulated in cisplatin-resistant cells (Table 1) and because collagens had previously been found generally upregulated in advanced ovarian cancer (Ismail et al., 2000; Hough et al., 2000), we hypothesized that collagen by itself might play a role in providing drug resistance to these tumors. This is particularly interesting, as little is known about the involvement of collagens in cancer drug resistance. *COL6A3* was one of only eight genes to be found upregulated in ACRP with $p < 0.001$. Human collagen VI protein was therefore used to coat tissue culture dishes to directly test its effects on cisplatin resistance. A2780 parental cells grown on collagen VI were found to be significantly more resistant to cisplatin than cells grown on untreated culture dishes at all cisplatin concentrations tested (Figure 4A). This phenomenon did not depend on the concentration of serum in the medium and was observed with media containing 1% or 10% serum during cisplatin treatment. Although plating on collagen I provided an increase in colony-forming ability in the absence of cisplatin, this phenomenon was not sufficient to explain the approximately 15-fold increase observed in cisplatin resistance at 1.0 μM on collagen VI in 1% serum (Figure 4B). While we observed the same pattern of resistance at higher concentrations of cisplatin, these experiments were performed at concentrations varying between 0.5 and 2 μM, a range more likely to be physiologically relevant and close to the $IC_{50}$ of A2780 (0.75 μM). In addition, while collagen I provided some protection against cisplatin treatment, the effect was much more pronounced with collagen VI, especially at higher cisplatin concentrations. The cisplatin resistance was not simply due to increased proliferation of the cells in the presence of collagen VI. Indeed, cells plated on collagens without cisplatin treatment did not exhibit increased proliferation as measured by MTS (Figure 4C). On the other hand, the resistance level of ACRP, a cell line overexpressing collagen VI, was not significantly affected by the presence of collagen VI (data not shown). This result with ACRP cells shows that the increase of cisplatin resistance does not represent a nonspecific effect that occurs in the presence of collagen regardless of the cell tested but is most pronounced in cisplatin-sensitive cells expressing low levels of collagen VI. A2780 cells were indeed shown to express and secrete less collagen VI protein compared to ACRP (see Supplemental Data on *Cancer Cell* website).

### Collagen VI is expressed in primary ovarian tumors

The previous results demonstrate that collagen VI can be expressed by ovarian tumor cells in vitro and promotes cisplatin resistance. In order to assess collagen VI expression in ovarian tumors in vivo, a panel of RNAs obtained from microdissected ovarian tumors (Hough et al., 2001) as well as our ovarian tumor microarray (Rangel et al., 2003) were analyzed by semi-quantitative real-time RT-PCR and immunofluorescence. Semi-quantitative RT-PCR analysis of collagen VI in the microdissected ovar-

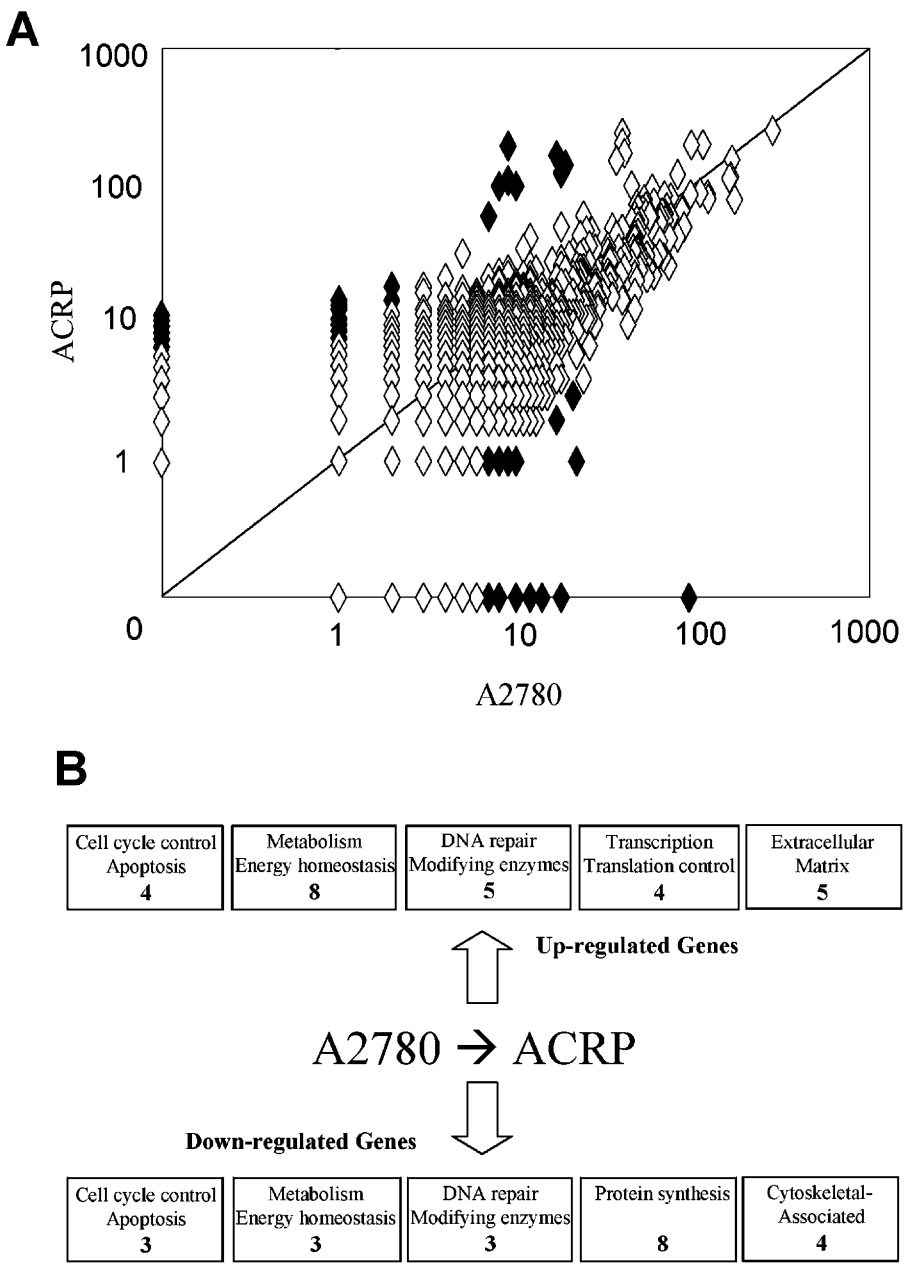


**Figure 2.** Many genes are differentially expressed between A2780 and the cisplatin-resistant cell line ACRP

**A:** Tag frequency scatter plot of ACRP versus A2780. The frequency of each tag (per 30,000) is plotted on a logarithmic scale. Tags that are significantly differentially expressed in ACRP compared to A2780 ($p < 0.05$) are indicated in black. Some diamonds may represent multiple tags that are expressed at identical levels.

**B:** Genes differentially expressed between A2780 and ACRP are grouped into main functional categories. The number of genes in each category is indicated.

ian tumors of various subtypes confirmed the expression of this gene in vivo. The levels of expression were highly and consistently elevated in all four major subtypes of ovarian cancer (serous, endometrioid, mucinous, and clear cell) when compared to cultures of human ovarian surface epithelial cells (Figure 5A). Interestingly, the levels of *COL6A3* expression in tumors were comparable to the levels observed in the ACRP cell line. *COL6A3* levels were most variable within the serous subtype, which led us to wonder whether *COL6A3* levels might be associated with other properties of these tumors. Interestingly, we found that the expression of *COL6A3* was associated with the grade of tumors. Highly or moderately differentiated (low grade) tumors expressed lower levels of *COL6A3* on average than poorly differentiated (high grade) tumors ($p = 0.018$) (Figure 5). This is particularly apparent as almost half of the poorly differentiated tumors expressed *COL6A3* above an arbitrary level of 325-fold (dotted line on Figure 5B) while none of the more differentiated tumors expressed this gene above that level. This correlation was particularly intriguing considering that ovarian tumor grade has previously been associated with response to therapy and overall patient survival (Ozols et al., 1980; Makar et al., 1995; Shimizu et al., 1998).

In order to show that collagen VI was present at the protein level in primary ovarian tumors, our ovarian tissue array was stained using a collagen VI antibody and Cy5-conjugated secondary antibody. Many of the tumors studied were positive for collagen VI. In these tumors, cytoplasmic staining of the tumor cells as well as extracellular staining in their immediate vicinity were frequently observed (Figures 6A and 6B). The staining sometimes extended somewhat into the stroma of the tumors

**Table 1.** Genes upregulated in cisplatin-resistant cells

| Transcript Tag[a] | Fold[b] | Gene | Location | Accession |
|---|---|---|---|---|
| **ACCCTTGGCC** | 22.6 | NADH dehydrogenase subunit 1 (*MTND1*) | Mito | XM_166858 |
| CAAAATCAGG | 15.0 | cyclin I (*CCNI*) | 4q13 | NM_006835 |
| ATTTTTTCAA | 14.0 | 3′-phosphoadenosine 5′-phosphosulfate synthase 1 (*PAPSS1*) | 4q24 | NM_005443 |
| GCAAAAGCTT | 13.0 | collagen, type XI, alpha 1 (*COL11A1*) | 1p21 | NM_001854 |
| **CACTACTCAC** | 12.9 | cytochrome b gene (*MTCYB*) | Mito | AF172368 |
| **ACTAACACCC** | 12.6 | NADH dehydrogenase subunit 2 (*MTND2*) | Mito | AF014899 |
| **ACTTTAGATG** | 12.0 | collagen, type VI, alpha 3 (*COL6A3*) | 2q37 | NM_004369 |
| **ATTGTGAGGC** | 11.0 | FLJ31373 | 9q31 | AK024090 |
| GCCCGCAAGC | 11.0 | bromodomain-containing 4 (*BRD4*) | 19p13 | NM_014299 |
| **CCTCAGGATA** | 10.3 | NADH dehydrogenase subunit 6 (*MTND6*) | Mito | AF510390 |
| **CTAAGACTTC** | 10.2 | mitochondrial match | Mito | |
| AAGATGAGGG | 10.0 | small nuclear ribonucleoprotein polypeptide B (*SNRPB2*) | 20p11 | NM_003092 |
| GTAGCAGGTG | 10.0 | mannose 6 phosphate receptor binding protein (*TIP47*) | 19p13.3 | XM_012862 |
| GTTTGGCAGT | 10.0 | hypothetical protein H41 (*H41*) | 3q22.2 | AF103803 |
| CATTTGTAAT | 9.5 | cytochrome c oxidase subunit I (*MTCO1*) | Mito | AF382012 |
| AAGCCTTGCT | 9.0 | HT027 (*FLJ20886*) | 17q25.3 | AF246238 |
| CTCACTTCTT | 9.0 | EST: clone 25076 | 3 | AF131807 |
| CTTGACACAC | 9.0 | eukaryotic translation initiation factor 5 (*EIF5*) | 14q32 | NM_001969 |
| **GAAGTTATAA** | 8.9 | paternally expressed 10 (*PEG10*) | 7q21 | NM_015068 |
| ATTTGAGAAG | 7.8 | RAD23 homolog B (*RAD23B*) | 9q31 | NM_002874 |
| CAATAGCTTA | 7.5 | cartilage linking protein 1 (*CRTL1*) | 5q14 | U43328 |
| GCAAGCCAAC | 7.5 | NADH dehydrogenase subunit 4 (*MTND4*) | Mito | NC_001807 |
| CACCTAATTG | 7.2 | ATP synthase 6 (*MATP6*) | Mito | AF368271 |
| AAAAGATACT | 7.0 | CBP/p300-interacting transactivator 2 (*CITED2*) | 6q23 | NM_006079 |
| AAGTTTCCAA | 7.0 | protein phosphatase 4 catalytic subunit (*PPP4C*) | 16p12 | NM_002720 |
| ACAGCTAATT | 7.0 | EUROIMAGE 39515 mRNA | X | AL079283 |
| ACTTATTATG | 7.0 | decorin (*DCN*) | 12q13.2 | NM_001920 |
| CGGTCTTATG | 7.0 | dual-specificity tyr-phospho regulated kinase 1A (*DYRK1A*) | 21q22 | NM_130436 |
| CTCAATGGCG | 7.0 | sal-like 4 (*SALL4*) | 20q13 | NM_020436 |
| GCCAACCTCC | 7.0 | NADH dehydrogenase subunit 1 (*MTND1*) | Mito | XM_166858 |
| TCACAATACA | 7.0 | cyclophilin D (*PPID*) | 4q31 | NM_005038 |
| TGAAGTAACA | 7.0 | putative translation initiation factor (*SUI1*) | 19p13 | NM_005801 |
| TGCAATAAGC | 7.0 | DKFZp434I0835 | ? | AL122072 |
| TGTTAGCAAA | 7.0 | EST | 11p13 | Z40582 |

[a] Tags in bold have a $p < 0.001$.
[b] The listed fold values are relative to A2780.

but the stromal areas and the normal ovarian surface epithelium were negative. This yielded a pattern of uniform staining in the tumor areas with collagen VI gradually decreasing away from the tumor cells to become undetectable in stromal area (Figures 6A and 6B). This pattern of staining is consistent with tumor cell secretion of collagen VI, leading to a remodeling of the ECM in the microenvironment of the tumors. Consistent with our semi-quantitative real-time RT-PCR findings, some of the tumors exhibited little or no detectable collagen VI staining (Figure 6C). Overall, 52% of the serous ovarian tumors exhibited positive collagen VI staining as measured by immunofluorescence. Highly differentiated, low grade tumors frequently did not express collagen VI, but in contrast to our observation using RT-PCR, no correlation between collagen VI protein and grade was observed. This could be due to the difficulties associated with quantification of immunofluorescence results. It will be important to study collagen VI expression in a large cohort of patients treated with cisplatin in order to tease out possible differences in chemotherapy response associated with collagen VI expression.

## Discussion

Platinum-based combination therapy is standard chemotherapy for ovarian cancer. Unfortunately, most tumors are intrinsically resistant or acquire resistance during treatment. The mechanisms of development of cisplatin resistance in vivo are poorly understood. Several models have been proposed but it remains unclear which process is sufficient for the development of drug-resistant tumors in patients and whether different tumors can use different strategies to develop resistance. Many studies have identified differential response of resistant cells to cisplatin challenge but little work has been done to identify intrinsic differences in cisplatin-resistant cells in the absence of drug. We reasoned that the resistant cells must possess inherent differences in gene expression and molecular circuitry, even in the absence of drugs, that will allow them to respond more efficiently to cisplatin exposure. In addition, we chose to generate a cisplatin resistance model using repeated short exposures to cisplatin, a situation similar to the type of natural selection that occurs in patients during chemotherapy. The cell lines generated exhibited significant increases in survival to cisplatin challenges.

Because we sought highly quantitative gene expression profiles, we chose SAGE to study our cisplatin resistance model. Of the approximately 18,000 tags representing transcripts expressed at levels higher than 0.01%, only 67 were found to be significantly different ($p < 0.05$). When a p value of 0.001 was used, only 16 genes were differentially expressed (see Tables 1 and 2). These results show that, despite exhibiting a significant

**Table 2.** Genes downregulated in cisplatin-resistant cells

| Transcript Tag[a] | Fold[b] | Gene | Location | Accession |
|---|---|---|---|---|
| **TTGGTGAAGG** | 95.0 | thymosin, beta 4 (*TMSB4X*) | Xq21.3 | NM_021109 |
| **CTGTTGGCAT** | 22.0 | ribosomal protein L21 (*RPL21*) | 10q26.13 | NM_000982 |
| **GTGAGCCCAT** | 18.0 | heat shock 90kD protein 1, beta (*HSPCB*) | 6p12 | NM_007355 |
| **TGGAGAATGT** | 14.0 | integrin beta-1 CD29; Fibronectin receptor (*ITGB1*) | 10p11.2 | NM_002211 |
| **TTACTTCCCC** | 14.0 | fatty acid desaturase 2 (*FADS2*) | 11q12 | NM_004265 |
| **CTCACCGCCC** | 12.0 | cellular retinoic acid-binding protein 2 (*CRABP2*) | 1q21.3 | NM_001878 |
| **GAATGAAGCT** | 12.0 | ESTs | ? | AA725362 |
| **TGAAGTTATA** | 12.0 | integrin beta-1 CD29; Fibronectin receptor (*ITGB1*) | 10p11.2 | NM_002211 |
| ATGGCGATCT | 10.0 | ribosomal protein S24 (*RPS24*) | 10q22 | NM_001026 |
| CTGTCCTTGT | 10.0 | thioredoxin related protein (MGC3178) | 6p25.2 | NM_030810 |
| GAAATACAGT | 10.0 | Cathepsin D (*CTSD* ) | 11p15.5 | NM_001909 |
| GCACCTCAGC | 10.0 | over-expressed breast tumor protein (*OBTP*) | 6p21.3 | NM_017601 |
| TACGTACTGC | 10.0 | translocase inner mito memb. 13 homolog (*TIMM13*) | 19p13.3 | NM_012458 |
| CACCCCTGAT | 9.0 | creatine kinase, brain (*CKB*) | 14q32 | L47647 |
| CCTGAACTGG | 9.0 | chromodomain helicase DNA binding protein 4 (*CHD4*) | 12p13 | NM_001273 |
| CGGCTGAATT | 9.0 | phosphogluconate dehydrogenase (*PGD*) | 1p36 | BC000368 |
| GCCCAAGGAC | 9.0 | filamin A, alpha (*FLNA*) | Xq28 | XM_048404 |
| GCCCCTGCCT | 9.0 | aminopeptidase B (*RNPEP*) | 1q32 | XM_036500 |
| TCTGGTTTGT | 9.0 | transmembrane trafficking protein (*TMP21*) | 14q24 | NM_006827 |
| TGCTGCCCTG | 9.0 | v-myb oncogene homolog-like 2 (*MYBL2*) | 20q13.1 | NM_002466 |
| CTTAAGGATT | 8.5 | PAI-1 mRNA-binding protein (*PAI-RBP1*) | 1p31-p22 | NM_015640 |
| CAGGCCCCAC | 8.0 | S100 calcium-binding protein A11 (*S100A11*) | 1q21 | NM_005620 |
| GGGTCTGCGG | 8.0 | copine VII (*CPNE7*) | 16q24.3 | NM_014427 |
| TCTTGATATT | 8.0 | collagen, type V, alpha 2 (*COL5A2*) | 2q14-q32 | NM_000393 |
| TGTGTGTTTG | 8.0 | H1 histone family, member 0 (*H1F0*) | 22q13.1 | BC000145 |
| CCCACGGTTA | 7.0 | methionine-tRNA synthetase (*MARS*) | 12q13.2 | NM_004990 |
| CTGTGCCCAG | 7.0 | ribosomal protein L30 (*RPL30*) | 8q22 | NM_000989 |
| GCACAATGGG | 7.0 | Growth arrest-specific 2 like 1 (*GAS2L1*) | 22q12.2 | NM_006478 |
| GGGCGGGGGC | 7.0 | DNA polymerase delta 1, (*POLD1*) | 19q13.3 | NM_002691 |
| GGTGAGACAC | 7.0 | solute carrier family 25 (*SLC25A6*) | Xp22;Yp | XM_114724 |
| TACTGGCCGC | 7.0 | ESTs | 6q22.33 | BG168419 |
| TCCCCGTACC | 7.0 | No matches | | |
| TTTGTCTGCT | 7.0 | No matches | | |

[a] Tags in bold have a $p < 0.001$.
[b] The listed fold values are relative to A2780.

difference in cisplatin response, these cell lines have very similar expression profiles. Importantly, many genes identified by SAGE were confirmed by semi-quantitative real-time RT-PCR and exhibited similar fold differences as found by SAGE.

Among the genes with significantly altered expression patterns between cisplatin-sensitive and cisplatin-resistant cells, many were predicted to encode proteins involved in ECM organization and/or signaling. This is particularly interesting since many collagen genes, including *COL6A3*, have previously been shown to be upregulated in ovarian cancer (Ismail et al., 2000). Collagen IV has been reported increased in the tumor microenvironment and has been hypothesized to be important for ECM-tumor interactions (Lohi et al., 1998; Sethi et al., 1999). In addition, the presence of ECM components and related signaling can reduce cell susceptibility to apoptosis and might be responsible for increased chemotherapeutic drug resistance in small cell lung cancer (Sethi et al., 1999; Rintoul and Sethi, 2001). From our study, one of the most highly upregulated genes in cisplatin-resistant cells is *COL6A3*, a gene encoding a chain of collagen VI, a microfibrillar collagen that contains subdomains similar to the type A domains of the von Willebrand factor, fibronectin type 3 domains, and a C-terminal Kunitz-type module (Mayer et al., 1994). Collagen VI is believed to be involved in cell anchoring as well as signaling through interactions with integrins (Pfaff et al., 1993) and possibly other receptors such as NG2 (Nishiyama and Stallcup, 1993) and DDR1/2 (Vogel et al., 1997). Collagen VI has been shown to bind to several proteins of the ECM, including decorin (Wiberg et al., 2001), another protein identified as upregulated in our study (Table 1). Collagen VI-decorin interactions may be important for the increased drug resistance that we observed.

When parental cells were exposed to purified collagen VI, we observed a specific and significant increase in cisplatin survival (Figure 4). Collagen VI was recently shown to inhibit apoptosis through reduction of Bax, and this effect was not observed with collagen I (Ruhl et al., 1999). There is therefore a precedent for collagen VI to affect signaling pathways important in survival and proliferation. It is still unclear whether these effects are mediated through the integrins or through other receptors. It will be important to determine the exact molecular pathways affected by collagen VI that influence the response of ovarian cancer cells to cisplatin. ECM proteins, and in particular collagen VI, may increase cisplatin resistance through their ability to regulate chemotherapy-induced apoptosis.

It is a well-accepted idea that the tumor microenvironment, and in particular the ECM, can have a profound effect on tumor cell sensitivity to cytotoxic stresses (Sutherland and Durand, 1972; Dalton, 1999; Sethi et al., 1999). Two forms of microenvironment-tumor interactions that can affect tumor cell sensitivity have been described: the interaction with soluble factors secreted by stromal cells and the direct interaction with ECM. A classic example of the first scenario is the secretion of IL-6 by

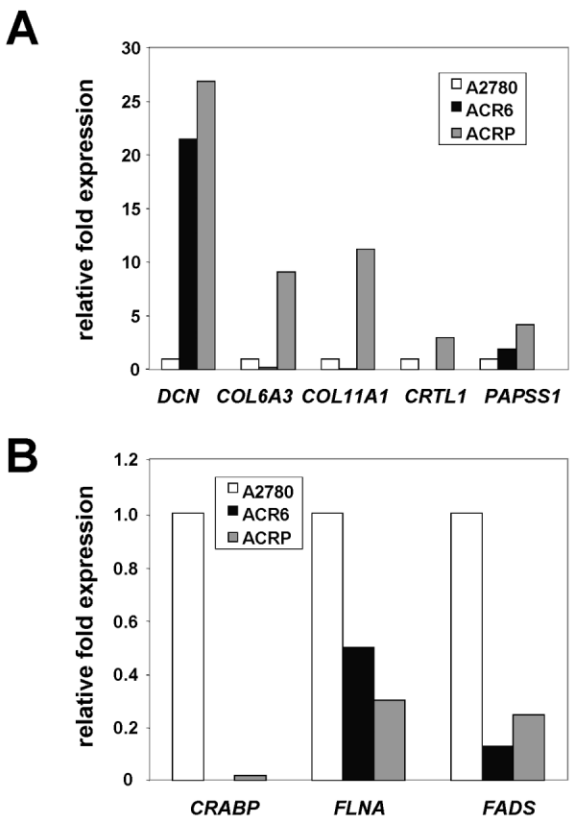


**Figure 3.** Semi-quantitative real-time RT-PCR validation of differentially expressed genes

**A:** Validation of selected upregulated genes in ACRP. The y axis represents the fold upregulation of the indicated genes in the cisplatin-resistant cell lines compared to parental A2780 cells.

**B:** Validation of selected downregulated genes in ACRP. The y axis represents the level of expression of the indicated genes in the cisplatin-resistant cell lines ACR6 and ACRP compared to parental A2780 cells.

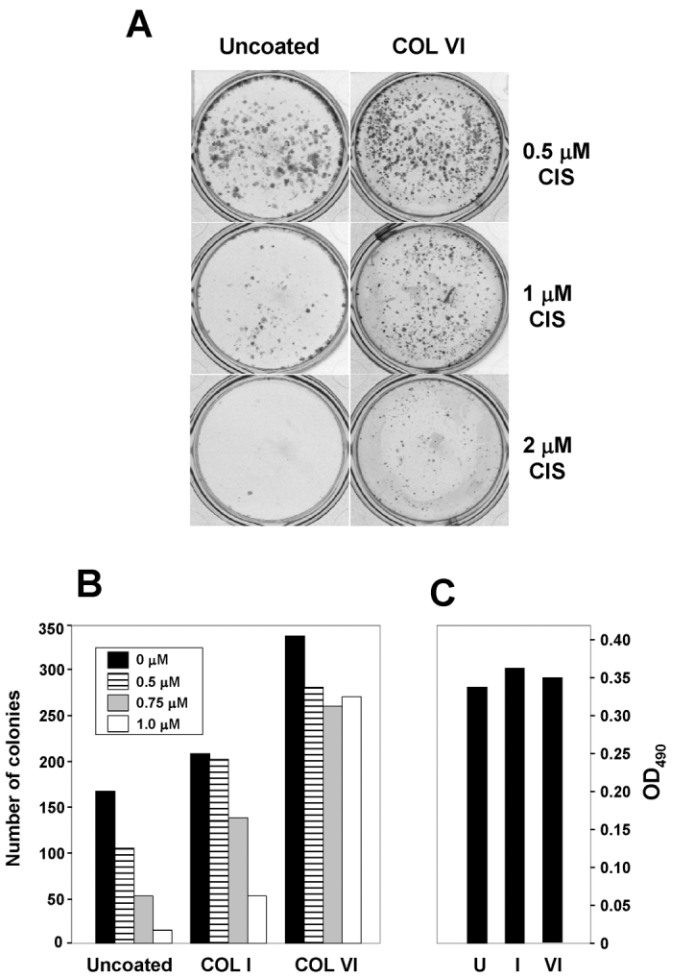


**Figure 4.** Collagen VI protein specifically increases cisplatin resistance of parental ovarian cancer cell line A2780

**A:** A2780 and ACRP cells were plated in 12-well dishes previously coated with collagen I, collagen VI, or left uncoated. Typical wells of A2780 clonogenic assays after treatment with the indicated concentrations of cisplatin for 24 hr are shown.

**B:** The results of a clonogenic assay of A2780 cells plated on various collagen substrates after cisplatin treatment (0, 0.5, 0.75, 1.0 μM) are shown. Number of surviving colonies are indicated.

**C:** MTS proliferation assays were performed on cells plated on collagen I (I), collagen VI (VI), or left uncoated (U).

stromal cells in the bone marrow that can affect drug response in myeloma cells (Lotem and Sachs, 1992). The second situation is exemplified by a large number of studies demonstrating that ECM and adhesion can significantly increase the resistance of tumor cells to anticancer agents (St Croix et al., 1996; Sethi et al., 1999; Damiano et al., 1999). In this report, we show that tumor cells themselves may play an active role in remodeling their microenvironment in order to maximize their survival in the presence of cytotoxic agents. In addition, in such circumstances, tumors expressing ECM proteins might have the ability to form drug-resistant distant metastases by taking advantage of optimal ECM-tumor cell interactions.

The fact that secreted factors may contribute to chemotherapeutic drug resistance has interesting clinical implications. In this scenario, only a fraction of the tumor population would need to secrete appropriate factors, creating a field effect. This mechanism would favor an increase in drug resistance in existing tumors by avoiding the requirement for a clonal expansion of a small number of resistant cells. Clonal expansion and field effect are clearly not mutually exclusive, and both mechanisms may be at work during treatment of cancer patients with chemotherapeutic drugs. Furthermore, it is likely that the drug response of a particular tumor is a result of a combination of many factors interacting in complex fashion. For example, increased drug inactivation, increased DNA repair, and other factors may all have additive or synergistic roles in cisplatin drug resistance. It will be important to perform detailed studies on the tumors of patients that respond to various degrees to chemotherapy in order to sort out these issues. The generation of mouse models of drug resistance may also help to resolve these questions.

In this paper, we show that ECM, and more specifically ECM remodeling through increased collagen VI expression by the tumor cells, may contribute to drug resistance. In this regard, it is particularly intriguing that *COL6A3* expression in primary ovarian tumors appears correlated with tumor grade, a marker that has been associated with ovarian cancer response to che-

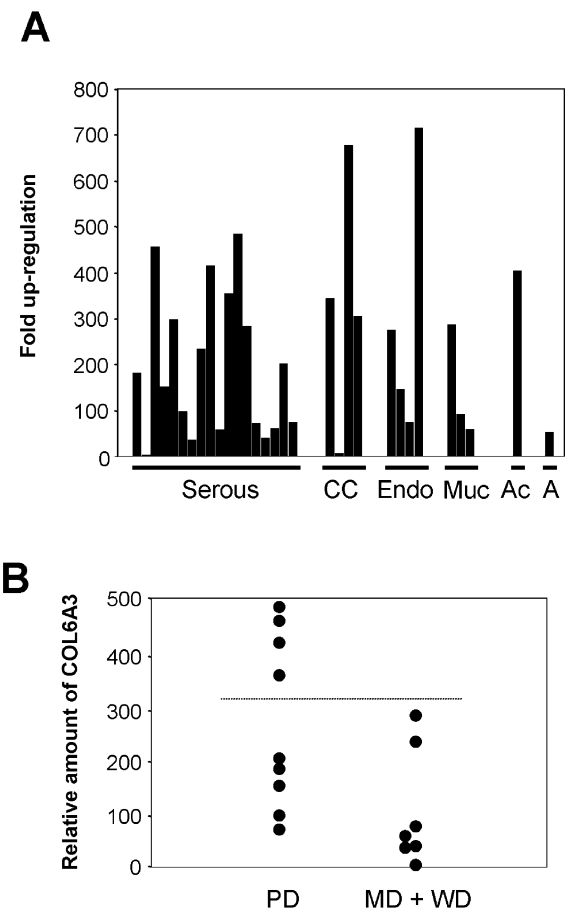


**Figure 5.** *COL6A3* expression in microdissected ovarian tumors

**A:** Fold overexpression of *COL6A3* compared to an ovarian surface epithelial culture is shown for ovarian tumors of various subtypes: serous, mucinous (Muc), endometrioid (Endo), and clear cell (CC). ACRP (Ac), the cisplatin-resistant cell line generated in this study, is included for comparison, along with A2780 (A), the parental line.

**B:** Expression levels of *COL6A3* in serous ovarian tumors as a function of tumor grade. The dotted line represents an arbitrary value emphasizing the difference in expression levels between poorly differentiated (PD) and moderately to well differentiated (MD + WD) ovarian serous tumors. The p value for PD versus MD + WD is 0.018.

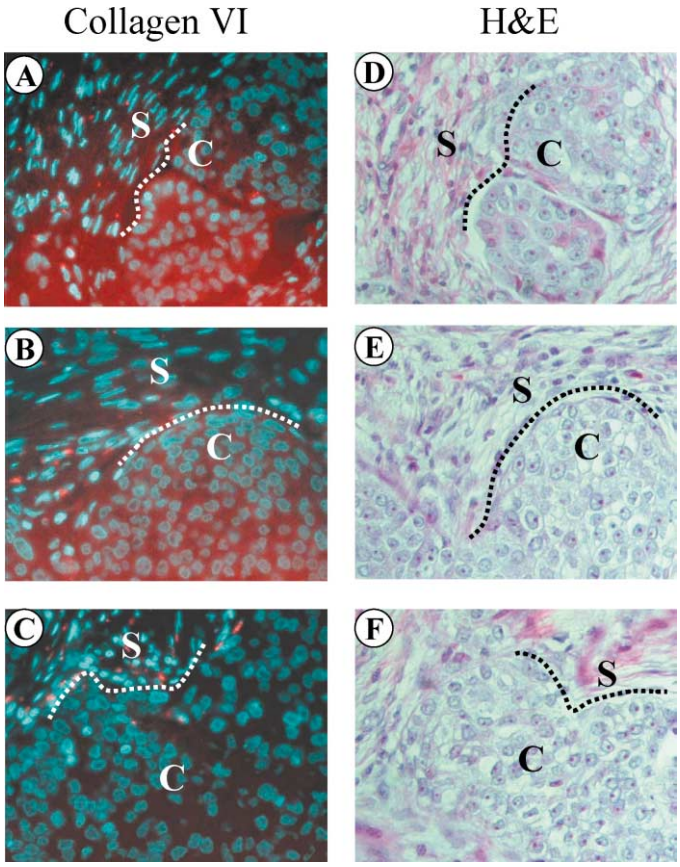


**Figure 6.** Collagen VI staining of human ovarian cancers

Cy-5-conjugated antibody against collagen VI was used to analyze ovarian tumor sections. H&E staining for three different representative tumors (**D–F**) is shown as well as the corresponding collagen VI staining (**A–C**). Red Cy-5 staining is apparent in panels (**A**) and (**B**). Tumor photographed in panel (**C**) did not exhibit collagen VI staining. Collagen VI is frequently overexpressed in the vicinity of the cancer cells but absent in the stromal areas. The approximate demarcations between stroma (S) and cancer (C) are shown by broken lines.

motherapy and overall survival. The increased drug resistance provided by the remodeled ECM may play a role in the failure of cancer therapy. Strategies involving inhibition of ECM-tumor interactions or resulting signaling may therefore improve efficiency of standard chemotherapy and may represent a promising new approach for cancer treatment.

### Experimental procedures

#### Generation of cisplatin-resistant cell lines

The cisplatin-sensitive serous adenocarcinoma cell line A2780 was generously provided by Dr. Vilhelm Bohr. This cell line was used to generate the cisplatin-resistant cell lines ACR6 and ACRP. A2780 was first treated with 5 μM cisplatin (Sigma, St. Louis, Missouri) for 24 hr followed by a recovery period. The remaining colonies were trypsinized, pooled, and passaged into several flasks until they were 90% confluent. The cisplatin treatment was repeated twice, once with 5 μM before a final exposure to 10 μM cisplatin. After the last treatment, nine individual colonies were subcloned and expanded (ACR1-9) along with another set, which were pooled and passaged together (ACRP). These clones were then tested individually for resistance to various chemotherapeutic drugs by a clonogenic assay. All the cell lines were grown in RPMI with 10% FBS and 4 μg/ml bovine insulin (Invitrogen Life Technologies, Carlsbad, California).

#### Clonogenic assays and growth on collagen

Cells were counted and plated in 6-well culture dishes. The cells were allowed to recover for 16 hr and then were treated for 24 hr with the indicated concentration of cisplatin (Sigma) or other chemotherapeutic agents (taxol and doxorubicin). The drug was removed and surviving cells were left to form colonies. This protocol was found to yield more reproducible results compared to methods involving the replating of cells following drug treatment. Colonies were stained with 0.25% Crystal violet/20% ethanol for 5 min, washed with HBSS, and allowed to dry. The well images were captured with a CCD camera and colonies were counted with the MCID M4 image analysis system version 3.0 (Imaging Research, Inc., St. Catharines, Ontario).

To study the effects of collagen, 12-well dishes were either coated with 10 μg of human placental collagen I or VI (Rockland Immunochemicals, Inc., Gilbertsville, Pennsylvania) in 250 μl of PBS or left uncoated. The different lines were plated at a density of $4 \times 10^5$ cells/well. 16 hr later, the cells were treated for a period of 24 hr with varying concentrations of cisplatin in the presence of 1% or 10% FBS. Cisplatin was removed, cells were washed

with HBSS, and surviving cells were cultivated in RPMI media containing 10% FBS until colonies could be counted.

**Serial analysis of gene expression (SAGE)**

Total RNA was obtained from guanidinium isothiocyanate cell lysates by centrifugation on CsCl. Polyadenylated mRNA was purified from total RNA obtained from A2780 and ACRP using the Messagemaker kit (Life Technologies, Gaithersburg, Maryland) and the cDNA generated using the cDNA Synthesis System (Life Technologies). SAGE was performed essentially as described (Velculescu et al., 1995). As part of the Cancer Genome Anatomy Project (CGAP) SAGE consortium (Lal et al., 1999), the A2780 library was arrayed at the Lawrence Livermore National Laboratories and sequenced at the Washington University Human Genome Center or NISC (NIH, Bethesda, Maryland). The ACRP library was arrayed in our laboratory and sequenced by the UCLA sequencing facility. Sequence data from each library were analyzed by the SAGE software (Velculescu et al., 1995) to quantify tags and identify their corresponding transcripts. Genes differentially expressed were identified and only those with a p value determined to be at least 0.05 (Madden et al., 1997) were considered statistically significant and included for further analysis.

**Semi-quantitative real-time RT-PCR**

One microgram of total RNA from A2780, ACRP, and ACR6 was used to generate cDNA using the Taqman Reverse Transcription Reagents (PE Applied Biosystems, Foster City, California). The SYBR Green I assay performed on the GeneAmp 5700 Sequence Detection System (PE Applied Biosystems) was used for detecting RT-PCR products as previously described (Hough et al., 2000). Primers for eight candidate genes (*COL6A3*, *COL11A1*, *DCN*, *CRTL1*, *PAPSS*, *FADS*, *FLNA*, and *CRABP*) were designed to cross intron-exon boundaries to distinguish PCR products generated from genomic versus cDNA template. Primers for *18S* ribosomal RNA were obtained from Ambion (Austin, Texas) and used for normalization purposes as described by the manufacturer. The primer pairs used as well as the sizes expected for each gene under study are available from the authors upon request. Each PCR reaction was optimized to ensure that a single band of the appropriate length (79–315 bp) was amplified and that no bands corresponding to genomic DNA amplification or primer-dimer pairs were present. The PCR cycling conditions were performed for all of the samples as follows: 2 min at 50°C for AmpErase UNG incubation; 10 min at 95°C for AmpliTaq Gold activation; and 40 cycles for the melting (95°C, 15 s) and annealing/extension (60°C for 1 min) steps. PCR reactions for each template were done in duplicate in one 96-well dish per gene-specific primer pair tested, except for the *18S* control, which was done in triplicate. The comparative $C_T$ method (Livak and Schmittgen, 2001) was used to quantitate the expression for each gene relative to the parental line A2780 using *18S* as a normalization control. The panel of RNAs from microdissected ovarian tumors of various subtypes has been described (Hough et al., 2001). For the particular experiments with the ovarian panel, the levels of *COL6A3* were expressed relative to HOSE, a culture of normal ovarian surface epithelial cells (Hough et al., 2000). Differences in the amounts of collagen VI between the high and low grades were calculated and statistical significance evaluated using a t test.

**Collagen VI staining of primary tumors**

The tissue Ovarray has been described and consists of two blocks of 60 cases each, three normal ovary samples, nine mucinous cystadenomas, ten serous cystadenomas, four mucinous borderline tumors, eleven serous borderline tumors, 57 serous adenocarcinomas, ten endometrioid adenocarcinomas, six clear cell adenocarcinomas, and kidney tissue punches included as internal control (Rangel et al., 2003). The specimens were sectioned and deparaffinized using a xylene (2×), 100% ETOH, 95% EtOH, 75% EtOH, $H_2O$ series, and antigens were retrieved by steaming samples in DAKO target retrieval buffer (DAKO) for 20 min. Sections were rinsed in PBS, incubated with a collagen VI antibody (clone V1-26, Chemicon International, Temecula, California) and a Cy5-conjugated secondary antibody, and were analyzed by immunofluorescence using a 12-bit CCD camera. As a positive control, CA125 staining was performed on all samples (not shown).

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