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Clusterin interacts with paclitaxel and confer paclitaxel resistance in ovarian cancer

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Abstract

Optimal debulking followed by chemotherapy is the standard treatment of managing late-stage ovarian cancer, but chemoresistance is still a major problem. In this study, we compared expression profiles of primary tumor tissue from five long-term (>8 years) and five short-term (years) ovarian cancer survivors and identified clusterin as one of the genes that were significantly up-regulated in short-term survivors. We then evaluated the prognostic significance of clusterin and its possible correlation with chemoresistance in ovarian cancer by immunohistostaining of clusterin in 62 tumor samples from patients with stage III, high-grade serous ovarian cancer. After adjusting for debulking status and age, Cox regression analyses showed that high levels of clusterin expression correlate with poor survival (hazard ratio, 1.07; 95% confidence interval, 1.002–1.443; P = .04). We also investigated clusterin in paclitaxel resistance by modulating the endogenous clusterin expression in ovarian cancer cells and treating the cells with purified clusterin. Results indicate that high-clusterin–expressing ovarian cancer cells are more resistant to paclitaxel. Moreover, exposing ovarian cancer cells to exogenous clusterin increases cells' resistance to paclitaxel. Finally, using size exclusion chromatography and fluorescently labeled paclitaxel, we demonstrated that clusterin binds to paclitaxel. In summary, our findings suggest that high levels of clusterin expression increase paclitaxel resistance in ovarian cancer cells by physically binding to paclitaxel, which may prevent paclitaxel from interacting with microtubules to induce apoptosis. Thus, clusterin is a potential therapeutic target for enhancing chemoresponsiveness in patients with a high-level clusterin expression.

Keywords

cancer, ovarian, clusterin, resistance, paclitaxel, confer, interacts, CMMB

Disciplines

Life Sciences | Physical Sciences and Mathematics | Social and Behavioral Sciences

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Clusterin Interacts with Paclitaxel and Confer Paclitaxel Resistance in Ovarian Cancer[1,2]

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Abstract

Optimal debulking followed by chemotherapy is the standard treatment of managing late-stage ovarian cancer, but chemoresistance is still a major problem. In this study, we compared expression profiles of primary tumor tissue from five long-term (>8 years) and five short-term (<2 years) ovarian cancer survivors and identified *clusterin* as one of the genes that were significantly up-regulated in short-term survivors. We then evaluated the prognostic significance of clusterin and its possible correlation with chemoresistance in ovarian cancer by immunohistostaining of clusterin in 62 tumor samples from patients with stage III, high-grade serous ovarian cancer. After adjusting for debulking status and age, Cox regression analyses showed that high levels of clusterin expression correlate with poor survival (hazard ratio, 1.07; 95% confidence interval, 1.002–1.443; $P = .04$). We also investigated clusterin in paclitaxel resistance by modulating the endogenous clusterin expression in ovarian cancer cells and treating the cells with purified clusterin. Results indicate that high-clusterin–expressing ovarian cancer cells are more resistant to paclitaxel. Moreover, exposing ovarian cancer cells to exogenous clusterin increases cells' resistance to paclitaxel. Finally, using size exclusion chromatography and fluorescently labeled paclitaxel, we demonstrated that clusterin binds to paclitaxel. In summary, our findings suggest that high levels of clusterin expression increase paclitaxel resistance

Abbreviations: shRNA, short hairpin RNA; CLU, clusterin; siRNA, small interfering RNA

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[2]This article refers to supplementary materials, which are designated by Table W1 and Figures W1 to W5 and are available online at www.neoplasia.com.

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in ovarian cancer cells by physically binding to paclitaxel, which may prevent paclitaxel from interacting with microtubules to induce apoptosis. Thus, clusterin is a potential therapeutic target for enhancing chemoresponsiveness in patients with a high-level clusterin expression.

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Introduction

Epithelial ovarian cancer, the most common human ovarian cancer [1], is also the most lethal, partly because 75% of ovarian cancers are detected as late-stage disease [2] and 25% of tumors do not respond to standard chemotherapy. Nevertheless, after surgical debulking of the tumor and standard chemotherapy, approximately 5% to 10% patients experience 5-year progression-free survival and are therefore considered long-term survivors [3]. Identifying new molecular therapeutic targets for the treatment of the disease should improve these outcomes, but to do so, we must first gain greater insight into the pathogenesis of ovarian cancer, perhaps by better understanding the molecular differences between tumors from short- and long-term ovarian cancer survivors.

Transcription profiling, one increasingly standard approach to investigating global gene expression differences in cancer samples [4–10], has already been used to identify many potential prognostic biomarkers and therapeutic targets. In this study, our primary purpose was to identify a list of genes that are significantly up-regulated in short-term survivors by transcription profiling. It is hoped that some of these genes might be potential therapeutic targets. *Clusterin* was found to be one of the genes that are significantly up-regulated in short-term survivors.

Although previous studies have established that high levels of clusterin expression correlate with the progression of various cancers, such as cancer of the bladder [11], breast [12], and prostate [13], and that clusterin may also be involved in chemoresistance in these cancers [14–16], the correlation of high clusterin expression in ovarian cancer cells with short-term survival and chemoresistance in ovarian cancer patients has not been established. Therefore, in this study, we sought to clarify the prognostic significance of clusterin in ovarian cancer and explore its role in chemoresistance. By manipulating clusterin expression and exposure in several ovarian cancer cell lines and using size exclusion chromatography and fluorescently labeled paclitaxel, we found that clusterin increases the resistance of ovarian cancer cells to paclitaxel by binding to it, both *in vivo* and *in vitro*. Furthermore, immunohistochemical analyses of 62 independent ovarian tumor samples from patients with stage III, high-grade serous ovarian cancer showed that high levels of clusterin expression correlate with poor overall survival rates.

Materials and Methods

Microarray Analysis

Affymetrix U133plus2 GeneChips (Santa Clara, CA) were used to generate expression profiles of microdissected tumor cells from five long-term (survival time, 95-192 months) and five short-term (survival time, 13-21 months) survivors with stage III, grade 3 serous ovarian cancer as described previously (Figure 1) [17]. All patients received an optimal debulking surgery and treatment with six cycles of paclitaxel and cisplatin. Raw images (".DAT" files) from an Affymetrix GeneChip scanner were processed with dChip software [18]. The raw signals of individual probes for all 10 arrays were normalized against the chip with the median raw signal intensity, and normalization was based on an "invariant set" of probes consisting of points from nondifferentially expressed genes. After normalization, the expression values of each gene in all the samples were computed using a perfect match-only model and an outlier detection algorithm. Normalized expression values from dChip analyses were used for a two-class unpaired SAM analysis. Differentially expressed genes

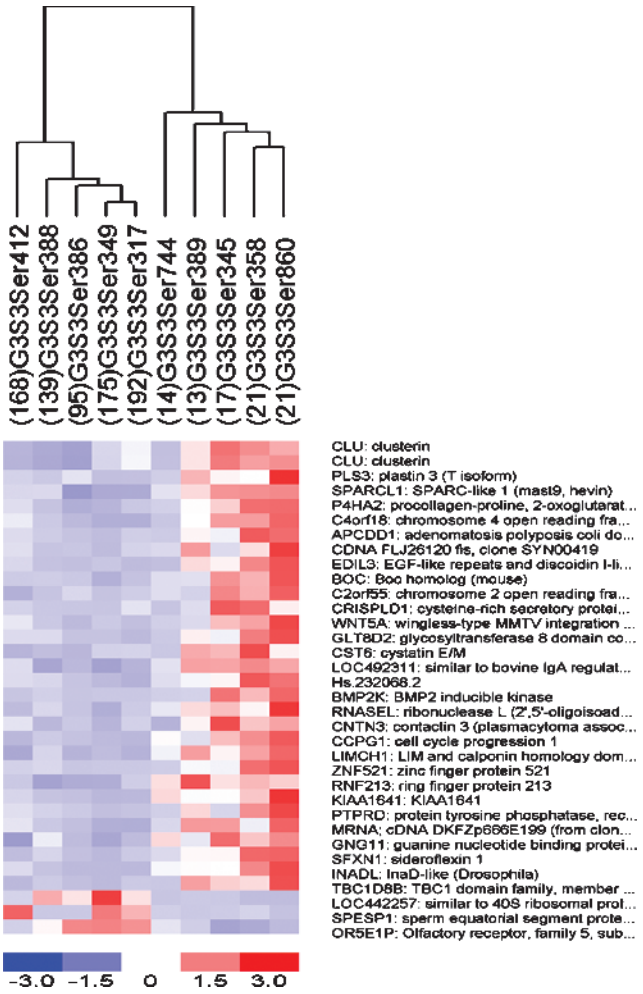

Figure 1. Partial list of differentially expressed genes in tumors from long- and short-term ovarian cancer survivors. The length of survival for each patient is shown within a labeled bracket. Red, white, and blue indicate a fold-change expression level above, at, and below the mean expression of a gene across all samples, respectively.

were identified by supervised analysis with Significance Analysis of Microarrays (SAM) software [19], which yields results similar to those obtained using Student's *t* test but includes permutations to calculate the false discovery rate, or a *q* value, for each gene. Each *q* value represents the probability that a gene is falsely identified as differentially expressed, with smaller *q* values indicating more significant differential expression levels.

Cell Lines and Tissue Samples

All 13 ovarian cancer cell lines (PEO4, CAOV3, OVCA420, TOV21G, RMUG-S, RMUG-L, OVCA822, 0V2008, ES2, TOV112, OVCA433, OVCA432, and SKOV3) were grown in a medium of equal parts M199 and MCDB105 supplemented with 10% fetal bovine serum as described previously [20]. All tumor specimens from patients were collected and archived according to protocols approved by the institutional review boards of the appropriate institutions that included an informed consent or a waiver. All the patients had stage III and grade III serous ovarian tumors and were treated uniformly with paclitaxel/carboplatin first-line chemotherapy regimen.

Immunohistochemistry

Five normal ovarian surface epithelial samples and 62 samples from previously untreated stage III, high-grade primary papillary serous carcinomas were immunostained using an avidin-biotin method with an anti–clusterin α chain mouse monoclonal antibody (5 μg/ml; Upstate Biotechnology, Lake Placid, NY), with clusterin expression scored as described previously [21]. Cox regression and Kaplan-Meier survival analyses were used to compare immunohistochemical results with patient survival data. Statistical significance was determined by using the log rank test with statistical software SPSS version 15.0.

Western Blot Analysis

Cell lysates were prepared from the 13 ovarian cancer cell lines [20] and subjected to Western blot analysis using an anti–clusterin mouse monoclonal antibody (Upstate Biotechnology) and an anti–β-actin monoclonal antibody for normalization of protein loading. Immunoreactivity was detected using the ECL chemiluminescence system (Amersham, Piscataway, NJ) and quantified using an imaging densitometer (Model GS-670; Bio-Rad, Hercules, CA).

IC_{50} and Cell Proliferation Assay

Cells (1×10^4 per well) were seeded in a 96-well plate in 0.1 ml of culture medium and allowed to grow overnight. The next day, the medium was supplemented with either vehicle control or 10^{-9} to 10^{-4} M paclitaxel (Sigma, St. Louis, MO). The 50% inhibitory drug concentration (IC_{50}) was determined using the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide (XTT) assay (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's instructions. The metabolism of XTT was quantified by measuring the absorbance at 450 nm, and the IC_{50} of paclitaxel for each of the cell lines was estimated from the semilogarithmic dose-response curves by linear interpolation.

DNA Fragmentation Assay for Apoptosis

Cells (1×10^4 per well) were seeded in a 96-well plate in 0.1 ml of culture medium and allowed to grow overnight. The next day, cells were treated with 10^{-7} M paclitaxel for 48 hours at 37°C in the presence or absence of clusterin. The amounts of cytoplasmic histone-associated DNA fragments (mononucleosome and oligonucleosomes) formed during apoptosis were measured using a cell death detection ELISA^{Plus} kit (Roche Molecular Biochemicals) according to the manufacturer's instructions. Each assay was performed in quadruplicate, and each experiment was repeated three times. The relative percentages of DNA fragmentation were calculated as the ratios OD_{405} readings from paclitaxel-treated to paclitaxel-untreated cells.

Generation of Stably Transfected Cells Overexpressing Clusterin

SKOV3 cells were transfected with full-length clusterin cDNA cloned into pcDNA3.1 expression vectors (pcDNA3.1/CLU) using FuGENE 6 transfection reagent according to the manufacturer's protocol (Roche Molecular Biochemicals). Mock transfection was performed the same way, using the vector pcDNA3.1. Stably transfected clones were selected 36 hours later by adding G418 (Roche Molecular Biochemicals) at 500 μg/ml. The stable clones were then evaluated for the expression of clusterin and paclitaxel resistance.

In Vitro *Small Interfering RNA–Mediated Gene Silencing*

Clusterin small interfering RNA (siRNA) duplex, consisting of nucleotides +85 to +96 (where the translation start site was defined as +1; Dharmacon Research, Lafayette, CO), or luciferase GL2 Duplex (Dharmacon) at 0.2 nmol/ml was transfected into ovarian cancer cells expressing high levels of clusterin (PEO4, RMUG-S, and TOV21G) using an oligofectamine reagent (Invitrogen, Carlsbad, CA) as described by the manufacturer's protocols (Life Technologies, Inc., Gaithersburg, MD). Transfected cells were evaluated for clusterin expression and sensitivity to paclitaxel.

Exogenous Clusterin Treatment

Clusterin was purified from human sera by immunoaffinity chromatography, using an anti–clusterin monoclonal antibody under native conditions at 4°C as described previously [22]. Analysis of clusterin purity by SDS-PAGE showed a major band (nonreduced) at 75 to 80 kDa [22]. To further ensure the purity of the product, we performed an additional purification step using ion-exchange chromatography. The eluate from the immunoaffinity column was dialyzed against 50 mM Tris buffer (pH 8.0), loaded onto a Q-Sepharose FF column, and eluted with a linear NaCl gradient in the same buffer. Clusterin-containing fractions (as judged by SDS-PAGE) were then pooled and concentrated. SKOV3 cells (1×10^4 cells per well) in 96-well plates were treated with 10^{-7} M paclitaxel in the presence of 0.5, 2.5, 5, and 7.5 and 15 μg/ml purified clusterin for 48 hours at 37°C. Cells were then harvested for XTT and apoptosis assays.

In Vivo *Short Hairpin RNA–Mediated Gene Silencing Using a Lentiviral Expression System*

A Lentiviral vector expressing short hairpin RNA (shRNA) targeting clusterin was constructed using the BLOCK-iT Lentiviral RNAi expression system (Invitrogen). DNA oligonucleotides encoding shRNA of clusterin (CLU) were designed using BLOCK-iT RNAi designer (Invitrogen) and inserted downstream of the human U6 pol III promoter and upstream of a pol III terminator in pENTR/U6 vector (Invitrogen) and then into the vector pLenti-GW/U6 as pLenti-GW/U6-CLUshRNA. Lentiviral particles were

then generated from pLenti-GW/U6-CLUshRNA by transfecting 293 FT cell lines with the shRNA construct and helper plasmids.

To investigate the effects of decreased clusterin expression on paclitaxel resistance in xenografted tumors, forty 6- to 8-week-old female nude mice (SLC; Shizuoka, Japan) were evenly distributed for generating xenograft mouse models using control cells (PEO4 transduced with Lentiviral particles carrying shRNA for the *LacZ* gene) or clusterin knockdown cells (PEO4 transduced with Lentiviral particles carrying shRNA for the *CLU* gene). After being transduced with Lentiviral particles carrying shRNA for the *LacZ* or *CLU* gene overnight, 5×10^5 paclitaxel-resistant PEO4 cells were used to inoculate subcutaneously into the posterior neck region of each nude mouse. When the xenografts grew to approximately 100 mm^3, mice were injected intraperitoneally with 0.2 mg/0.1 ml per 10 g of paclitaxel three times per week for 3 to 4 weeks. Tumor volumes were measured twice every week and calculated using the formula: length × width × depth × 0.5236. The mice were euthanized at day 27, and the levels of clusterin expression in xenografted tumors were determined by Western blot analysis. Animals were housed in pathogen-free units in compliance with institutional animal care and use committee regulations.

Effects of Clusterin on the Binding of Paclitaxel to Microtubules in Ovarian Cancer Cells

PEO4 and SKOV3 cells were trypsinized, inoculated into chambered coverglasses (Fisher Scientific, Pittsburgh, PA), and incubated for 12 hours at 37°C. The cells were washed three times with 2% BSA/PBS, 10^{-6} M Oregon Green (OG) 488–labeled paclitaxel (paclitaxel-OG; Invitrogen) was added, and the cells were incubated for 1 hour at 37°C in PEM buffer (50 mM PIPES, 2 mM EGTA, 2 mM $MgCl_2$, pH 7.4), with or without 7.5 mg/ml purified clusterin. After incubation with the 10^{-6} mg/ml Hoechst 33258 pentahydrate (Molecular Probes, Eugene, OR) for 15 minutes at 37°C, the cells were examined using a Leica DMIRE2 inverted fluorescence microscope with appropriate filters.

Measurement of Paclitaxel-Clusterin Binding

Solutions of clusterin or control protein glutathione-*S*-transferase (GST) at 5 mM, alone or in the presence of a 1:1 molar ratio of paclitaxel-OG, phalloidin-OG, or OG in PEM were incubated for 1 hour at 37°C. Then, solutions were fractionated using size exclusion chromatography with a Biosep SEC S4000 column (Phenomenex, Sydney, Australia) and an AKTA FPLC system (GE Healthcare, Sydney, Australia); the A_{280} of the eluate, as an indication of protein elution, was measured continuously. Collected fractions (200 ml each) were transferred into the wells of black 96-well plates (Greiner, Frickenhausen, Germany), and the fluorescence of paclitaxel-OG, phalloidin-OG, and OG was measured using a Fluostar microplate reader (BMG Labtech, Melbourne; excitation, 485 ± 5 nm; emission, 520 ± 5 nm). Other control proteins (superoxide dismutase and hemoglobin) were also tested.

Results

Expression Profiling of Microdissected Tumors from Short- and Long-term Survivors

Using SAM analysis to compare the expression profiles of tumors from five short- and five long-term ovarian cancer survivors, we identified 77 probe sets with greater than threefold expression in short- than in long-term survivors (Table W1; $q < 15\%$). Two of these probe sets encode clusterin that was up-regulated 3.8-fold more in short- than in long-term survivors (Figure 1).

Clusterin Expression Correlates with Poor Survival and Chemoresponse

To evaluate whether clusterin expression correlates with poor survival, clusterin immunostaining was performed on an independent set of 62 ovarian cancer samples from patients with stage III, grade 3 serous ovarian cancers. After adjustment for optimal debulking and age, Cox regression analyses showed that high clusterin expression correlates with poor survival [hazard ratio (HR), 1.07; 95% confidence interval (CI), 1.002–1.443; $P = .04$]. In addition, using median weight scores as cutoffs, Kaplan-Meier survival analyses showed that patients with positive clusterin expression had significantly reduced survival times in both optimally and suboptimally debulked

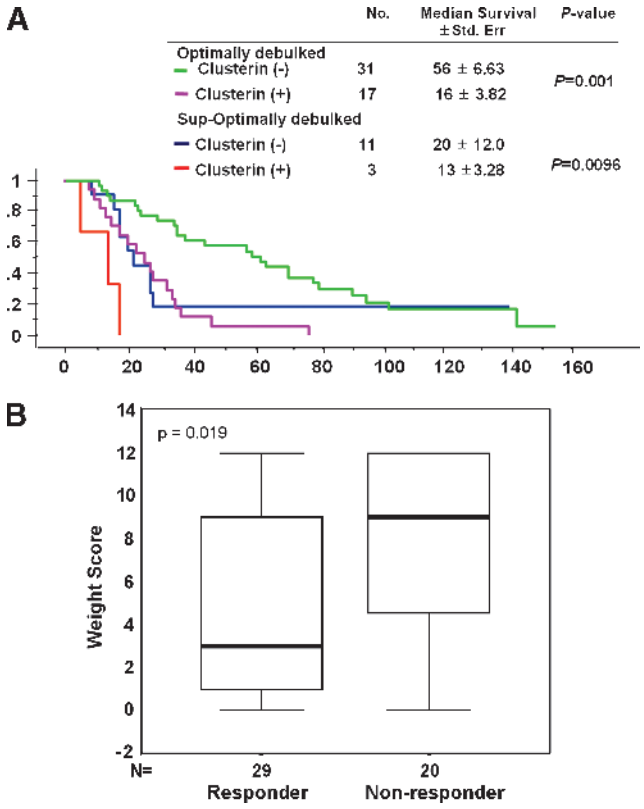

Figure 2. Clusterin expression correlates with overall survival and chemoresponse. (A) Kaplan-Meier survival curves for patients with stage III, high-grade serous ovarian adenocarcinomas. Patients who had tumors with positive clusterin expression (+) had significantly poorer prognoses than those who had negative clusterin expression (−), whether their debulking was optimal (i.e., the largest residual tumor was <2 cm) or suboptimal (i.e., the largest residual tumor was >2 cm). (B) Box-plot showing clusterin protein expression in chemoresponders and nonresponders. The box is bounded above and below by the 75th and 25th percentiles, and the median is the line in the box. Whiskers are drawn to the nearest value not beyond a standard span from the quartiles; points beyond (outliers) are drawn individually, where the standard span is 1.5 × (interquartile range).

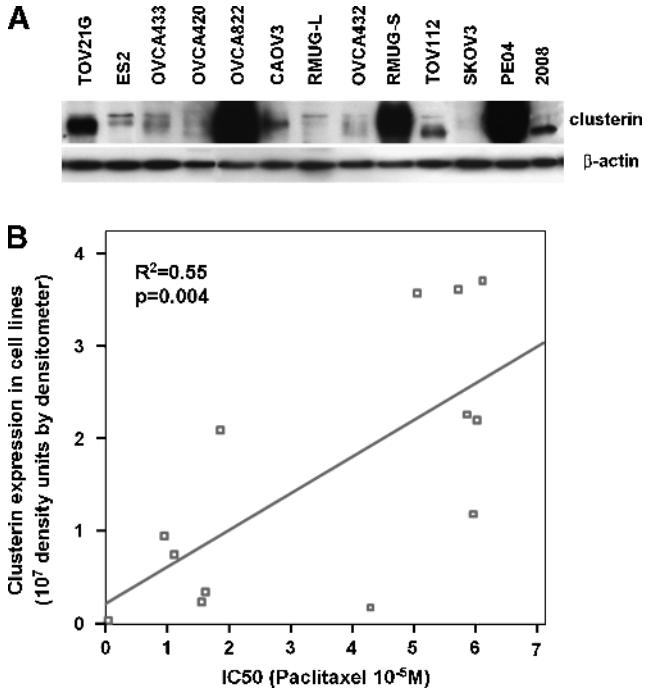

Figure 3. Correlation of clusterin expression and paclitaxel resistance. (A) Western blot analysis of clusterin expression in 13 epithelial ovarian cancer cell lines. (B) Scatter plot showing correlation between clusterin expression and paclitaxel IC_{50} for the 13 ovarian cancer cell lines tested. High IC_{50} values denote paclitaxel resistance, whereas low IC_{50} values indicate paclitaxel sensitivity. A Spearman correlation test showed that clusterin overexpression significantly correlated with IC_{50} for paclitaxel ($R^2 = 0.55$, $P = .004$).

cases ($P = .001$ and $P = .0096$, respectively; Figure 2*A*). Normal ovarian surface epithelial cells had no immunostaining of clusterin. The immunostaining intensities of clusterin (weight score) in the nonresponder group was significantly ($P = .019$) higher than those in the responder group (Figure 2*B*).

Clusterin Expression Correlates with Paclitaxel Resistance in Ovarian Cancer Cell Lines

To test whether high clusterin expression is associated with chemoresistance, we determined the level of clusterin protein expression and paclitaxel IC_{50} values in 13 ovarian cancer cell lines. The Western blot showed that four cell lines (PEO4, RMUG-S, OVCA822, and TOV21G) had very high levels of clusterin expression (Figure 3*A*) and that these cell lines were the most resistant to paclitaxel. A significant correlation between clusterin expression and paclitaxel IC_{50} is illustrated in Figure 3*B* (Spearman's test, $R^2 = 0.55$, $P = .004$). We have also determined the IC_{50} values for cisplatin, carboplatin, topotecan, and doxorubicin for these cell lines; however, no significant correlation was found.

Up-regulation of Clusterin Expression Increases Paclitaxel Resistance of SKOV3 Cells

Paclitaxel-sensitive SKOV3 cells, which express low levels of clusterin, were stably transfected with a full-length human clusterin cDNA construct or with the vector alone. Cells transfected with the stable SKOV3/pcDNA3.1/CLU expressed high levels of clusterin and were more resistant to paclitaxel than those transfected with the vector control SKOV3/pcDNA3.1 (Figure 4, *A* and *B*; $P < .05$).

Exogenous Clusterin Enhances Paclitaxel Resistance of SKOV3 Ovarian Cancer Cells

Because clusterin is a secreted protein, we evaluated the effects of exogenous clusterin on paclitaxel resistance. In the presence of paclitaxel, the number of surviving SKOV3 cells increased with the amount of exogenous clusterin (Figure 5; $P < .05$). As a control, we also tested the effect of clusterin alone on cell proliferation. There is no significant effect on cell proliferation when SKOV3 was treated with 7.5 μg/ml clusterin for 48 hours (Figure W1).

Clusterin Expression Knockdown by siRNA Reduces Paclitaxel Resistance of PEO4 Ovarian Cancer Cells

PEO4 is a paclitaxel-resistant cell line that expresses high levels of clusterin. Clusterin protein levels were significantly lower both 24 and 48 hours after PEO4 cells were transfected with clusterin siRNA (Figure 6*A*). No change in clusterin expression was observed in cells transfected with the luciferase siRNA control. After 24 hours of transfection with clusterin or luciferase siRNA, all PEO4 cells were treated with 10^{-7} M paclitaxel for 48 hours. A correlation between reduced clusterin expression and increased paclitaxel sensitivity was observed (Figure 6*B*). Moreover, paclitaxel-induced apoptosis also significantly increased when clusterin expression was knocked down in PEO4 cells (Figure W2). Similar results were obtained in two other paclitaxel-resistant cell lines, RMUG-S and TOV21G, which express high levels of clusterin (Figure W3).

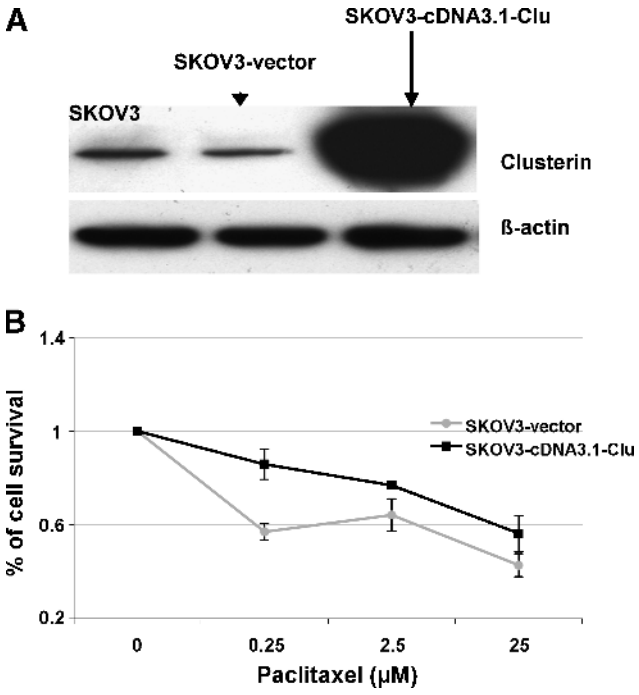

Figure 4. Increased paclitaxel resistance in clusterin transfectants. (A) Western blot analysis showing overexpression of clusterin in SKOV3 cells stably transfected with plasmid pcDNA3.1 with clusterin (cDNA3.1-Clu). (B) SKOV3-cDNA3.1-Clu stably transfected expressing clusterin had higher survival rates than the control cells SKOV3-vector, which were transfected with pcDNA3.1 vector only.

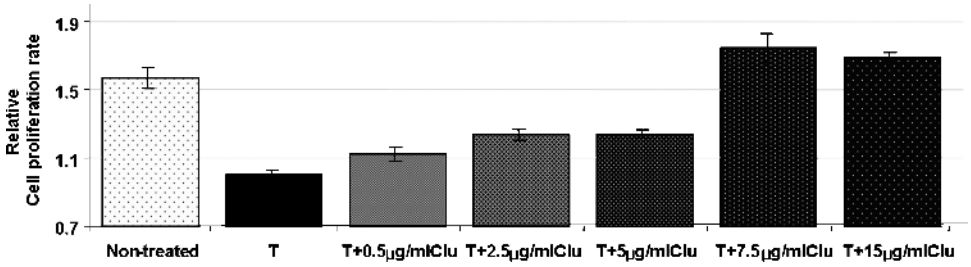

Figure 5. Exogenous clusterin enhances the paclitaxel resistance of SKOV3 cells. Cell proliferation was measured by XTT assay in SKOV3 cells treated with 10^{-7} M paclitaxel (T) for 48 hours in the presence of the indicated concentrations of clusterin. The baseline used for calculating relative cell proliferation rate was set by adjusting the proliferation rate of paclitaxel-treated SKOV3 cells without exogenous clusterin to 1.

Clusterin Expression Knockdown in Ovarian Cancer Cells by shRNA Reduces Tumor Size in Xenograft Mouse Models When Treated with Paclitaxel

PEO4 cells transduced with Lentivirus carrying clusterin or LacZ shRNA were xenografted into mice, and the effects of paclitaxel were determined. After 22 days, the tumors that had developed from the xenografted cells transduced with clusterin shRNA were significantly smaller than those of the control groups (Figure 7*A*). Decreased clusterin expression in xenografted tumor masses after autopsy was confirmed by Western blot analysis and densitometer quantification. The difference between shRNA-clusterin–treated tumor and shRNA-control was significant (twofold decrease) with $P = .037$ (Figure 7*B*).

Effect of Clusterin on Microtubule Binding

To evaluate whether clusterin confers paclitaxel resistance by preventing the binding of paclitaxel to microtubules, both paclitaxel-resistant PEO4 cells and paclitaxel-sensitive SKOV3 cells were treated with identical concentrations of paclitaxel-OG. PEO4 cells had minimal microtubule staining in the absence or presence of exogenous clusterin (Figure 8, *A* and *B*). In contrast, SKOV3 cells had strong cytoplasmic staining in the absence of clusterin, whereas no staining was observed in the presence of exogenous clusterin, suggesting that exogenous clusterin prevents the binding of paclitaxel to microtubules (Figure 8, *C* and *D*). To clarify the role of endogenous clusterin, we infected PEO4 cells with Lentivirus carrying shRNA-clusterin (Lenti-shRNA-clusterin) to knockdown the endogenous clusterin expression. The result showed that PEO4 cells infected with Lenti-shRNA-clusterin had strong cytoplasmic stain by paclitaxel-OG, whereas PEO4 cells infected with Lentivirus vector control had undetectable staining similar to Figure 8, *C* and *B*, respectively (Figure W4).

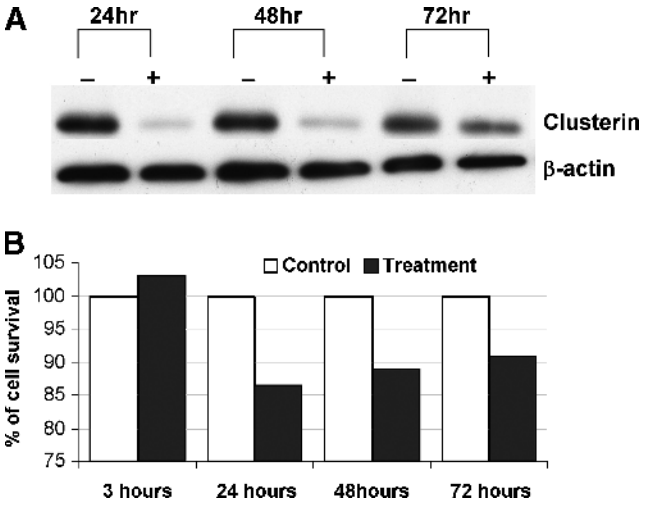

Figure 6. PEO4 became sensitive to paclitaxel after silencing clusterin expression with siRNA. (A) Western blot analyses show the time course of clusterin silencing in PEO4 cells transfected with siRNA for clusterin (+) or luciferase (−). After transfection, the cells were cultured for 2 days in medium containing 10^{-7} M paclitaxel. (B) Cell survival after paclitaxel treatment as determined by XTT assay.

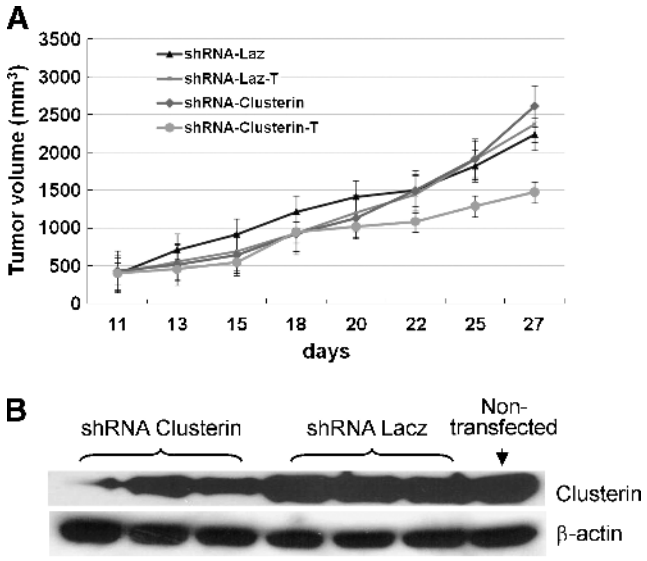

Figure 7. Effects of decreased clusterin expression on paclitaxel resistance in xenografted tumors. (A) When xenografts in nude mice resulting from the inoculation of paclitaxel-resistant cells transduced with shRNA for *clusterin* or the *Lacz* gene grew to approximately 100 mm^3, mice were injected with 0.2 mg/0.1 ml per 10 g of paclitaxel intraperitoneally. Tumor volumes were measured twice every week. (B) Western blot analysis of clusterin expression from xenografted tumors from mouse autopsies. Tumors from three separate mice from both the shRNA-Lacz and shRNA-clusterin groups were analyzed.

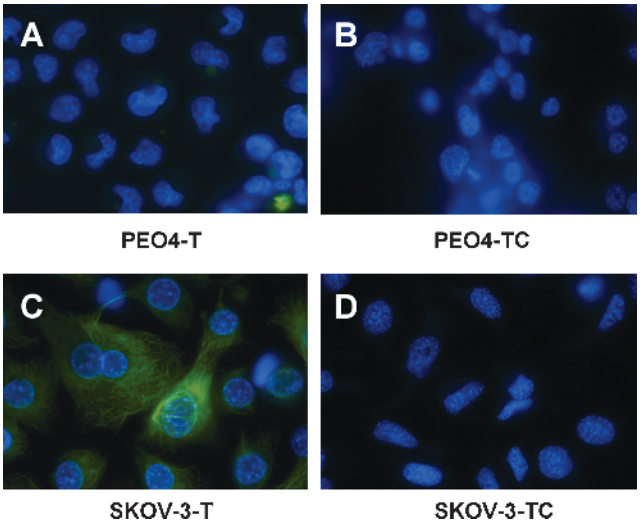

Figure 8. Exogenous clusterin prevents the binding of paclitaxel to microtubules in SKOV3 cells. PEO4 (A and B) and SKOV3 (C and D) cells cultured in chamber coverglasses were treated with 10^{-6} M OG-labeled paclitaxel in the absence (A and C) or presence (B and D) of 7.5 μg/ml clusterin for 30 minutes. Cells were also simultaneously incubated with the Hoechst stain for nuclei visualization, and fluorescent images were captured by an inverted fluorescence microscope equipped with a digital camera using the same setting. The binding of OG-labeled paclitaxel to microtubules is shown in panel C.

Binding Interactions between Clusterin and Paclitaxel

To evaluate whether clusterin binds specifically to paclitaxel, equimolar mixtures of clusterin or GST as a control and paclitaxel-OG, phalloidin-OG, or OG were incubated together and then fractionated by size exclusion chromatography. After incubation with clusterin, the elution profile of paclitaxel-OG adopted a pattern similar to that of clusterin alone (Figure 9, *A* and *B*). In contrast, after incubation with GST, the elution profile of paclitaxel-OG was similar to that of paclitaxel-OG alone, implying that under these conditions, the small molecule remained unbound. Similar results were obtained with two other control proteins, superoxide dismutase and hemoglobin (data not shown), and OG and phalloidin-OG, the control dyes (Figure W5).

Discussion

By comparing the expression profiles generated from short- and long-term ovarian cancer survivors, we found that clusterin expression was significantly higher in short- than in long-term survivors (Figure 1). Also, Kaplan-Meier survival analyses of results from clusterin immunostaining in 62 samples of late-stage, high-grade ovarian serous carcinomas indicated that the overexpression of clusterin correlates with poor survival (Figure 2*A*). This is consistent with reports that clusterin is a marker of poor prognosis in other cancer types [23–27]. Moreover, overexpression of clusterin also correlates with chemoresistance (Figure 2*B*).

Clusterin, also known as testosterone-repressed prostate message-2 (TRPM-2), sulfated glycoprotein-2 (SGP-2), SP-40, and apolipoprotein J (ApoJ), is a secreted heterodimeric glycoprotein encoded by a single gene on chromosome 8p21-p12. When analyzed by SDS-PAGE, clusterin migrates as a broad band at 70 to 80 kDa but has an actual mass of approximately 61 kDa (determined by mass spectrometry) [28]. Clusterin is present in various biologic fluids [29] and has been implicated in several physiological processes, including cell adhesion and aggregation [30], complement inhibition [31], lipid transport, membrane protection, sperm maturation, and endocrine secretion [31,32]. Clusterin expression has also been implicated in chemoresistance in several other cancer types [14,15]. Because the resistance of tumor cells to various available chemotherapeutic agents such as paclitaxel has been one of the major factors leading to poor survival in ovarian cancer patients, we therefore hypothesized that clusterin expression confers chemoresistance to ovarian cancer cells.

In this study, we demonstrated that clusterin expression correlated with paclitaxel resistance both *in vitro* and *in vivo*. We found that high levels of clusterin expression in ovarian cancer cell lines correlated with paclitaxel resistance, although a few cell lines with low levels of clusterin expression were also resistant to paclitaxel (Figure 3). These cell lines might use mechanisms other than overexpression of clusterin to confer paclitaxel resistance such as the expression of metabolizing enzyme cytochrome P450 or cellular efflux ABC transporter [33]. To demonstrate the role of clusterin in paclitaxel resistance, we manipulated the endogenous level of clusterin expression in a paclitaxel-sensitive cell line (SKOV3, low level of endogenous clusterin expression) and a paclitaxel-resistant cell line (PEO4, high level of endogenous clusterin expression). We found that paclitaxel-sensitive SKOV3 cells became more resistant to paclitaxel when endogenous clusterin expression level was up-regulated (Figure 4). Conversely,

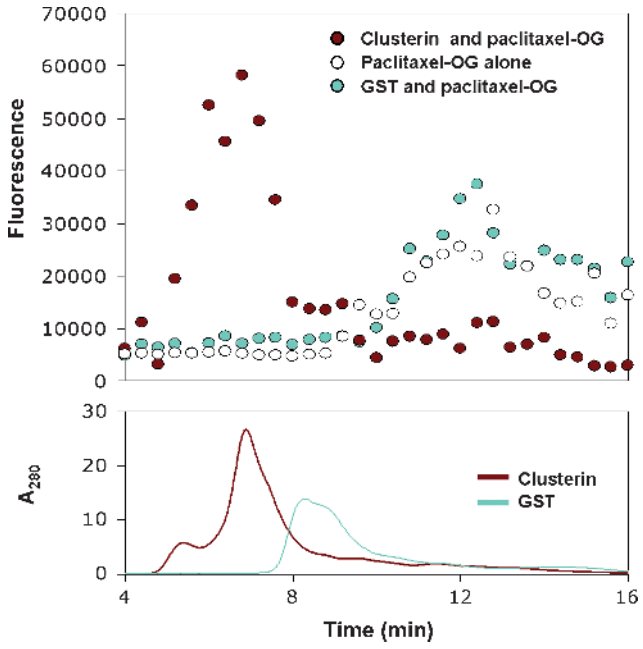

Figure 9. Coelution of clusterin and OG-labeled paclitaxel (paclitaxel-OG) in size exclusion chromatography. Solutions containing clusterin and paclitaxel-OG mixture, paclitaxel-OG alone, or a GST and paclitaxel-OG mixture (all at 5 mM) were fractionated by size exclusion chromatography in PEM buffer, and the OG fluorescence and A_{280} of the eluate were measured. The upper panel shows OG fluorescence as a function of elution time; the lower panel shows the corresponding A_{280} profile. The result shown is representative of three independent experiments.

paclitaxel-resistant PEO4 cells became more sensitive to paclitaxel and more apoptotic *in vitro* and *in vivo* when endogenous clusterin expression level was down-regulated. These results indicated that high levels of endogenous clusterin expression were involved in the paclitaxel resistance of ovarian cancer cells.

In addition to the endogenous clusterin, the exogenous clusterin added in our experiments enhanced the paclitaxel resistance of SKOV3 cells, as indicated by the cells' increased survival rate and the reduction in paclitaxel-induced apoptosis (Figure 5). The extent of both the increased survival and the reduced apoptosis correlated with the increase in concentration of exogenous clusterin. This suggests that exogenous clusterin binds to extracellular paclitaxel and subsequently prevents it from entering the cells and exerting toxicity. To test the hypothesis that the exogenous clusterin secreted by ovarian cancer cells inhibits the binding of paclitaxel to microtubules inside the cells, we determined the level of microtubule-associated fluorescence in paclitaxel-OG–treated ovarian cancer cells with or without clusterin. Significant microtubule-associated fluorescence was observed in SKOV3 cells (Figure 8*C*) but not in PEO4 cells, which expressed high levels of endogenous clusterin (Figure 8, *A* and *B*). However, when SKOV3 cells were coincubated with both exogenous clusterin and paclitaxel-OG, negligible microtubule-associated fluorescence was observed (Figure 8*D*).

Using size exclusion chromatography to directly test whether clusterin bound specifically to paclitaxel in solution, we found that clusterin did bind to paclitaxel-OG but not to phalloidin-OG or OG alone and that GST and other control proteins did not bind to paclitaxel-OG. In addition, ELISA confirmed that clusterin bound with high affinity to paclitaxel but not at all to other therapeutic agents, such as cisplatin and carboplatin (data not shown). This is the first time we are aware of that the binding of clusterin and paclitaxel has been clearly demonstrated, although clusterin's role in chemoresistance has been suggested by previous reports in other cancer types [14–16]. Thus, our results suggest that clusterin, which is generally a secreted molecule, may protect cancer cells from paclitaxel toxicity by binding to the agent in the extracellular microenvironment and preventing it from entering the cell to exert its toxic effects.

In conclusion, clusterin overexpression significantly correlates with decreased survival in ovarian cancer patients, and clusterin confers resistance to paclitaxel-induced apoptosis in ovarian cancer cells. The mechanism of resistance may involve clusterin (potentially in both extra- and intracellular locations) complexing with paclitaxel to inactivate the agent's cytotoxic activity. Therefore, targeting the reduction of clusterin expression may be a worthwhile new therapeutic modality for treating ovarian tumors expressing high levels of clusterin.

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Table W1. List of Genes Up-regulated in Short-term Survivors.

ProbeSet ID	Gene Name	Fold Change	*q* (%)
205051_s_at	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	4.2	0
207480_s_at	Meis1, myeloid ecotropic viral integration site 1 homolog 2 (mouse)	3.3	0
226977_at	similar to bovine IgA regulatory protein	3.7	4.5
230000_at	chromosome 17 open reading frame 27	3.1	4.5
212327_at	hypothetical protein	3.5	11.6
200795_at	SPARC-like 1 (mast9, hevin)	3.4	11.6
204115_at	guanine nucleotide binding protein (G protein), gamma 11	3.4	11.6
227354_at	phosphoprotein associated with glycosphingolipid microdomains 1	3.3	11.6
209348_s_at	v-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian)	3.3	11.6
210346_s_at	CDC-like kinase 4	3.2	11.6
227131_at	mitogen-activated protein kinase kinase kinase 3	3	11.6
206595_at	cystatin E/M	5.2	11.7
223204_at	chromosome 4 open reading frame 18	4.4	11.7
238067_at	TBC1 domain family, member 8B (with GRAM domain)	4.2	11.7
220940_at	KIAA1641	4.2	11.7
213905_x_at	biglycan /// teashirt family zinc finger 1	4.1	11.7
211458_s_at	GABA(A) receptor–associated protein like 1 /// GABA(A) receptors associated protein like 3	4	11.7
1559060_a_at	*KIAA1961* gene	4	11.7
228256_s_at	erythrocyte membrane protein band 4.1 like 4A	4	11.7
204777_s_at	mal, T-cell differentiation protein	4	11.7
1559360_at	Ephrin-A5	3.9	11.7
208791_at	clusterin	3.8	11.7
208792_s_at	clusterin	3.8	11.7
201261_x_at	biglycan	3.8	11.7
233952_s_at	zinc finger protein 295	3.7	11.7
241893_at	Mannosyl (alpha-1,6-)-glycoprotein beta-1,6-*N* acetyl-glucosaminyltransferase	3.6	11.7
214705_at	InaD-like (*Drosophila*)	3.6	11.7
225990_at	Boc homolog (mouse)	3.6	11.7
202733_at	procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha polypeptide II	3.6	11.7
229285_at	ribonuclease L (2′,5′-oligoisoadenylate synthetase–dependent)	3.5	11.7
214246_x_at	misshapen-like kinase 1 (zebrafish)	3.4	11.7
224831_at	cytoplasmic polyadenylation element binding protein 4	3.3	11.7
223681_s_at	InaD-like (*Drosophila*)	3.3	11.7
225688_s_at	pleckstrin homology-like domain, family B, member 2	3.2	11.7
222154_s_at	DNA polymerase-transactivated protein 6	3.1	11.7
202430_s_at	phospholipid scramblase 1	3.1	11.7
202007_at	nidogen 1	3.1	11.7
218656_s_at	lipoma HMGIC fusion partner	3.1	11.7
218353_at	regulator of G-protein signaling 5	3	11.7
229795_at	Transcribed locus	3	11.7
201865_x_at	nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)	3	11.7
236313_at	cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	3	11.7
225275_at	EGF-like repeats and discoidin I–like domains 3	7	14.3
37170_at	BMP2 inducible kinase	5.8	14.3
229831_at	contactin 3 (plasmacytoma-associated)	4.8	14.3
230869_at	Transcribed locus, moderately similar to NP_775622.1 transmembrane protein 28 [*Mus musculus*]	4.6	14.3
211675_s_at	MyoD family inhibitor domain containing /// MyoD family inhibitor domain containing	4.4	14.3
201438_at	collagen, type VI, alpha 3	4.3	14.3
212764_at	—	4.2	14.3
230865_at	Lix1 homolog (mouse)	4	14.3
228067_at	similar to 2010300C02Rik protein	3.9	14.3
223343_at	membrane-spanning 4-domains, subfamily A, member 7	3.8	14.3
224724_at	sulfatase 2	3.8	14.3
206167_s_at	Rho GTPase activating protein 6	3.8	14.3
212328_at	hypothetical protein	3.8	14.3
237001_at	NIK and IKK{beta} binding protein	3.7	14.3
221538_s_at	plexin A1	3.5	14.3
212240_s_at	phosphoinositide-3-kinase, regulatory subunit 1 (p85 alpha)	3.5	14.3
230069_at	sideroflexin 1	3.5	14.3
212980_at	ubiquitin-specific peptidase 34	3.5	14.3
204872_at	transducin-like enhancer of split 4 (E(sp1) homolog, *Drosophila*)	3.5	14.3
212586_at	calpastatin	3.4	14.3
236241_at	Mediator of RNA polymerase II transcription, subunit 31 homolog (*Saccharomyces cerevisiae*)	3.4	14.3
227955_s_at	CDNA: FLJ22256 fis, clone HRC02860	3.3	14.3
201185_at	HtrA serine peptidase 1	3.3	14.3
206101_at	extracellular matrix protein 2, female organ and adipocyte-specific	3.3	14.3
231853_at	tubulin, delta 1	3.2	14.3

Table W1. (*continued*)

ProbeSet ID	Gene Name	Fold Change	*q* (%)
223843_at	scavenger receptor class A, member 3	3.2	14.3
219489_s_at	nucleoredoxin	3.1	14.3
212757_s_at	calcium/calmodulin–dependent protein kinase (CaM kinase) II gamma	3.1	14.3
202878_s_at	CD93 molecule	3.1	14.3
235085_at	homolog of rat pragma of Rnd2	3.1	14.3
224565_at	trophoblast-derived noncoding RNA	3.1	14.3
202086_at	myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	3.1	14.3
219777_at	GTPase, IMAP family member 6	3	14.3
224837_at	forkhead box P1	3	14.3
224694_at	anthrax toxin receptor 1	3	14.3

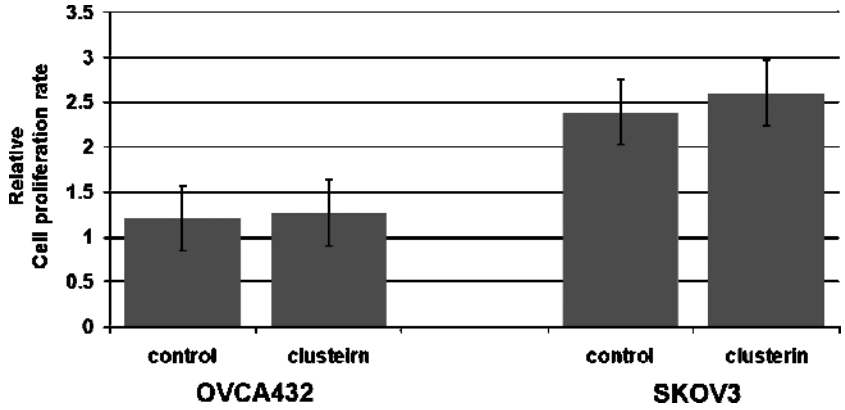

Figure W1. Two ovarian cancer cell lines, OVCA432 and SKOV3 were treated with 7.5 μg/ml clusterin for 48 hours. Cell proliferation was measured with XTT assay.

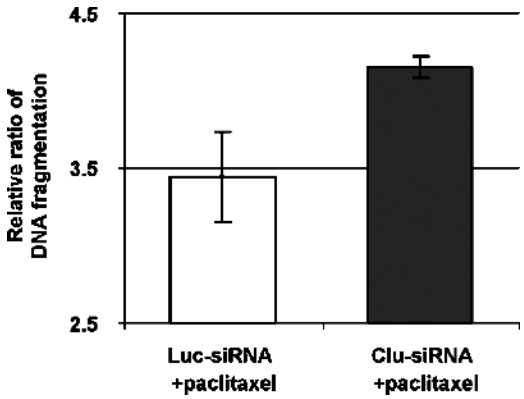

Figure W2. Relative ratio of paclitaxel-induced DNA fragmentation in PEO4 cells transfected with luciferase siRNA or clusterin siRNA. DNA fragmentation, as assayed by quantitative cell death detection ELISA, indicated the extent of apoptosis. Values displayed are the mean of three experiments, each conducted with quadruplicated samples.

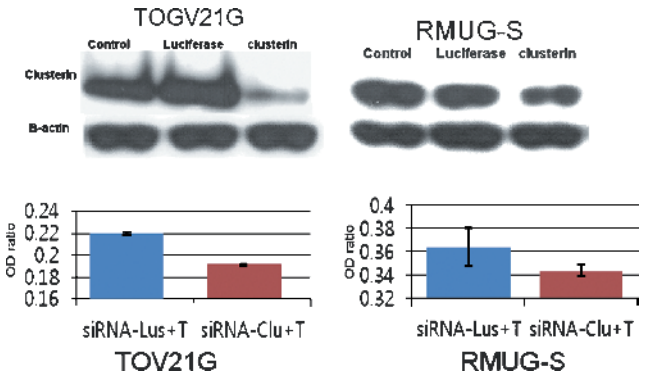

Figure W3. siRNA knockdown experiments on three additional ovarian cancer cell lines. Cells were transduced with Lentiviral vectors with siRNA for luciferase and clusterin. Western blot analysis (upper panel) was used to confirm clusterin silencing in two ovarian cancer cell lines with high levels of clusterin expression. Treatment with paclitaxel (T) decreased the relative cell proliferation, i.e., the number of viable clusterin siRNA-transfected cells, significantly more than it decreased the number of Luciferase siRNA-transfected cells (lower panel).

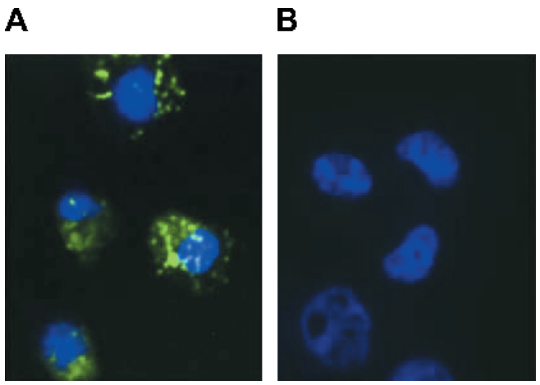

Figure W4. Cytoplasmic staining of OG-labeled paclitaxel in PEO4 cells after clusterin expression was silenced by Lentivirus carrying shRNA-clusterin. PEO4 cells were infected with Lentivirus with shRNA-clusterin (A) or vector control (B) for 48 hours in chamber slides. Subsequently, cells were treated with 10^{-6} M OG-labeled paclitaxel for 2 hours. Cells were counterstained with DAPI for nuclei and fluorescent images were captured.

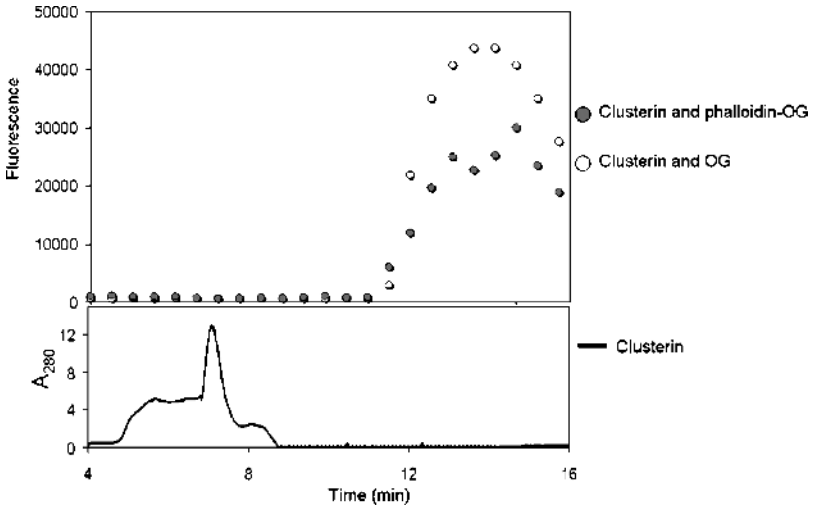

Figure W5. Solutions containing clusterin and either OG or phalloidin-OG (all at 5 μM) were fractionated by size exclusion chromatography in PEM buffer, and the OG fluorescence and A_{280} of the eluate were measured as described in the Materials and Methods section. The upper panel shows OG fluorescence as a function of elution time; the lower panel shows the corresponding A_{280} profile. The identity of individual traces is indicated in the corresponding key.