Nucleotide-based therapies targeting clusterin chemosensitize human lung adenocarcinoma cells both \textit{in vitro} and \textit{in vivo}

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Abstract

Introduction: Lung cancer is highly lethal and resistant to most anticancer interventions. Treatment resistance is mediated, in part, by enhanced expression of cell survival proteins that help facilitate tumor progression. Clusterin is a stress-associated cytoprotective protein up-regulated by various apoptotic triggers in many cancers and confers treatment resistance when overexpressed. The objectives in this study were to evaluate clusterin expression levels in human lung cancer tissue, and to test effects of clusterin silencing using antisense oligonucleotides (ASOs) and short interfering double-stranded RNAs (siRNAs) on chemosensitivity in human lung cancer A549 cells.

Methods: Clusterin immunostaining was evaluated in a tissue microarray of 149 spotted human lung cancers. The effects of clusterin ASO or siRNA treatment on clusterin expression and chemosensitivity to paclitaxel was examined in A549 cells \textit{in vitro} while the ability of clusterin ASO to chemosensitize \textit{in vivo} was evaluated in immunocompromised mice bearing A549 tumors. Results: More than 80% of human non-small cell lung cancers are immunoreactive for clusterin. Clusterin ASO or siRNA decreased clusterin mRNA expression in A549 cells >75% in a dose-dependent, sequence-specific manner, and significantly enhanced chemosensitivity to paclitaxel \textit{in vitro}. Characteristic apoptotic DNA laddering was observed after combined treatment with ASO plus paclitaxel, but not with either agent alone. \textit{In vivo} administration of clusterin ASO, compared to mismatch control oligonucleotide, synergistically enhanced the effects of paclitaxel or gemcitabine to significantly delay A549 tumor growth. Conclusion: These findings identify clusterin as a valid therapeutic target in strategies employing novel multimodality therapy for advanced lung cancer.


Introduction

Lung cancer is the leading cause of death from malignancy in both males and females with more than 200,000 new cases diagnosed in North America last year. Over 85% of these patients eventually die from disseminated disease accounting for 29% of all cancer deaths, more than prostate, colorectal, and breast cancers combined (1). Sixty percent of patients are considered inoperable at the time of diagnosis and 20% are poor surgical candidates. The overall 5-year survival rate of 10–13% has not changed appreciably over the last two decades. Adjutant radiotherapy improves local control but has no effect on survival (2). Traditional chemotherapeutic agents, such as alkylating agents or antimetabolites, produce response rates (RR) of <15% in non-small cell lung cancer (NSCLC) patients and do not improve survival rates (3). Cisplatin is the first chemotherapeutic agent shown to improve survival in patients with all stages of disease, improve quality of life, alleviate symptoms, and possibly induce radiosensitization (3–5). However, RR with single agent cisplatin are only 20% and toxicity limits its use to approximately 50% of patients with good pretreatment functional status (6). Several promising new agents, including taxanes and gemcitabine, are being studied in Phase II/III trials with combination regimens including cisplatin or carboplatin yielding responses in 40–50% of patients (7). Two-drug taxane/platinum combinations have consistently doubled median survival times in multicenter Phase III trials with 1-year survival rates of 35–38% compared to 25% with older cisplatin combinations (8). Advanced disease that is resistant to conventional therapies remains the primary obstacle to improved survival in patients with lung cancer. The development of novel therapeutic options is imperative, particularly those employing combination strategies targeting genes involved in apoptosis and tumor progression.

Advances in the field of nucleic acid chemistry offer one attractive strategy to silence gene products mediating tumor progression and treatment resistance. Antisense oligonucleotide (ASO)-based agents specifically hybridize with complementary mRNA regions of a target gene to form RNA/DNA duplexes and thereby inhibit gene expression (9). Several ASO targeted against specific genes involved in neoplastic progression have been evaluated as potential therapeutic agents (10–13). Collectively, these studies identify ASO as a novel class of antineoplastic agents when designed for appropriate molecular targets. However, because numerous genes are involved in tumor progression, simultaneous inhibition of multiple target genes...
genes may be necessary to optimally inhibit tumor progression. In fact, combined use of ASO with other compounds, such as chemotherapeutic agents, has demonstrated synergistic antineoplastic effects in several tumor models (14–17). Isis 3521, a phosphorothioate ASO targeting PKCα, is currently in Phase III trials in combination with docetaxel in patients with lung cancer (18).

Another novel mechanism of gene down-regulation is the use of RNA interference (RNAi). When introduced into cells, short interfering double-stranded RNAs (siRNAs) can potently suppress gene expression by mechanisms such as mRNA degradation or inhibition of translation. Inhibition of several targets using siRNA has been reported to suppress growth of various cancer cell lines, and hence offers a second nucleotide-based approach at suppressing gene products for loss-of-function analyses (19). Furthermore, siRNAs have been shown to enhance sensitivity to different chemotherapeutic agents in vitro (20).

Tumor progression and drug resistance results, in part, from increased expression of cell survival genes that collectively regulate the apoptotic ‘rheostat’ of cancer cells. Clusterin, also known as testosterone-repressed prostate message-2 (TRPM-2), and sulfated glycoprotein-2 (SGP-2), is a highly conserved disulphide-linked heterodimeric sulfated glycoprotein originally isolated from ram rete testes fluid (21) and first cloned from regressing rat ventral prostate tissue (22). It is present in most animal tissues and body fluids and is implicated in a wide variety of physiological and pathological processes including tissue remodeling, lipid transport, phagocyte recruitment signaling, complement cytolysis inhibition, and apoptosis (23–25). Because clusterin expression is enhanced in various normal and malignant tissues undergoing apoptosis (26–29), it has been regarded as a marker for cell death and a possible mediator of apoptosis. Although clusterin was initially reported as an androgen-repressed gene in prostate tissue (30), its functional role in apoptosis was poorly defined.

Recent studies, however, identify a cytoprotective, anti-apoptotic function of clusterin in several tissues, including prostate epithelial cells following androgen ablation (31, 32), renal tubular cells after oxidative stress (26), and thymocytes under negative selection (33). In general, clusterin is down-regulated during cell proliferation (34), but up-regulated during conditions of cell stress, cell atrophy, and organ involution (23, 35). More specifically, clusterin appears to display a chaperone-like activity similar to small heat shock proteins (sHSP) important for cytoprotection in various disease states and during periods of pathological stress (36, 37). In prostate cancer, clusterin expression is associated with development of androgen resistance and plays a protective role against treatment-induced cell death. For example, overexpression of clusterin in stably transfected human prostate LNCaP cells renders them resistant to androgen ablation in vitro (38), cytotoxic chemotherapy (39), apoptosis induced by tumor necrosis factor α (TNFα) (40, 41), and radiotherapy (42). Systemic administration of clusterin ASO in the androgen-dependent Shionogi tumor model results in earlier onset of castration-induced apoptosis, more rapid tumor regression following castration, and a significant delay in progression to androgen independence (38). Furthermore, combined treatment of Shionogi or human androgen-independent PC3 tumors with clusterin ASOs enhanced the cytotoxic effects of taxol or mitoxanthrone chemotherapy (39, 43). Clusterin is expressed at higher levels in renal cell cancer compared to benign renal tubular cells, and ASO-induced inhibition of clusterin chemosensitized human Caki-2 renal cell cancer cells (44). Clusterin overexpression also correlates with metastatic potential and prognosis in patients with renal cell cancer (45). Collectively, these findings identify that clusterin is a survival protein up-regulated by androgen ablation and chemotherapy that confers resistance to hormone therapy, radiotherapy, and chemotherapy, and that clusterin ASOs enhance cell death following treatment with androgen ablation and chemotherapy.

The presence of clusterin in lung cancer cells was first demonstrated in 1988 (46). While other members of the anti-apoptotic gene family have been extensively studied, the function and potential clinical significance of clusterin in lung cancer have not been characterized. In the present study, we identify clusterin to be prevalently expressed in human NSCLC and report for the first time that down-regulation of clusterin using sequence-specific ASOs and siRNA enhances paclitaxel chemosensitivity in human lung cancer A549 cells.

Materials and Methods

Tumor Cell Line

A549 cells, derived from human lung adenocarcinoma, were purchased from the American Type Culture Collection (Rockville, MD). Cells were maintained in Ham’s F12K medium supplemented with 2 mm l-glutamine, 1.5 g/l sodium bicarbonate, 10% fetal bovine serum, and penicillin/streptomycin.

Chemotherapeutic Agents

Paclitaxel was purchased from Sigma Chemical Co. (St. Louis, MO). Stock solutions of paclitaxel were prepared with DMSO, and diluted with PBS to the required concentrations before each in vitro experiment. Polymeric micellar paclitaxel used in the in vivo studies was generously supplied by Dr. Helen M. Burt (Faculty of Pharmaceutical Sciences, University of British Columbia, Vancouver, Canada). Gemcitabine (Lilly Pharmaceuticals, Indianapolis, IN) was obtained from the B.C. Cancer Agency, Vancouver, BC, Canada.

Antisense Clusterin Oligonucleotides (Clusterin ASOs)

Second generation 2'-methoxyethyl (MOE) gapmer oligonucleotides (OGX-011) used in this study were supplied by OncoGenex Technologies (Vancouver, BC, Canada). The sequence of clusterin ASO corresponding to the human clusterin translation initiation site was 5’-CAGCAGCA-GAGTCTTCATCAT-3’. A two-base clusterin mismatch
oligonucleotide (5'-CAGCAGCGAGGAATTTATCAT-3') and a scrambled oligonucleotide (5'-CAGCGCTGACAA-CAGTTTCAT-3') were used as controls. Dual controls were used for in vitro studies while mismatch control alone was employed in the in vivo studies.

Small Interfering RNA for Use in RNA Interference of Clusterin

The siRNA sequence used was supplied by Dharmacon Research Inc. (Lafayette, CO). The sequence of siRNA corresponding to the human clusterin initiation site was 5'-GCAGCAGACGUUCAUAU-3'. A scrambled control siRNA was used with sequence 5'-CAGCGCGACACACUGUUAU-3'.

Treatment of Cells with ASO and RNAi

Lipofectin, a cationic lipid (Invitrogen Life Technologies, Inc., Burlington, Ontario, Canada) was used to enhance transfection of cells with the oligonucleotides in vitro. A549 cells were treated with 10–1000 nM ASO and RNAi after 20 min preincubation with 4 μg/ml lipofectin in serum free OPTI-MEM (Invitrogen). Four hours after starting the incubation, the medium containing oligonucleotides and lipofectin was replaced with standard culture medium described above. Cells were treated once daily for three consecutive days then harvested 24–48 h following the final treatment.

Northern Blot Analysis

Total RNA was isolated from A549 cells using the RNeasy Mini Kit (Qiagen Inc., Basel, Switzerland). Ten-microgram aliquots from each sample were then subjected to electrophoresis on 1.2% agarose-formaldehyde gels and transferred to nylon membranes (Amersham Life Science, Arlington Heights, IL) overnight according to standard procedure. Following prehybridization in ULTRAhyb solution (Ambion, Austin, TX), the RNA blots were hybridized with human clusterin cDNA probe labeled with 32P-deoxyctydinetriphosphate by random primer labeling. After stripping, membranes were rehybridized with a human glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA probe. Probes were generated by reverse transcription-PCR from human kidney total RNA of using primers 5'-AAGGAAA-TCAAATGCTGCAA-3' (sense) and 5'-ACAGACAAAGATCTCCCGACACT-3' (antisense) for clusterin, and 5'-TGCTTTTAACTCTGGTAAGT-3' (sense) and 5'-ATAATTGGCAGGTTTTCTGA-3' (antisense) for GAPDH. Density of bands for clusterin was normalized against that of GAPDH by densitometric analysis.

Real-Time PCR

Total RNA was isolated from A549 cells using the Rneasy Mini Kit (Qiagen). For cDNA synthesis, reverse transcription-PCR was carried out in the standard fashion using 2 μg of extracted RNA per sample. The Applied Biosystems 5700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA) was used for real-time monitoring of PCR amplification of the cDNA following the Taqman Universal PCR Master Mix protocol (47). The amplification of clusterin cDNA was performed using primers and Taqman probes (Nucleic Acids Protein Services [NAPS] Unit, University of British Columbia Biotechnology Laboratory, Vancouver, BC, Canada) consisting of 5'-GAGCAGCTGAACAGCTGTT-3' as a forward primer, 5'-CTTGCCTTGGCTGAG-3' as a reverse primer, and 5'-VIC-ACATCCCTGTCGCT-GGCA-TAMRA-3' as the Taqman probe. Relative quantification of gene expression was performed using rRNA as a control. Ribosomal cDNA was amplified separately on a duplicate set of samples using standard primers and Taqman probe (Perkin-Elmer). The comparative CT (cycle threshold) method was used for relative quantification of clusterin mRNA.

MTT Assay

The effects of ASO, siRNA, and control oligonucleotides on paclitaxel sensitivity in A549 cells in vitro were assessed using the MTT assay as described previously (48). Briefly, 3 × 10⁴ cells were plated in 96-well plates and allowed to attach overnight. The next day, cells were treated with either clusterin ASO, scrambled or MM (500 nM), or RNAi (50 nM) oligonucleotide for 4 h daily for 2 days. Cells were then treated with various concentrations of paclitaxel ranging from 0 to 500 nM. After 72 h of incubation, 20 μl of 10 mg/ml dimethylthiazolid-2-yl 2,5-diphenyltetrazolium bromide (Sigma) in PBS were added to each well, followed by incubation for 4 h at 37°C. The formazan crystals were dissolved in DMSO. The absorbance was determined with a microculture plate reader (Becton Dickinson Labware, Lincoln Park, NJ) at 560 nm. Absorbance values were normalized to the values obtained for the vehicle-treated cells to determine the percentage of survival. There were six identical wells per assay.

DNA Fragmentation Analysis

The nucleosomal DNA degradation was analyzed as described previously with a minor modification (48). Briefly, 1 × 10⁶ A549 cells were plated in 10-cm culture dishes and allowed to adhere overnight. After treatment with ASO plus paclitaxel using the same schedule as described above, cells were harvested and then lysed in a hypotonic lysing buffer containing 10 mM Tris (pH 7.5), 10 mM EDTA, and 0.5% Triton. After centrifugation at 12,000 rpm for 15 min, the supernatants, containing the fragmented DNA, were incubated with 400 μg/ml proteinase K for 3 h at 65°C. The DNA was extracted by the addition of phenol-chloroform (1 volume). Following centrifugation, the aqueous upper layer was treated with 2.5 M sodium acetate and 1 volume of isopropanol. The DNA precipitates were pelleted, air-dried, and resuspended in 10 mM Tris and 1 mM EDTA (pH 7.4). Following treatment with 100 μg/ml RNase A for 1 h at 37°C, the samples were electrophoresed on a 2% agarose gel and the DNA visualized with ethidium bromide.

Tissue Microarray and Immunohistochemistry

Paraffin blocks from 149 lung cancer specimens were generously provided by the Department of Pathology at the Vancouver Hospital and Health Sciences Centre. Patient demographics, pathology number, paraffin block label, diagnosis, and clinical correlative data were entered in a
with clusterin ASO alone, clusterin ASO plus paclitaxel, or A549 tumors grew to 1 cm in diameter, usually 8–10 weeks (5% induction- and 1.5% maintenance-concentration). When Dawley Inc., Indianapolis, IN) under halothane anesthesia male athymic mice (BALB/c strain; Harlan Sprague-s.c. in each of two sites in the flank region of 6- to 8-week-old (Becton Dickinson Labware, Bedford, MA) were inoculated with goat polyclonal antibody against clusterin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was carried out overnight at room temperature. The next day, the primary antibody was carefully washed 3 times with PBS and then blocked with 5% bovine serum albumin (Promega, Madison, WI) for 30 min. Sections were then washed again and incubated with 3% H2O2 in PBS for 15 min to block endogenous peroxidase activity. Incubation with goat polyclonal antibody against clusterin was independently evaluated and scored by two pathologists (L.F., J.E.). Specimens were graded from 0 to +3 intensity representing the range from no staining to heavy staining (0–100%) was indicated. All comparisons of staining and the overall percentage of cancer cells show-ning staining (0–100%) was indicated. All comparisons of staining intensity and percentages were made at 400 × magnification. All tissue sections contained non-tumor cells such as nerves, vascular wall, and stromal elements that served as internal positive and negative controls for the assessment of antibody specificity and epitope immunopreservation.

Assessment of in Vivo Tumor Growth

Approximately 6 × 106 A549 cells with 0.1 ml of Matrigel (Becton Dickinson Labware, Bedford, MA) were inoculated s.c. in each of two sites in the flank region of 6- to 8-week-old male athymic mice (BALB/c strain; Harlan Sprague-Dawley Inc., Indianapolis, IN) under halothane anesthesia (5% induction- and 1.5% maintenance-concentration). When A549 tumors grew to 1 cm in diameter, usually 8–10 weeks after injection, mice were randomly selected for treatment with clusterin ASO alone, clusterin ASO plus paclitaxel, or mismatch control oligonucleotides plus paclitaxel. Each experimental group consisted of seven mice. Following randomization, 12.5 mg/kg clusterin ASO or mismatch control oligonucleotide was administered i.p. once daily for the first week then 3 times per week until the completion of chemotherapy. Longer treatment intervals were used as the MOE backbone significantly increases tissue half-life of the oligonucleotides (49). From days 7 to 11, and from days 21 to 25, 0.5 mg polymeric micellar paclitaxel was administered 3×/week by i.v. injection. Tumor volume was measured once weekly and calculated by the formula length × width × depth × 0.5236 (49). Data points were reported as mean tumor volumes ± SD. The above protocol was then repeated in a second set of mice using non-obese diabetic severe combined immunodeficient (NOD SCID) mice (Prostate Center, Vancouver General Hospital). Finally, experiments were designed to determine whether clusterin ASO could enhance the activity of gemcitabine, another commonly used cytotoxic for patients with NSCLC. A total of 22 mice was given injections of A549 cells as described above and randomly assigned to 12.5 mg/kg clusterin ASO (n = 12) or mismatch control oligonucleotide (n = 10) once daily for the first week then 3 times per week while 120 mg/kg of gemcitabine was administered i.p. every third day for 5 weeks. Full Institutional Review Board and University of British Columbia Committee on Animal Care approval was granted for this study.

Statistical Analysis

The in vitro cytotoxic effects of antisense or mismatch oligonucleotide and paclitaxel were analyzed with the use of a repeated-measure ANOVA model. All other data were analyzed by Student’s t test. The levels of statistical significance were set at P < 0.05 (two-sided), and all statisti-cal calculations were done using Statview 5.0 software (Abacus Concepts, Inc., Berkeley, CA).

Results

Clusterin Immunostaining in Lung Cancer Tissue Microarray

Table 1 summarizes clusterin immunostaining in differ-ent lung tumor types. Clusterin immunoreactivity was observed in 82% of the lung cancer specimens while 18% of the cases were scored as negative. No association was observed between clusterin level and tumor type. Twenty-seven percent of the total tumor cores in this microarray displayed glandular or papillary structure consistent with adenocarcinoma. The remainder of the tumor cores either showed squamous differentiation or were large cell type cancers. One case of small cell carcinoma is also included. The staining pattern of clusterin was granular and confined to the cytoplasm. The intensity of clusterin immunostaining was slightly higher in less differentiated tumors compared to well-differentiated tumors but this difference did not achieve statistical significance (Fig. 1, Table 1).

Sequence-Specific, Dose-Dependent Suppression of Clusterin Expression by ASO and siRNA

Northern blot analysis was used to determine the effect of treatment with clusterin ASO on clusterin mRNA
expression in A549 cells. As shown in Fig. 2, A and B, daily treatment of A549 cells with clusterin ASO (10, 50, 100, 500, or 1000 nM) for 3 days reduced clusterin mRNA levels by 0%, 45%, 75%, 75%, and 70%, respectively, while clusterin mRNA expression was not affected by mismatch control oligonucleotides at any of the employed concentrations. These findings were confirmed by real-time PCR demonstrating a 60% reduction of mRNA levels in cells treated with clusterin ASO in higher concentrations ranging from 100 to 1000 nM compared with mismatch or vehicle-treated cells (data not shown). Similarly, real-time PCR of clusterin siRNA-treated cells (50–100 nM) detected a 60–80% reduction of mRNA levels compared with scramble siRNA control (data not shown). Western analysis of clusterin siRNA-treated A549 cells (10, 50, 100 nM) demonstrated a dose-dependent down-regulation of protein expression of clusterin (Fig. 2C). Clusterin levels were not affected by scrambled siRNA treatment.

Enhanced Chemosensitivity in Vitro after Clusterin ASO or RNAi Treatment

To determine whether treatment with clusterin ASO enhances the cytotoxic effects of paclitaxel, A549 cells were treated with 500 nM clusterin ASO or mismatch control oligonucleotides once daily for 2 days and then incubated with medium containing various concentrations of paclitaxel for 3 days. The MTT assay was then performed to determine cell viability. As shown in Fig. 3A, clusterin ASO treatment significantly enhanced chemosensitivity of paclitaxel in a dose-dependent manner, reducing the IC₅₀

Table 1. Clusterin immunostaining in lung cancer tissue microarray

<table>
<thead>
<tr>
<th>Tumor Type</th>
<th>Grade of Differentiation</th>
<th>No. of Cases Examined/No. of Total Cases</th>
<th>Clusterin-Positive Cells Mean Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenocarcinoma</td>
<td>Well differentiated</td>
<td>18/44</td>
<td>40.9% 1.2</td>
</tr>
<tr>
<td></td>
<td>Moderately differentiated</td>
<td>13/44</td>
<td>29.5% 1.3</td>
</tr>
<tr>
<td></td>
<td>Poorly differentiated</td>
<td>13/44</td>
<td>29.5% 1.3</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>Well differentiated</td>
<td>12/79</td>
<td>13.5% 1.0</td>
</tr>
<tr>
<td></td>
<td>Moderately differentiated</td>
<td>31/79</td>
<td>41.5% 1.2</td>
</tr>
<tr>
<td></td>
<td>Poorly differentiated</td>
<td>36/79</td>
<td>35% 1.2</td>
</tr>
<tr>
<td>Large cell carcinoma</td>
<td>N/A</td>
<td>25/25</td>
<td>100% 1.24</td>
</tr>
<tr>
<td>Small cell carcinoma</td>
<td>N/A</td>
<td>1/1</td>
<td>0 0.00</td>
</tr>
</tbody>
</table>

Figure 1. Immunohistochemical analysis of clusterin in human lung cancer tissue microarray. Human non-small cell lung tumors originating from the bronchial epithelial lining are immunoreactive for clusterin. A, moderately differentiated adenocarcinoma of the lung. C, moderately differentiated squamous cell carcinoma of the lung. B and D, antibody specificity was confirmed by using clusterin antigen to block antibody staining.
(i.e., the concentration that reduces cell viability by 50%) of paclitaxel by 75% (70–17 nM), whereas mismatch or scrambled oligonucleotides had no effect. Clusterin RNAi also enhanced the chemosensitivity of paclitaxel in a dose-dependent manner, reducing the IC50 by 98% (250–5 nM) (Fig. 3B).

A DNA fragmentation assay was performed to compare induction of apoptosis after combined treatment with 500 nM clusterin ASO and 10, 25, or 50 nM concentrations of paclitaxel (Fig. 3C).

**Figure 2.** Sequence-specific inhibition of clusterin expression by ASO and siRNA in A549 tumor cells. **A,** A549 cells were treated daily with various concentrations of clusterin ASO or a two-base clusterin mismatch control oligonucleotide for 3 days. Total RNA was extracted from cultured cells then clusterin and GAPDH levels analyzed by Northern blotting. **Lipo,** vehicle control-treated cells. **B,** laser densitometry was used to quantify clusterin mRNA levels after normalization to GAPDH mRNA levels in A549 cells after treatment with various concentrations of ASO or mismatch oligonucleotides. Points, means of triplicate analyses; bars, SD. *, differs from control (P < 0.001). **C,** Western blot and densitometry (normalized to vinculin) analysis of clusterin protein levels in A549 cells following treatment with various doses of clusterin and scrambled siRNA.

**Figure 3.** Effect of clusterin suppression on paclitaxel sensitivity in A549 cells. **A,** cells were treated daily with 500 nM clusterin ASO or scramble control oligonucleotide for 2 days. Following treatment, cells were trypsinized, replated, allowed to adhere overnight, then treated with various concentrations of paclitaxel. After 72 h of incubation, cell viability was determined by in vitro mitogenic assay. Clusterin ASO pretreatment decreased the IC50 by >70%. Points, means of three independent experiments; bars, SD. Similar results were obtained with mismatch control oligonucleotides (not shown). **B,** A549 cells treated with 50 nM clusterin or scrambled siRNA for 2 days and various concentrations of paclitaxel. siRNA pretreatment decreased the IC50 by >90% with cell viability determined by MTT assay. **C,** DNA fragmentation assay was performed to compare induction of apoptosis after combined treatment with 500 nM clusterin ASO and various concentrations of paclitaxel. Following the same treatment schedule described above, DNA was extracted from cultured cells, electrophoresed in a 2% agarose gel, and visualized by ethidium bromide staining and UV transillumination. Characteristic DNA laddering was observed only after combined treatment with clusterin ASO and higher concentrations of paclitaxel.
paclitaxel. After the same treatment schedule described above, characteristic apoptotic DNA laddering was observed only after combined treatment with clusterin ASO and paclitaxel in 25 or 50 nm concentrations (Fig. 3C).

Enhanced Chemosensitivity of A549 Tumors in Vivo after Clusterin ASO Treatment

In two separate sets of in vivo experiments, male athymic nude and NOD-SCID mice bearing A549 tumors approximately 1 cm in diameter were randomly selected for treatment with clusterin ASO plus paclitaxel, mismatch control oligonucleotides plus paclitaxel, or clusterin ASO alone. Mean tumor volume was similar at the beginning of treatment in nude (643–688 mm³) and SCID (416–452 mm³) mice. After randomization, 12.5 mg/kg clusterin ASO or mismatch control oligonucleotides were injected i.p. 3 times per week for 21 days. From days 7 to 11, and from days 21 to 25, 0.5 mg polymeric micellar paclitaxel was administered 3 times per week by i.v. injection. As shown in Fig. 4, A and B, clusterin ASO enhanced micellar paclitaxel chemosensitivity in A549 tumors in both nude and SCID mice, causing a 54% reduction in mean tumor volume in both nude and SCID mice by 5 weeks following initiation of treatment. Clusterin ASO also enhanced the in vivo activity of gemcitabine, causing a 60% reduction in mean tumor volume by 5 weeks after initiation of treatment (Fig. 5). Under the experimental conditions used in the above in vivo experiments, no side effects associated with ASO treatment and/or chemotherapy were observed.

Discussion

Despite significant advances in oncology over the last several decades, lung cancer remains highly lethal. Most patients present with advanced disease and are often inoperable at the time of diagnosis. Radiotherapy has no effect on survival, and cisplatin is the first agent demonstrated to increase survival with a 20% RR (6). Five promising new drugs have been shown to achieve survival rates equivalent or superior to cisplatin, and when used in combination with cisplatin or carboplatin, RRs are as high as 40–50%. These agents include paclitaxel, docetaxel, vinorelbine, irinotecan, and gemcitabine (7). The limited efficacy of cytotoxic chemotherapy and radiotherapy remains a major obstacle for the treatment of patients with advanced lung cancer.

Lung cancer progression is a complex process involving disruption of key cell-cycle regulators and signal transduction cascades (50, 51). Studies on the mechanisms of chemoresistance have focused on the regulation of drug transport and metabolism such as the up-regulation of the toxin transport pump MDR1 (52). Chemoresistance may also develop from alterations in the apoptotic machinery, secondary to increased activity of anti-apoptotic pathways or the expression of anti-apoptotic genes. Ironically, agents used to destroy malignant cells may also induce the
expression of genes that mediate radiation- and chemoresistance. Survival proteins up-regulated after apoptotic triggers that function to inhibit cell death include anti-apoptotic members of the bcl-2 protein family, clusterin, HSPs, and survivin (53).

Bcl-2 is a potent anti-apoptotic protein capable of protecting cells from a diverse array of apoptotic stimuli. The bcl-2 protein family comprises several anti-apoptotic proteins (e.g., bcl-2, bcl-x, A1) and pro-apoptotic proteins (e.g., bax, bad, diva), the interactions of which define the cell’s susceptibility to apoptosis (53, 54). HSPs not only protect cells from heat-induced stress, but also from anticancer drugs and other apoptotic stimuli. Hsp70 and Hsp27 are often overexpressed in cancer and appear to function downstream of cytochrome c release and caspase activation (53, 55). The inhibitor of apoptosis (IAP) protein family suppresses apoptosis primarily by direct pro-caspase and effector caspase (caspases 3 and 7) inhibition (56). The most notable IAP, survivin, while absent in differentiated adult tissues, is preferentially expressed in the majority of cancer cell lines. Finally, clusterin is a sulfated glycoprotein capable of inhibiting apoptosis by several mechanisms, including inhibition of stress-induced protein precipitation (36, 39).

Clusterin expression is highly up-regulated in various tissues undergoing apoptosis, including normal and malignant breast and prostate tissues following hormone withdrawal (26–29). While initially considered an androgen-repressed gene and marker of apoptosis after castration in the involuting rat prostate gland (30), clusterin up-regulation following castration is inhibited when apoptosis is attenuated by pretreatment with calcium channel blockers (38). Under these conditions, clusterin levels remained unchanged, confirming that clusterin is an apoptosis-related gene rather than androgen-repressed gene (38, 57). Furthermore, clusterin up-regulation following androgen ablation in xenograft tumor models accelerates progression to the androgen-independent phenotype and renders cells resistant to various apoptotic stimuli including taxane chemotherapies (38, 39). Clusterin exhibits chaperone-like activity, inhibiting stress-induced protein precipitation in a manner analogous to sHSPs, and its promoter contains a 14-bp element recognized by transcription factor heat shock factor 1 (HSF1) (36, 37). Recent preclinical studies provide proof of principle evidence that targeting cell survival genes, such as clusterin or bcl-2, with ASOs enhances apoptosis induced by conventional chemotherapies (17, 38, 39), and has led to clinical trials testing ASO therapy in combination with chemotherapy at several institutions (15, 58, 59).

Antisense strategies have been applied against various targets in lung cancer including PKCa, C-raf kinase, thrombomodulin, C-raf kinase, THP-1, and various cell survival genes (18, 60–62). Bcl-2 and bcl-xL are highly expressed in SCLC and NSCLC respectively. Induction of apoptosis has been demonstrated with single-agent bcl-2 ASO treatment in SCLC and with bcl-xL ASO treatment in NSCLC and mesotheliomas (51, 63, 64). A novel bspecific ASO that down-regulates bcl-2 and bcl-xL expression has activity in both SCLC and NSCLC cell lines, as well as breast and colorectal carcinomas (65, 66). Synergistic effects have been noted with the addition of bcl-2 or survivin ASO therapy to conventional lung cancer chemotherapy with cisplatin or etoposide (67, 68). Finally, Phase I/II studies combining PKCa phosphorothioate ASO (Isis 3521) with carboplatin and paclitaxel in patients with stage IIIB or IV NSCLC showed a 1-year survival rate of 78% and 18-month median survival compared with an 8-month survival in patients receiving chemotherapy alone (69). Recent reports of a Phase III trial of PKCa ASO (Affinitak) plus carboplatin and paclitaxel revealed no improved overall survival.

No studies have evaluated the role of clusterin in lung cancer progression since its presence was initially described 1988 (46). An initial objective of the present study was to characterize clusterin expression levels in human NSCLC using a tissue microarray of various human lung cancers. These data confirm that clusterin is present in >80% of tumors originating from bronchial epithelial lining. To clarify the functional role of increased clusterin expression in lung cancer, we then tested whether suppression of clusterin using ASO or RNAi could enhance the cytotoxic effects of paclitaxel in human A549 lung adenocarcinoma cells and tumors. Our results demonstrate that the MOE-gamper clusterin ASO and RNAi used in this study inhibited expression of clusterin mRNA in A549 cells, and sequence specificity was confirmed using appropriate control oligonucleotides, which had no effects on clusterin expression in A549 cells. When used alone, ASO treatment had no effect on A549 cell growth. However, when administered in combination with paclitaxel, clusterin ASO enhanced A549 cell apoptosis both in vitro and in vivo. Pretreatment of A549 cells with clusterin ASO or RNAi reduced the IC50 of paclitaxel by 75% and 98% respectively. Consistent with these in vitro results, synergistic effects of combined use of clusterin ASO plus paclitaxel was also observed in vivo. Systemic administration of clusterin ASO plus paclitaxel (or gemcitabine) suppressed A549 tumor growth compared to treatment with mismatch control oligonucleotides plus paclitaxel. Detection of increased apoptosis after combined ASO and chemotherapy by DNA fragmentation analysis in A549 cells suggests that decrease in tumor progression rates after combined clusterin ASO plus paclitaxel resulted from enhanced chemotherapies-induced apoptosis rather than decreased cell proliferation. These findings may have implications with respect to development of future therapeutic strategies. Indeed, the 2’ MOE-gamper clusterin ASO used in this study, also referred to as OGX-011, is currently being evaluated in Phase I trials in combination with docetaxel.

In summary, these data demonstrate that oligonucleotide-induced suppression of clusterin expression attenuates its anti-apoptotic effects and enhances chemosensitivity. These experimental data support the development of targeted strategies employing clusterin ASO complementary to conventional cytotoxic therapies for advanced lung cancer.
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References


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Laura V. July, Eliana Beraldi, Alan So, et al.


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