Induction of Apoptosis in Mesothelioma Cells by Antisurvivin Oligonucleotides

Chunyao Xia, Zhidong Xu, Xiaocheng Yuan, Kazutsugu Uematsu, Liang You, Kai Li, Li Li, Frank McCormick, and David M. Jablons[1]

Department of Surgery, Comprehensive Cancer Center, University of California, San Francisco, California 94115

Abstract

Malignant pleural mesothelioma is a rare and aggressive tumor characterized by rapid progression, late metastases, and poor prognosis. In this study, we investigated the expression of *survivin,* a member of the inhibitors of apoptosis protein gene family, in mesothelioma and an antisense oligonucleotide-based gene therapy for mesothelioma using *survivin* as a target. Initially, we documented the expression of *survivin* in human mesothelioma cell lines and fresh tissues using reverse transcription-PCR and Western blot analysis. Our results showed that survivin was overexpressed in 7 of 8 (87.5%) mesothelioma cell lines assayed and in all (12 of 12; 100%) freshly resected mesothelioma tissues analyzed. To investigate the use of survivin as a therapeutic target on mesothelioma, we carried out transfections with antisurvivin oligonucleotides to induce apoptosis in mesothelioma cell lines MS-1 and H28. Results from cellular transfection and subsequent analysis using the flow cytometry demonstrated that antisurvivin oligonucleotides induced significantly greater apoptosis rates in the survivin-positive mesothelioma cell line H28 (42.5%) as compared with the control oligonucleotides (16.2%; $P < 0.001$). The *survivin*-negative cell line LRK1A (*survivin*−/−) did not apoptose with antisense oligonucleotides. Furthermore, time course evaluation by Western blot analysis showed that *survivin* was inhibited by antisurvivin oligonucleotides within 12 h after transfection. Our results show, for the first time, that *survivin*, an inhibitors of apoptosis protein family gene member, is highly overexpressed in malignant pleural mesothelioma. Down-regulation of *survivin* by a targeted antisense oligonucleotide appears to be an effective gene therapy approach to the treatment of mesothelioma.

Introduction

Apoptosis is the carefully regulated process of programmed cell death that is critical for maintaining normal cell and tissue homeostasis. Dysregulation of cell death pathways often results in tumor initiation, progression, and drug resistance in many human cancers. *Survivin,* a member of the IAP[2] gene family, is of interest because it is specifically up-regulated in cancer cells and completely down-regulated and undetectable in normal adult tissues (1). Although its exact mechanism of action remains unclear, *survivin* has been implicated in the control of cell division and apoptotic cell death (2, 3). Recently, studies using a transgenic mouse model that selectively expresses *survivin* in the skin confirmed that *survivin* selectively inhibits the intrinsic, caspase-9-dependent apoptotic pathway (4).

The high prevalence of *survivin* in many human cancers has prompted studies using *survivin* as a therapeutic target in the treatment of cancer and as a prognostic marker for cancer. Previous studies have shown that reduction of *survivin* expression achieved by antisense strategies can cause apoptotic cell death and sensitization to anticancer drugs in several tumor cell lines (5). These results suggest that *survivin* expression is likely important for cell survival or resistance to chemotherapy in carcinomas.

Different approaches have been described for blocking *survivin* expression in tumor cells. Generation of *survivin*-specific $CD8^+$ T effector cells pulsed with *survivin* peptides was reported to be effective in suppressing *survivin* expression (6). A *survivin* antisense cDNA was shown to down-regulate *survivin* action (5, 7, 8). Antisense oligonucleotides of 17-mer to 20-mer were shown to effectively down-regulate *survivin* expression (7, 9, 10) and to sensitize tumor cells to cytotoxic chemotherapy (10). Moreover, infection of cancer cell lines with a replication-deficient adenovirus encoding a *survivin* mutant (pAdT34A) resulted in a 2–3-fold increase in apoptosis and further enhanced the levels of apoptotic cell death in combination with chemotherapeutic drugs (11).

Malignant pleural mesothelioma is a rare (<4000 United States cases/year) and inexorably fatal tumor characterized by rapid local progression, late metastases, and poor prognosis. Standard cytotoxic chemotherapy and radiation therapy have had limited effectiveness (12, 13), although multimodality therapy (surgery, radiation, and chemotherapy) may increase short-term survival. Molecular genetic changes in mesothelioma development and progression have been reviewed (14). Mutations in the *p53*, *Ras*, and *pRB* genes, which are found in the majority of human tumors, are uncommon in mesothelioma. Instead, homozygous deletion of the *INK4a/ARF* locus is detected in the majority of mesothelioma (15, 16). This deletion results in the loss of $p14^{ARF}$, an increase in *MDM2*, and the functional inactivation of *p53*. In our previous studies, we have showed that reintroduction of

Received 1/30/02; revised 5/9/02; accepted 5/14/02.
[1] To whom requests for reprints should be addressed, at Department of Surgery, Cancer Center, 1600 Divisadero Street, C322C, Box 1674, San Francisco, CA 94115. Phone: (415) 885-3882; E-mail: jablonsd@surgery.ucsf.edu.

[2] The abbreviations used are: IAP, inhibitors of apoptosis protein; RT-PCR, reverse transcription-PCR.

p14^{ARF} into mesothelioma cells leads to the overexpression of p14^{ARF}, which results in G_1-phase arrest and apoptotic cell death (16). Recently, using cDNA microarray hybridization, we have detected a number of IAPs, including *Bcl-2* and *survivin*, that are overexpressed in primary freshly resected human mesothelioma.[3] This has led us to analyze *survivin* expression and function in mesothelioma. In light of up-regulation in other human cancers, *survivin* may also play an important role in preventing apoptosis and cell proliferation in mesothelioma. In the present study, we examined *survivin* expression in multiple mesothelioma cell lines and freshly resected mesotheliomas. We also investigated the efficacy of antisurvivin oligonucleotides in inducing apoptosis in mesothelioma cell lines in which *survivin* was up-regulated.

Materials and Methods

Tumor Tissues and Cell Lines. Freshly resected mesothelioma tumor samples were obtained with consent from patients undergoing resection and snap-frozen in liquid nitrogen tank. Mesothelioma cell lines, including H28, H290, MSTO-211H (211H), MS-1, LRK1A, H513, Met5A, and H2052, breast carcinoma cell line MCF7, lung adenocarcinoma cell line A549, and glioma cell lines U87 and CF210 were obtained from American Type Culture Collection (Manassas, VA). Cells were maintained in RPMI 1640 or MEM complete medium supplemented with 2 mM L-glutamine and 10% FCS at 37°C in a humidified atmosphere containing 5% CO_2.

RNA Isolation. Total RNA was isolated from cell cultures and mesotheliomas using Trizol reagents according to the manufacturer's instructions (Life Technologies, Inc.). Human normal lung total RNA was obtained from Ambion Inc.

cDNA Microarray Hybridization. Incorporation of amino-allyl dUTP into cDNA was conducted with the FairPlay Microarray Labeling kit (Stratagene, La Jolla, CA) using total RNA (10 μg each) from mesothelioma and normal pleura. Fluorescent dyes (Amersham Pharmacia Biotech) were coupled to amino-allyl dUPT-labeled tumor (Cy3) and normal pleural cDNAs (Cy5), respectively, and cohybridized to the cDNA microarray slide (HPLower9k.7) according to DeRisi *et al.*[4] Slides were scanned with the GenePix 4000A scanner (Axon), and acquired images were analyzed with the software GenePix Pro3.0 and Microsoft Excel.

RT-PCR. RT-PCR was performed in a GeneAmp PCR System 9700 using a one-step RT-PCR kit (Life Technologies, Inc.) according to the manufacturer's instructions. Briefly, 1 μg of total RNA was used as template that was mixed with reaction buffer, 10 pmol of sense and antisense survivin gene primers, and 1 μl of reverse transcriptase/platinum Taq mixture in a PCR tube, respectively, in a 50-μl volume. The cDNA synthesis and predenaturation were performed as follows: 1 cycle of 50°C for 30 min and 94°C for 2 min. PCR amplification was continued for 30–35 cycles of 94°C for 15 s, 60°C for 30 s, and 72°C for 1 min. A final extension was performed for 7 min at 72°C. RT-PCR primers were ordered from Life Technologies, Inc. The primer sequences for a 436-bp fragment of *survivin* gene were as follows: survivin-S, 5′-ATGGGTGCCCCGACGTTG-3′; and survivin-A, 5′-AGAGGCCTCAATCCATGG-3′. A 395-bp fragment of the *L19* ribosomal protein gene was used as an internal control (L19-S, 5′-GAAATCGCCAATGCCAACT-3; L19-A, 5′-TCTTAGACCTGCGAGCCTCA-3′).

Western Blotting. After medium was removed, cells were rinsed once with PBS solution at room temperature. After that, all of the following steps were performed on ice. Appropriate amounts of cold radioimmunoprecipitation assay buffer containing proteinase inhibitors were added to the cell culture plate. Cells were removed from the plate and transferred to a 1.5-ml microcentrifuge tube. The cell lysate was passed through a 21-gauge needle to shear the DNA. After centrifugation at 10,000 $\times$ g for 10 min, protein concentration was measured using Bio-Rad Protein Assay reagent. Whole cell lysate protein (30 μg) was boiled for 5 min and separated by 10–20% SDS-PAGE. Proteins were transferred to an Immobilon-P membrane (Millipore Corp., Bedford, MA) using semi-dry transfer cell (Bio-Rad). The membrane was blocked with 5% nonfat milk powder and 0.1% Tween 20 in Tris-buffer saline overnight at 4°C and then incubated with primary antibody for 1 h at room temperature. Membrane was washed in 5% nonfat milk powder and 0.1% Tween 20 in Tris-buffer saline for three 10-min periods. Primary antibodies for survivin and actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated goat antirabbit or donkey antigoat antibodies were used as secondary antibodies. Proteins were visualized with chemiluminescence luminol reagents (Santa Cruz Biotechnology).

Antisurvivin and Control Oligonucleotides. Antisurvivin 20-mer phosphorothioate antisense oligonucleotide targeting nucleotides 232–251 of *survivin* mRNA was used based on the oligonucleotide 4003 sequence (10). The control oligonucleotide was the reverse of the antisense sequence. The antisurvivin oligonucleotide sequence was 5′-CCCAGCCTTCCAGCTCCTTG-3′, and the control primer was 5′-GTTCCTCGACCTTCCGACCC-3′. The primers were ordered from Oligos Etc Inc. (Wilsonville, OR) using the second-generation processing procedure and purified by level 1 high-performance liquid chromatography.

Treatment of Cells with Antisense and Control Oligonucleotides. Two *survivin*-positive mesothelioma cell lines, H28 and MS-1, and one *survivin*-negative cell line, LRK1A, were used for transfection with oligonucleotides. One day before transfection, 2 $\times$ 10^5 cells/well were plated in 6-well tissue culture plates. Cells were rinsed with 2 ml of Opti-MEM I medium before transfection. Oligonucleotides were delivered in the form of complexes with Lipofectin (Life Technologies, Inc.) as follows: 20 μl of Lipofectin were mixed with oligonucleotides (100–600 nM) in 2 ml of Opti-MEM I reduced serum medium and added to prerinsed cells. After culturing for 5 h, the oligonucleotide-Lipofectin mixture was replaced with 2 ml of RPMI 1640, and cells were cultured for an additional 19 h or longer.

[3] L. You and D. M. Jablons. Profiling of differentially expressed genes in human malignant pleural mesotheliomas, submitted for publication.
[4] www.microarrays.org.

Dose-dependent Analysis. To evaluate the effects of oligonucleotides or Lipofectin on cells after transfection, we assessed cell viability using an inverted phase-contract microscope (Leica) and trypan blue exclusion assays. Cells were treated with different concentrations of oligonucleotides (100–600 nM) in 6-well cell culture plates for 5 h. After the medium was changed, cells were continued in culture to 12–36 h. Cells were harvested after trypsinization and resuspended in PBS. An equal volume of 0.4% trypan blue solution (Sigma, St. Louis, MO) was added to the cell suspension. Viable and dead cells were counted with a hemocytometer. All cell counts were done on triplicate samples.

Caspase-3 Activity Assay. The caspase-3 colorimetric activity assay kit was used for measurement of caspase activation essentially according to the manufacturer's instructions (Chemicon International, Inc., Temecula, CA). Briefly, approximately 1×10^6 cells/10-cm dish were treated with antisurvivin oligonucleotides and controls. Cells were harvested at 26 h after the start of treatment and lysed in 200 μl of cell lysis buffer. After incubation for 5 min on ice, lysates were centrifuged at $10{,}000 \times g$ at 4°C for 5 min. Cytosolic protein (200 μg) was mixed with 30 μg of caspase-3 substrate (ac-DEVD-pNA) and incubated at 37°C for 4–5 h. The reaction was monitored at 405 nm using a SPECTRAmax microplate reader and analyzed using Softmax PRO software (Molecular Devices). Human caspase-3 (active) recombinant protein was used as a positive control, and a caspase-3-specific inhibitor (ac-DEVD-CHO) was used as a negative control according to the instructions provided. Fold increase in caspase-3 activity was determined by comparing the absorbance readings from the induced samples with those of untreated controls.

Annexin V Apoptosis Analysis. Approximately 1×10^6 H28 cells were plated in 10-cm dishes and incubated overnight (16 h) at 37°C. Cells were rinsed with Opti-MEM I medium, transfected with 500 nM antisurvivin or control oligonucleotide-Lipofectin complex in 5 ml of serum reduced Opti-MEM I medium for 5 h, and continued to grow for 19 h after changing the medium. H28 cells without oligonucleotide treatment were used as mock control. Cells were harvested after trypsinization, and apoptotic cells were assayed with an annexin V-FITC apoptosis detection kit (Oncogene, Cambridge, MA). Briefly, 5×10^5 cells in 0.5 ml of PBS were incubated with 10 μl of medium-binding reagent and 1.25 μl of annexin V-FITC for 30 min at room temperature in the dark. After centrifugation at $1000 \times g$ for 5 min, the medium was removed, and cells were gently suspended in 0.5 ml of ice-cold 1× binding buffer, and then 10 μl of propidium iodide were added immediately before the flow cytometry analysis (FACScan; Becton Dickinson, Franklin Lake, NJ). Early apoptotic cells with exposed phosphatidylserine but intact cell membranes bound to annexin V-FITC but excluded propidium iodide. Cells in necrotic or late apoptotic stages were labeled with both annexin V-FITC and propidium iodide.

Statistical Analysis. Results were expressed as means ± SD. All statistical analyses were made with a two-sided Student's t test. $P < 0.05$ was considered to be statistically significant.

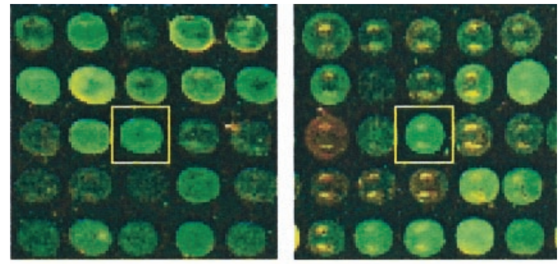


Fig. 1. Representative cDNA microarrays showing overexpressed *Bcl-2* and *survivin* genes in human mesothelioma. Fluorescence-labeled mesothelioma (Cy3) and normal pleural (Cy5) cDNAs were cohybridized to a cDNA microarray slide containing 9000 genes as described in "Materials and Methods." The expanded view shows mRNA expression of the Bcl-2 (block 11, column 15, row 11) and survivin (block 4, column 21, row 11). *Bcl-2* and *survivin* were overexpressed in mesothelioma 5.1- and 4.6-fold, respectively, as compared with normal pleura.

Results

Detection of *Survivin* Gene Expression in Mesothelioma Cell Lines and Tissues. High levels of *survivin* expression have been detected in multiple human cancers, including cancers of the lung, colon, pancreas, prostate, and breast. *Survivin* expression has not been described in human mesothelioma. Using cDNA microarray hybridization, we have recently detected overexpression of the *Bcl-2* and *survivin* genes in a number of mesothelioma tissues. A representative cDNA microarray in Fig. 1 showed that *Bcl-2* and *survivin* genes were both up-regulated 5.1- and 4.6-fold, respectively, in mesothelioma as compared with normal pleura. Considering the overwhelming expression of the IAP gene family member *survivin* in many common cancers, we then extended our screening for *survivin* expression in mesothelioma cells.

We screened 8 human mesothelioma cell lines and 12 fresh human mesothelioma tissues using RT-PCR and Western blot analysis. Our results showed that *survivin* was expressed in both mesothelioma cell lines and freshly resected mesothelioma tissues. As shown in Fig. 2*a,* the *survivin* gene was detected in all but the LRK1A mesothelioma cell line (seven of eight cell lines). It was reported previously that the LRK1A cell line did not express *Bcl-2* (17). Expression of the *survivin* gene in LRK1A was not detected in this study by RT-PCR, and these results were confirmed by Western blot analysis (Fig. 2*a*). This cell line was therefore used as a negative control for antisurvivin oligonucleotide transfection experiments in our study.

Results from screening of freshly resected mesothelioma tissues showed that *survivin* was expressed in all samples screened (12 of 12 samples; Fig. 2*b*). With RT-PCR, we also detected *survivin* gene expression in four other cell lines including breast carcinoma MCF7, lung carcinoma A549, and glioma cells CF210 and U87. *Survivin* expression was not detected in normal human lung RNA, which is consistent with the notion that *survivin* is not expressed in normal and differentiated tissues.

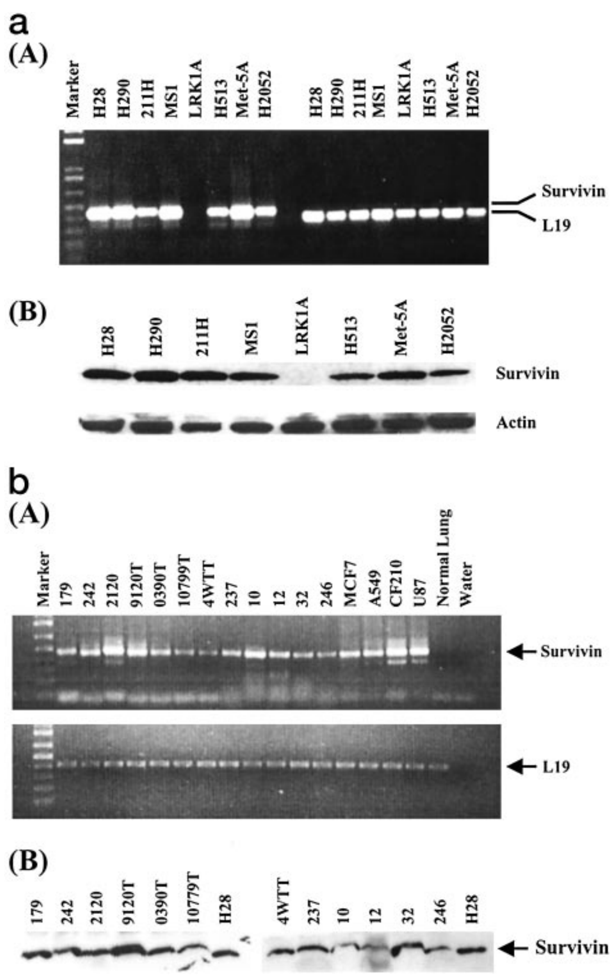


Fig. 2. *a,* detection of *survivin* gene expression in mesothelioma cell lines. *A,* RT-PCR analysis. *Survivin* cDNA primers and *L19* ribosomal protein cDNA primers used as internal control were described in "Materials and Methods." PCR products were electrophoresed on 1% agarose gel stained with ethidium bromide. Mesothelioma cell lines are indicated at the *top*. Relative sizes of RT-PCR products are as follows: *survivin*, 436 bp; and *L19*, 395 bp. Molecular size markers are the 1-kb ladders. *B,* Western blot analysis of *survivin* expression. For each cell line, 30 μg of soluble proteins were analyzed by Western blot and detected by antisurvivin monoclonal antibody, and β-*actin* antibody was used as control for equal sample loadings. *b,* detection of *survivin* expression in freshly resected human mesothelioma tissues. *A,* RT-PCR analyses. Total RNA was isolated from 12 mesothelioma tissues (*179, 242, 2120, 9120T, 0390T, 10799T, 4WTT, 237, 10, 12, 32,* and *246*) and analyzed by RT-PCR using *survivin*- and *L19*-specific primers. Four cell lines (*MCF7, A549, CF210,* and *U87*) were used for comparison. A normal human lung total RNA was used as a negative control for *survivin* expression, and water was used as a negative control for PCR. *B,* Western blot analysis. Soluble proteins (30 μg) from each mesothelioma tissue were analyzed by Western blot and immunochemistry as described above. The mesothelioma sample case numbers are shown on the *top*. The H28 cell line was used as a positive control. β-*Actin* antibody was used as a control for equal sample loading (data not shown).

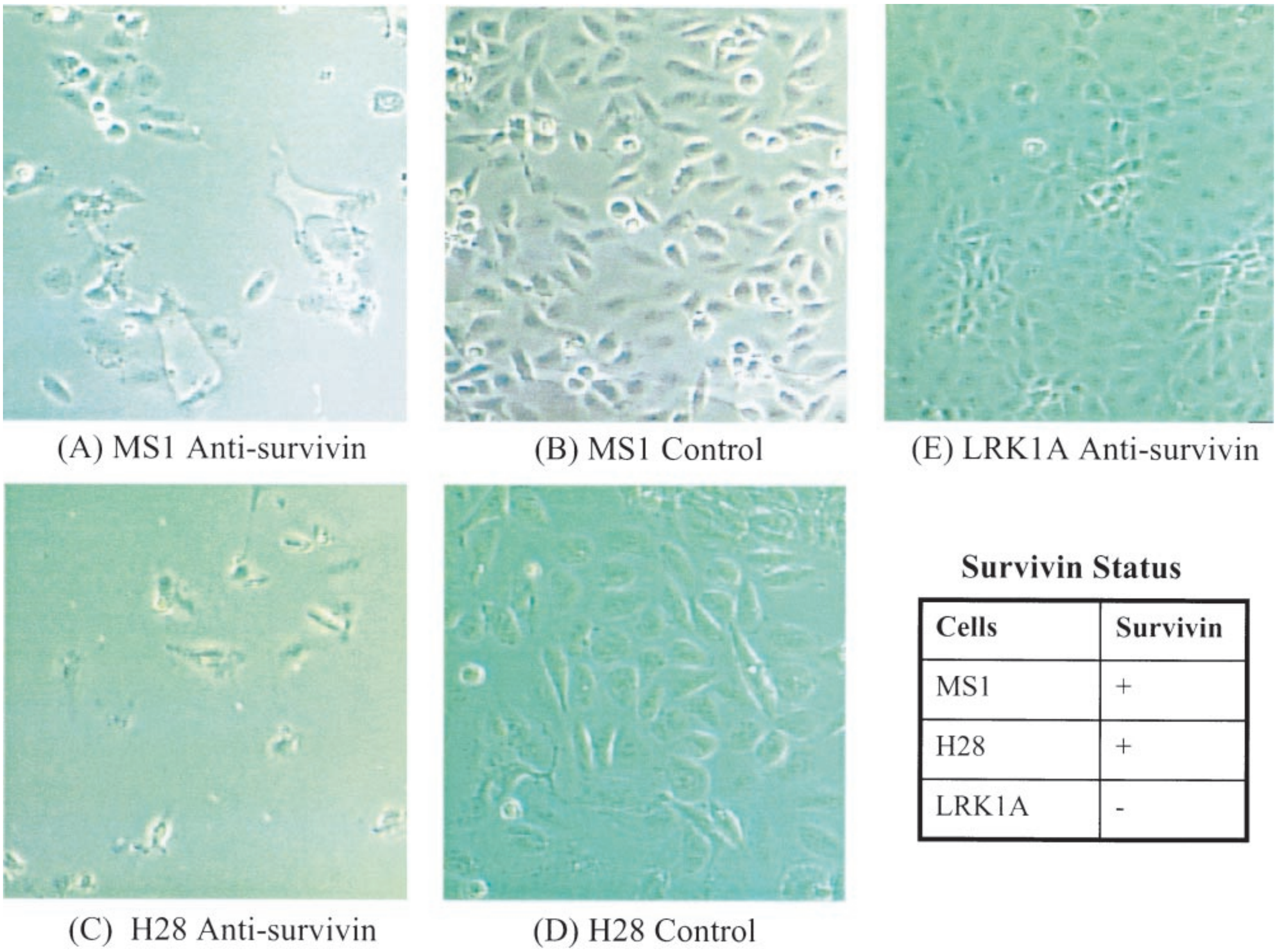
Effects of Antisurvivin Oligonucleotides on Mesothelioma Cell Growth. We first examined the effects of antisurvivin oligonucleotides on cell growth. Two *survivin*-positive mesothelioma cell lines, H28 and MS-1, were treated with 500 nM antisurvivin oligonucleotides and control oligonucleotides. The LRK1A cell line (*survivin* negative) was used as control. As shown in Fig. 3, transfection of antisurvivin oligonucleotide caused cell death in both H28 and MS-1 cells 24 h after transfection (Fig. 3, *A* and *C*). Transfection using control oligonucleotides did not significantly affect cell growth in these two cell lines (Fig. 3, *B* and *D*). In contrast, the LRK1A cell line was not susceptible to apoptotic cell death by antisurvivin oligonucleotides (Fig. 3*E*).

Dose-dependent Analysis of the Effect of Antisurvivin Oligonucleotides on Cell Viability. To investigate the biological effect on cell viability of down-regulation of *survivin,* we treated H28 cells (*survivin* positive) with antisurvivin oligonucleotide 4003 (100–600 mM). The percentage of dead cells was counted using the trypan blue staining method 24 h after the start of transfection. Untreated cells, cells treated with Lipofectin alone, and cells treated with the control oligonucleotide were used for comparison. The apoptotic effects of antisurvivin oligonucleotides on H28 cells appeared from 200 nM and exhibited a dose-dependent manner until 600 nM (Fig. 4). Treatment of cells with antisurvivin oligonucleotides at concentrations higher than 600 nM resulted in severe cell death.

Increased Caspase-3 Activity by Treatment with Antisurvivin Oligonucleotides. We tested whether cell death in MS-1 and H28 cells was due to the induction of apoptosis by antisurvivin oligonucleotide treatment using a caspase-3 activity assay (Fig. 5). Lysates from antisurvivin oligonucleotide-treated cells showed increased caspase-3 activity (7–9-fold higher than that of control groups). These results suggest that the observed cell death in mesothelioma cell lines after treatment with antisurvivin oligonucleotide is likely caused by apoptosis signaling pathways.

Apoptosis Analysis by Flow Cytometry. To further verify that apoptotic cell death resulted from transfection of antisurvivin oligonucleotides, we analyzed H28 cells 24 h after transfection with 500 nM oligonucleotides with an annexin V-FITC apoptosis detection kit. The results showed that antisurvivin oligonucleotides induced significantly greater apoptotic cell death in the *survivin*-positive mesothelioma cell line H28 (42.5%) as compared with the control oligonucleotides (16.2%; $P < 0.001$; Fig. 6). No treatment and treatment with Lipofectin alone did not have a significant apoptotic effect on these cells.

Time Course Inhibition of *Survivin* Expression by Antisurvivin Oligonucleotides. To investigate the duration of *survivin* expression inhibition by antisense oligonucleotides, we carried out a time course analysis by Western blot. H28 and MS-1 cells were transfected for 5 h with 500 nM antisurvivin oligonucleotides, and cells were collected at 12 and 24 h, respectively, after the start of transfection. *Survivin* expression was inhibited in samples harvested at 12 and 24 h after transfection in both cell lines (Fig. 7). Transfection using control oligonucleotides and Lipofectin alone did not affect *survivin* expression. These results indicate that treatment with antisurvivin oligonucleotides can specifically inhibit survivin protein synthesis and lower survivin protein levels within 12 h.



Cells	Survivin
MS1	+
H28	+
LRK1A	-

Fig. 3. Effects of antisurvivin oligonucleotides on mesothelioma cell growth. Cells were treated with oligonucleotide (500 μM)-Lipofectin complex for 5 h, medium was removed, and cells were cultured in fresh medium for an additional 19 h. Cell viability was examined, and photographs were taken under an inverted phase-contract microscope. *A,* MS-1 with antisurvivin oligonucleotide; *B,* MS-1 with control oligonucleotide; *C,* H28 with antisurvivin oligonucleotide; *D,* H28 with control oligonucleotide; *E,* LRK1A with antisurvivin oligonucleotide. The status of survivin expression for each cell line is shown in the *inset*. Magnification, ×400.

Discussion

Expression of the IAP family member *survivin* is up-regulated in a variety of human cancers, with high levels documented in breast and lung cancer (18). Overexpression of *survivin* in tumors correlates with resistance to chemotherapy and poor prognosis in patients with non-small cell lung cancer (19), colorectal cancer (20), and neuroblastoma (21). In this study, we demonstrate, for the first time, that *survivin* is up-regulated in both mesothelioma cell lines (7 of 8 cell lines; 87.5%) and freshly resected mesotheliomas (12 of 12 samples; 100%). These results confirmed our previous observation by cDNA microarray analysis that *survivin* was overexpressed in mesothelioma and may play an important role in tumor development, progression, and, importantly, resistance to chemotherapy.

Therapeutic strategies using antisense oligonucleotides have been found to be an effective way to down-regulate *survivin* and reduce the apoptotic threshold in tumor cells. A number of antisurvivin oligonucleotides have been tested for

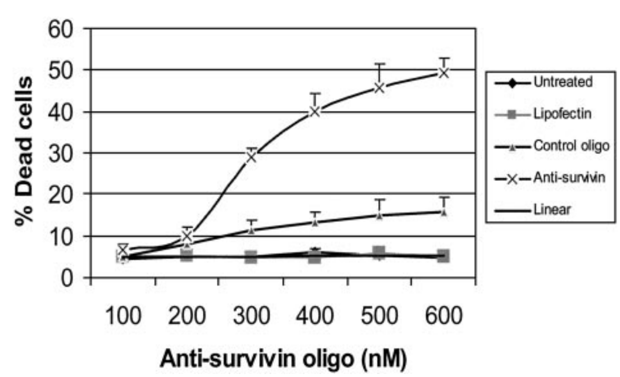


Fig. 4. Dose-response curve of the effect of antisurvivin oligonucleotides on the growth and viability of H28 cells. Cells were treated for 5 h with an increasing concentration of antisurvivin oligonucleotide 4003 (10). Untreated cells, cells treated with Lipofectin alone, and cells treated with the control oligonucleotide were used for comparison. Cell viability was determined in triplicate cultures 24 h after the start of transfection using the trypan blue staining method. Each value represents the means ± SD of three independent experiments.

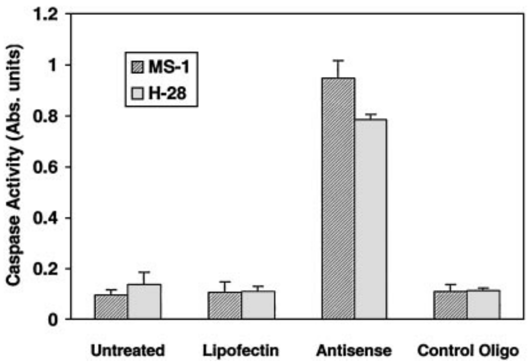


Fig. 5. Caspase-3 activity assay in lysates of MS-1 and H28 cells that were untreated or treated with Lipofectin, 500 nM antisurvivin oligonucleotide, or 500 nM control oligonucleotide. Cells were harvested 26 h after the start of transfection and subjected to caspase-3 activity assays as described in "Materials and Methods." Data are presented as the mean ± SD of three independent experiments.

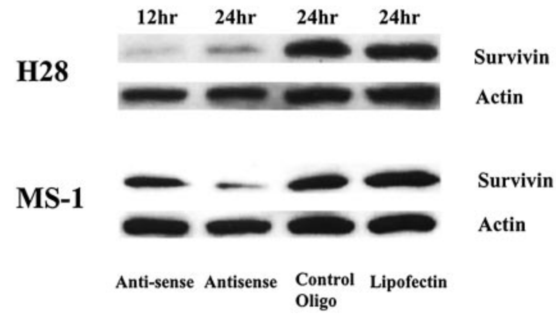


Fig. 7. Western blot analysis of *survivin* expression in the mesothelioma cell lines H28 and MS-1 after transfection with antisurvivin oligonucleotides. Cells were treated for 5 h with 500 nM antisurvivin oligonucleotides and harvested at 12 and 24 h after the start of transfection. Western blots were hybridized with *survivin* and *actin* monoclonal antibodies and visualized by immunochemistry staining.

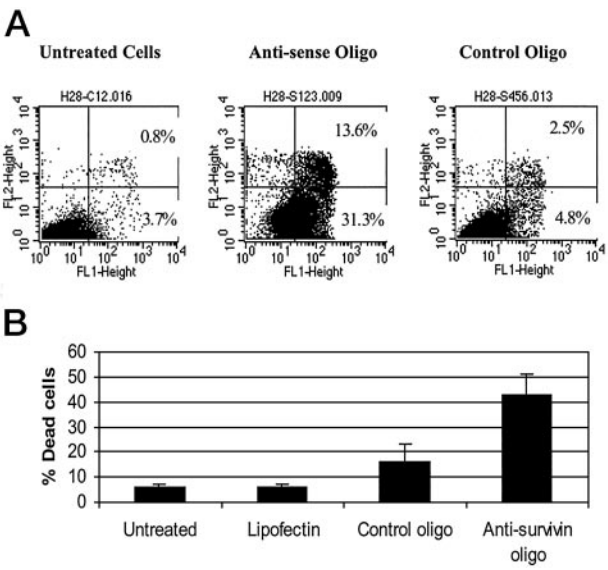


Fig. 6. Apoptosis induction by antisurvivin oligonucleotides in H28 mesothelioma cells. *A,* example of a flow cytometry analysis. Cells were transfected with antisurvivin oligonucleotide or control oligonucleotide or mock-transfected and then harvested and analyzed by flow cytometry. *B,* percentage of dead cells induced by antisurvivin oligonucleotide transfection. Data represent the mean ± SD of three independent experiments.

their ability to block *survivin* expression in tumor cell lines primarily for the elucidation of the biological function of *survivin* during cell division and apoptosis (22). Olie *et al.* (10) tested six antisurvivin oligonucleotides, of which antisense oligonucleotide 4003 was found to be the most effective in inducing growth inhibition and apoptosis in a lung carcinoma cell line A549. This same antisurvivin oligonucleotide used in the present study effectively down-regulated *survivin* expression in two survivin-positive cell lines, H28 and MS-1. Cell viability was significantly decreased in the cells after antisurvivin oligonucleotide treatment as compared with untreated controls. Results from caspase-3 activity assay and flow cytometry analysis further confirmed that the reduced cell viability in mesothelioma cells was due to apoptotic cell death induced by antisurvivin oligonucleotides. These results are consistent with previous studies using the A549 cell line that showed elevated caspase-3-like protein activity after antisense treatment (10). Previous studies have showed that *survivin* can inhibit a number of effector caspases, including caspase-3, -7, and -9 (4, 18), blocking the apoptotic cell death pathways. Immunoprecipitation study and protein structure indicate that the antiapoptotic function of survivin results from an indirect inhibitory role on caspase-3, possibly by promoting a pro-caspase-3-p21 complex (23, 24).

In the present study, we also found that *survivin* expression could be suppressed within 12 h after antisense oligonucleotide transfection (Fig. 7). We have detected a significant reduction of *survivin* expression by Western blot analysis as early as 12 h after transfection that correlates with the induction of apoptosis in both H28 and MS-1 cell lines. Olie *et al.* (10) observed reduction of *survivin* mRNA 20 h after transfection and apoptosis 64–72 h after transfection with antisense oligonucleotides. Our results suggested that transfection of antisurvivin oligonucleotides was efficient in inhibiting *survivin* expression in mesothelioma cells.

Mesothelioma is highly resistant to chemotherapy and radiotherapy (12, 13) and is resistant to apoptosis (17). Resistance to apoptosis may contribute to the overall insensitivity of mesothelioma to standard therapies. Because mesothelioma typically harbors wild-type *p53* (25, 26), a major modulator of apoptosis, its resistance to apoptosis would be expected to arise downstream from *p53,* such as the *Bcl-2* family of proteins that regulate cell death (27). Overexpression of *Bcl-2*, a protein that suppresses apoptosis in response to a variety of treatments, has been found to correlate with poor prognosis in several solid tumors including breast, prostate, and lung cancer (28–30). However, expression of *Bcl-2* varied in different tumors and was limited to a fraction of cases. For examples, *Bcl-2* expression was detected in 3 of 14 cases of mesothelioma (17) and in only 7.5% of 174 cases of gastric carcinomas (31). Thus, overexpression of *Bcl-2* alone cannot account for the resistance to apoptosis in mesothelioma and other tumors.

In contrast to the level of *Bcl-2* expression, we have determined that *survivin* is expressed overwhelmingly in both mesothelioma cell lines and freshly resected tumors in the present study (87.5% and 100%, respectively). Furthermore, we have shown that targeted down-regulation of *survivin* expression by antisense oligonucleotides results in increased cell death and enhanced apoptosis in mesothelioma cells. These findings are consistent with others in different cell systems (5, 10). Our studies suggest that the overexpression of *survivin* plays a more important role than *Bcl-2* in developing resistance to apoptosis and contributes to the poor response of mesothelioma cells to chemotherapy and radiation therapy.

These results suggest that targeting *survivin* expression using antisense oligonucleotides may hold promise as an effective therapy for mesothelioma. Previous studies have showed synergic effects of antisurvivin oligonucleotides in sensitizing cancer cells to chemotherapeutic drugs (10, 22, 32). Experiments are now under way in our laboratory to test the efficacy of survivin antisense oligonucleotides in combination with cytotoxic chemotherapy *in vitro* and *in vivo*. It is hoped that translation of this strategy to the clinic may improve the efficacy of treatment of patients with mesothelioma.

In conclusion, we have shown that *survivin* is overexpressed in both mesothelioma cell lines and fresh tumor samples. *Survivin* antisense oligonucleotides efficiently down-regulate the expression level of *survivin* and cause apoptotic cell death *in vitro*. *Survivin* overexpression in mesothelioma may play an important role in the development of resistance to apoptosis and thus to insensitivity to standard chemotherapy and radiation therapy. Targeting *survivin* expression using an antisense strategy in combination with chemotherapy may increase clinical effectiveness in mesothelioma treatment.

Acknowledgments

We acknowledge the use of fresh mesothelioma tissues collected by the Cell and Tissue Bank through the Thoracic Oncology Program, Comprehensive Cancer Center at University of California, San Francisco.

References

1. Ambrosini, G., Adida, C., and Altieri, D. C. A novel anti-apoptosis gene, *survivin*, expressed in cancer and lymphoma. Nat. Med., *3:* 917–921, 1997.

2. Reed, J. C. The survivin saga goes *in vivo*. J. Clin. Investig., *108:* 965–969, 2001.

3. Deveraux, Q., and Reed, J. C. IAP family proteins: suppressors of apoptosis. Genes Dev., *13:* 239–252, 1999.

4. Grossman, D., Kim, P. J., Blanc-Brude, O. P., Brash, D. E., Tognin, S., Marchisio, P. C., and Altieri, D. C. Transgenic expression of survivin in keratinocytes counteracts UVB-induced apoptosis and cooperates with loss of p53. J. Clin. Investig., *108:* 991–999, 2001.

5. Ambrosini, G., Adida, C., Sirugo, G., and Altieri, D., Induction of apoptosis and inhibition of cell proliferation by *survivin* gene targeting. J. Biol. Chem., *18:* 11177–11182, 1998.

6. Schmitz, M., Diesterlkoetter, P., Weigle, B., Schmachtenberg, F., Stevanovic, S., Ockert, D., Rammersee, H. G., and Rieber, E. P. Generation of survivin-specific $CD8^+$ T effector cells by dendritic cells pulsed with protein of selected peptides. Cancer Res., *60:* 4845–4849, 2000.

7. Li, F., Ambrosini, G., Chu, E. Y., Plescia, J., Tognin, S., Marchisio, P. C., and Altieri, D. C. Control of apoptosis and mitotic spindle checkpoint by survivin. Nature (Lond.), *396:* 580–584, 1998.

8. Grossman, D., McNiff, J. M., Li, F., and Altieri, D. C. Expression of the apoptosis inhibitor, survivin, in nonmelanoma skin cancer and gene targeting in a keratinocyte cell line. Lab. Investig., *79:* 1121–1126, 1999.

9. Chen, J., Wu, W., Tahir, S. K., Kroeger, P. E., Robenberg, S. H., Cowsert, L. M., Bennett, F., Krajewski, S., Krajewska, M., Welsh, K., Reed, J. C., and Ng, S. C. Down-regulation of survivin by antisense oligonucleotides increases apoptosis, inhibits cytokinesis and anchorage-independent growth. Neoplasia, *2:* 235–241, 2000.

10. Olie, R. A., Simoes-Wust, A. P., Baumann, B., Leech, S. H., Fabbro, D., Stahel, R. A., and Zangemeister-Wittke, U. A novel antisense oligonucleotide targeting survivin expression induces apoptosis and sensitizes lung cancer cells to chemotherapy. Cancer Res, *60:* 2805–2809, 2000.

11. Mesri, M., Wall, N. R., Li, J., Kim, R. W., and Altieri, D. Cancer gene therapy using a survivin mutant adenovirus. J. Clin. Investig., *108:* 981–990, 2001.

12. Bowman, R. V., Manning, L. S., Davis, M. R., and Robinson, B. W. S. Chemosensitivity and cytokine sensitivity of malignant mesothelioma. Cancer Chemother. Pharmacol, *28:* 420–426, 1991.

13. Ong, S. T., and Vogelzang, N. J. Chemotherapy in malignant pleural mesothelioma: a review. J. Clin. Oncol., *14:* 1007–1017, 1996.

14. Lechner, J. F., Tesfaigzi, J., and Gerwin, B. I. Oncogens and tumor-suppressor genes in mesothelioma: a synopsis. Environ. Health Perspect., *105* (Suppl. 5)*:* 1061–1067, 1997.

15. Cheng, J. Q., Jhanwar, S. C., Klein, W. M., Bell, D. W., Lee, W. C., Altomare, D. A., Nobori, T., Olopade, O. I., Buckler, A. J., and Testa, J. R. p16 alterations and deletion mapping of 9p21–p22 in malignant mesothelioma. Cancer Res., *54:* 5547–5551, 1997.

16. Yang, C-T., You, L., Yet, C-C., Chang, J. W-C., Zhang, F., McCormick, F., and Jablons, D. M. Adenovirus-mediated $p14^{ARP}$ gene transfer in human mesothelioma cells. J. Natl. Cancer Inst. (Bethesda), *92:* 636–641, 2000.

17. Narasimhan, S. R., Yang, L., Gerwin, B. I., and Broaddus, V. C. Resistance of pleural mesothelioma cell lines to apoptosis: relation to expression of Bcl-2 and Bax. Am. J. Physiol., *275:* L165–L171, 1998.

18. Tamm, I., Wang, Y., Sausville, E., Scudiero, D. A., Vigna, N., Oltersdorf, T., and Reed, J. C. IAP-family protein survivin inhibits caspase activity and apoptosis induced by Fas (CD95), Bax, caspases, and anticancer drugs. Cancer Res., *58:* 5315–5320, 1998.

19. Nonzo, M., Rosell, R., Felip, E., Astudillo, J., Sanchez, J. J., Maestre, J., Martin, C., Font, A., Barnadas, A., and Abad, A. A novel anti-apoptosis gene: re-expression of survivin messenger RNA as a prognostic marker in non-small cell lung cancer. J. Clin. Oncol., *17:* 2100–2104, 1999.

20. Kawasaki, H., Altieri, D. C., Lu, C-D., Toyoda, M., Tenjo, T., and Tanigawa, N. Inhibition of apotosis by survivin predicts shorter survival rates in colorectal cancer. Cancer Res., *58:* 5071–5074, 1999.

21. Nakagawara, A. Molecular basis of spontaneous regression of neuroblastoma: role of neurotrophic signals and genetic abnormalities. Hum. Cell, *11:* 115–124, 1998.

22. Li, F., Ackermann, E. J., Bennett, C. F., Rothermel, A. L., Plescia, J., Tognin, S., Villa, A., Marchisio, P. C., and Altieri, D. C. Pleiotropic cell-division defects and apoptosis induced by interference with survivin function. Nat. Cell. Biol., *1:* 461–466, 1999.

23. Verdecia, M. A., Huang, H., Dutil, E., Kaiser, D. A., Hunter, T., and Noel, J. P. Structure of the human anti-apoptotic protein survivin reveals a dimeric arrangement. Nat. Struct. Biol., *7:* 602–608, 2000.

24. Suzuki, A., Ito, T., Kawano, H., Hayashida, M., Hayasaki, Y., Tsutomi, Y., Akahane, K., Nakano, T., Miura, M., and Shiraki, K. Survivin initiates procaspase 3/p21 complex formation as a result of interaction with Cdk4 to resist Fas-mediated cell death. Oncogene, *19:* 1346–1353, 2000.

25. Metcalf, R. A., Welsh, J. A., Bennett, W. P., Seddon, M. B., Lehman, T. A., Pelin, K., Linnainmaa, K., Tammilehto, L., Mattson, K., and Gerwin, B. I. p53 and Kirsten-ras mutations in human mesothelioma cell lines. Cancer Res., *52:* 2610–2615, 1992.

26. Mor, O., Yaron, P., Huszar, M., Yellin, A., Jakobovitz, O., Brok-Simoni, F., Rechavi, G., and Reichert, N. Absence of p53 mutations in malignant mesotheliomas. Am. J. Respir. Cell Mol. Biol., *16:* 9–13, 1997.

27. Reed, J. C. Bcl-2 and the regulation of programmed cell death. J. Cell Biol., *124:* 1–6, 1994.

28. Jiang, S. X, Kameya, T., Sato, Y., Yanase, N., Yoshura, H., and Kodama, T. Bcl-2 protein expression in lung cancer and close correlation with neuroendocrine differentiation. Am. J. Pathol., *148:* 837–846, 1996.

29. McDonnell, T. J., Troncoso, P., Brisbay, S. M., Logothetis, C., Chung, L. W. K., Hsieh, J-T., Tu, S. M., and Campbell, M. L. Expression of the proto-oncogene bcl-2 in the prostate and its association with emergence of androgen-independent prostate cancer. Cancer Res, *52:* 6940–6944, 1992.

30. Silvestrini, R., Benini, E., Veneroni, S., Daidone, M. G., Tomasic, G., Squicciarini, P., and Salvadori, B. p53 and bcl-2 expression correlates with clinical outcome in a series of node-positive breast cancer patients. J. Clin. Oncol., *14:* 1604–1610, 1966.

31. Lu, C-D., Altieri, C., and Tanigawa, N. Expression of a novel antiapoptosis gene, *survivin*, correlated with tumor cell apoptosis and p53 accumulation in gastric carcinomas. Cancer Res., *58:* 1808–1812, 1998.

32. Jiang, X., Wilford, C., Duensing, S., Munger, K., Jones, G., and Jones, D. Participation of survivin in mitotic and apoptotic activities of normal and tumor-derived cells. J. Cell. Biochem., *83:* 342–354, 2001.