The Oncoprotein SF2/ASF Promotes Non–Small Cell Lung Cancer Survival by Enhancing Survivin Expression

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Abstract

Purpose: SF2/ASF is a splicing factor recently described as an oncoprotein. In the present work, we examined the role of SF2/ASF in human non–small cell lung cancer (NSCLC) and analyzed the molecular mechanisms involved in SF2/ASF-related carcinogenesis.

Experimental Design: SF2/ASF protein levels were analyzed in 81 NSCLC patients by immunohistochemistry. SF2/ASF downregulation cellular models were generated using small interfering RNAs, and the effects on proliferation and apoptosis were evaluated. Survivin and SF2/ASF expression in lung tumors was analyzed by Western blot and immunohistochemistry. Survival curves and log-rank test were used to identify the association between the expression of the proteins and time to progression.

Results: Overexpression of SF2/ASF was found in most human primary NSCLC tumors. In vitro downregulation of SF2/ASF induced apoptosis in NSCLC cell lines. This effect was associated with a reduction in the expression of survivin, an antiapoptotic protein widely upregulated in cancer. In fact, SF2/ASF specifically bound survivin mRNA and enhanced its translation, via a mammalian target of rapamycin complex 1 (mTORC1) pathway-dependent mechanism, through the phosphorylation and inactivation of the translational repressor 4E-BP1. Moreover, SF2/ASF promoted the stability of survivin mRNA. A strong correlation was observed between the expression of SF2/ASF and survivin in tumor biopsies from NSCLC patients, supporting the concept that survivin expression levels are controlled by SF2/ASF. Furthermore, combined expression of these proteins was associated with prognosis.

Conclusion: This study provides novel data on the mTORC1- and survivin-dependent mechanisms of SF2/ASF-related carcinogenic potential, and shows that SF2/ASF and survivin expression is involved in NSCLC progression.

Regulation of mRNA metabolism (processing, export, localization, surveillance, translation, and decay) contributes more relevantly than previously suspected to the genesis of a great variety of diseases, including cancer (1–7). Modifications in the levels of mRNA processing proteins can elicit global changes in the profile of mRNA expression, affecting a number of cancer-associated genes. In lung cancer, the leading cause of cancer mortality (8), several studies have shown changes in the expression of members of two families of mRNA processing proteins: heterogeneous nuclear ribonucleoproteins (hnRNP) and serine/arginine (SR)-rich proteins (9–11). Elevated expression of hnRNP A1 and the SR protein SF2/ASF has been related to the presence of metastasis-associated alternative splicing isoforms of CD44 in a mouse model of lung cancer (10). SF2/ASF has been recently found to be upregulated in several human neoplasias, and has been described as an oncoprotein with roles in the establishment and maintenance of transformation (12). SF2/ASF plays a role in several key features of RNA metabolism, such as mRNA constitutive and alternative splicing, nuclear export, stability, and translation (13–17).

Recently, the mechanism by which SF2/ASF activates mRNA translation has been elucidated. SF2/ASF promotes phosphorylation of 4E-BP1, a translational repressor protein that binds and sequesters eukaryotic translation initiation factor 4E (eIF4E). Hyperphosphorylation of 4E-BP1 liberates eIF4E from this inhibitory complex and allows its incorporation to eIF4F, which initiates translation (18). SF2/ASF triggers this mechanism by activating mammalian target of rapamycin complex 1 (mTORC1), which, in turn, phosphorylates 4E-BP1 (19).

Some of the molecular details in the transformation mechanism driven by SF2/ASF have already been proposed,
SF2/ASF is a pre-mRNA splicing factor recently described as an oncoprotein, with roles both in the establishment and in the maintenance of the transformed phenotype. In our study we show that SF2/ASF is overexpressed in non–small cell lung cancer (NSCLC) compared with normal lung, and we also provide novel data on the mammalian target of rapamycin– and survivin-dependent mechanisms of SF2/ASF-related carcinogenic potential. We show that SF2/ASF enhances the expression of survivin, an antiapoptotic protein widely overexpressed in several types of cancer, whose association with prognosis has been extensively analyzed. Finally, the potential role of survivin and SF2/ASF as prognostic factors is analyzed in a series of 81 NSCLC patients. We show that the combined expression levels of SF2/ASF and survivin are associated with poor prognosis.

Materials and Methods

Cell culture

Human NSCLC cell lines were obtained from the American Type Culture Collection, the German Collection of Microorganism and Cell Culture, and the European Collection of Cell Cultures. Cell lines (LOU-NH91, EPLC-272H, SK-MES-1, NCI-H157, NCI-H322, NCI-H358, A549, NCI-H441, HCC-827, NCI-H1299, and NCI-H727) were grown in RPMI 1640 medium (Gibco) supplemented with 10% Fetalclone III (Hyclone), penicillin, and streptomycin (Gibco). Cell lines were authenticated by analysis of their genetic alterations. NHBE and SAEC cells were purchased from Cambrex and were grown with Clonetics BEGM and SAGM Bullekits (Cambrex), respectively. Cells were maintained at <80% confluence under standard conditions (37°C, humidified atmosphere, 95% air, 5% CO₂).

Immunohistochemical analysis

Indirect immunoperoxidase staining was carried out on formalin-fixed paraffin-embedded tissue sections, using the DakoAutostainer (Dako). Endogenous peroxidase activity was quenched with 3% hydrogen peroxide for 10 minutes. Microwave antigen retrieval was carried out with EDTA buffer (1 mmol/L, pH 8) for 30 minutes, and nonspecific binding sites were blocked with 5% goat normal serum in TBS-Tween (Wash buffer, Dako) for 30 minutes. Afterwards, sections were incubated with primary antibodies for 30 minutes at room temperature: anti-SF2/ASF (1:75; Zymed) or anti-survivin (1:2,000; Cell Signaling Technology). After applying the EnVision+ System-HRP (Dako) for 30 minutes, immunostaining was shown by incubation with 3-3′-diaminobenzidine and H₂O₂. Tissues expressing known levels of the tested antigens were included in each immunohistochemical run to unify the possible intensity discordance between experiments. Negative controls consisted in omission of the primary antibody. Staining scores were established by semiquantitative analysis (23). The extension and intensity of the staining was evaluated by two observers, independently and blindly. The extension was scored as percentage

Translational Relevance

SF2/ASF is a pre-mRNA splicing factor recently described as an oncoprotein, with roles both in the establishment and in the maintenance of the transformed phenotype. In our study we show that SF2/ASF is overexpressed in non–small cell lung cancer (NSCLC) compared with normal lung, and we also provide novel data on the mammalian target of rapamycin– and survivin-dependent mechanisms of SF2/ASF-related carcinogenic potential. We show that SF2/ASF enhances the expression of survivin, an antiapoptotic protein widely overexpressed in several types of cancer, whose association with prognosis has been extensively analyzed. Finally, the potential role of survivin and SF2/ASF as prognostic factors is analyzed in a series of 81 NSCLC patients. We show that the combined expression levels of SF2/ASF and survivin are associated with poor prognosis.
of positive cells (0-100%), and the intensity of staining was assessed compared with a known external positive control (1, weak; 2, moderate; 3, strong staining). A final score, called the H-score, was calculated by adding the products of the percentage cells stained at a given staining intensity (0-100) and the staining intensity (0-3).

**Immunoblotting**

Cells and tumors containing at least 70% tumor cells were lysed in radioimmunoprecipitation assay buffer containing a cocktail of protease inhibitors. For mTOR inhibition experiments, cells were treated with rapamycin (25, 50, 100, and 150 nmol/L) 24 hours prior to protein extraction. Total protein (20-30 μg) from each lysate was boiled at 95°C for 5 minutes, separated by SDS-PAGE, and transferred onto a nitrocellulose membrane. The membranes were blocked and probed with antibodies by using enhanced chemiluminescence detection. Primary antibodies were SF2/ASF (1:10,000; Zymed), β-actin (1:5,000; Sigma), caspase-3 (1:2,000; Cell Signaling Technology), cleaved caspase-3 (1:2,000; Asp175, Cell Signaling Technology), and survivin (1:1,000; Cell Signaling Technology). Secondary antibody was horseradish peroxidase–conjugated donkey anti-rabbit or sheep anti-mouse IgG (1:4,000; Amersham).

**RNA interference**

Twenty-four hours prior to transfection, cells were seeded in 6-well plates in antibiotic-free medium, at the appropriate density to achieve 40% to 50% confluency. Cells were transfected with 75 pmol of small interfering RNAs (siRNA; Dharmacon) per well using Lipofectamine-2000 (Invitrogen). The transfection efficiency was determined by flow-cytometry detection of positive cells transfected with FAM-labeled nontargeting siRNA (Ambion). siRNA target sequences were: GAAAGAAGATATGACCTAT (SF2/ASF siRNA 1); TGAAGCAGGTGATGTATGT (SF2/ASF siRNA 2); CACCGCATCTCTACATTCA (survivin siRNA 1); CCACGTGAAACGCCAGCA (survivin siRNA 2). A negative control (nontargeting siRNA 1, Dharmacon) was also used.

**Reverse transcriptase-PCR**

Total RNA was extracted 3 days after siRNA transfection, using the RNeasy Kit (Qiagen). Reverse transcription was done using 1.5 μg of total RNA, SuperScript III (Invitrogen), and random hexamers (Applied Biosystems). PCR was done using AmpliTaq Gold DNA polymerase (Applied Biosystems). PCR cycling conditions were: 95°C for 5 minutes, followed by 30 cycles (30 seconds at 95°C, 30 seconds at 58°C, and 50 seconds at 72°C). Primers sequences are included in Supplementary Table S2. PCR products were separated on agarose gels and stained with ethidium bromide. For analysis of mRNA stability, cells were treated with 2 or 5 μmol/L actinomycin D (Sigma) 2 days after siRNA transfection. Afterwards, cells were harvested at 3-hour intervals. Survivin and c-Jun mRNAs were analyzed by semiquantitative PCR, using TaqMan Gene Expression Assays (Applied Biosystems). IPO8 mRNA expression was used as the endogenous control (24).

**Cell proliferation assays**

Six hours after siRNA transfection, cells were harvested and seeded for the following experiments. Anchorage-dependent cell proliferation was determined by MTT cell proliferation assay. Briefly, 2,000 cells per well were seeded in 96-well plates and proliferation was measured at 24-hour intervals by MTT conversion (Sigma), analyzed at 540/690 nm on a SunRise enzyme-linked immunosorbent assay plate reader (Tecan). Assays were conducted in sextuplicate in three independent experiments. Anchorage-independent cell proliferation was determined by colony formation in soft agar. A549 and NCI-H157 cells (1,000 and 2,500, respectively) were plated in 6-well plates with a bottom layer of 0.6% agar and a top layer of 0.3% agar, and were incubated at 37°C for 8 days. After incubation, 500 μL of MTT labeling reagent were added per well. Four hours later, DMSO (500 μL) was applied to each well and the plates were further incubated overnight. Resulting colonies were counted. Assays were conducted in triplicate in three independent experiments.

**Cell cycle analysis**

The distribution of cells in different phases of the cell cycle was measured by flow cytometry analysis. Cells were transfected as described above, and the medium was replaced after 6 hours. Then, at 24-hour intervals, floating and attached cells were harvested and fixed with 70% ethanol for at least 1 hour at 4°C. Fixed cells were treated with 0.2 mg/mL RNase A (Sigma) for 1 hour at 37°C, stained with 10 μg/mL of propidium iodide (PI; Sigma) and analyzed on a FACScan Flow cytometer (Becton Dickinson). Percentages of cells in the Sub-G0, G0-G1, S, and G2-M phases were determined. Assays were conducted in three independent experiments.

**Apoptosis analysis by flow cytometry**

The percentage of apoptotic cells after SF2/ASF or survivin downregulation was analyzed using the FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen). Cells were transfected as described above, and the medium was replaced after 6 hours. Then, at 24-hour intervals, floating and attached cells were harvested, processed according to the manufacturer's guidelines, and analyzed on a FACScan Flow cytometer (Becton Dickinson). Annexin V–FITC- and PI-positive cells were considered apoptotic cells.

**Vector construction and transfection**

Survivin cDNA was cloned into pcDNA3.1(-) vector (Invitrogen). The cDNA was obtained from A549 cells by reverse transcriptase-PCR (RT-PCR) using the following primers: CCGCTCGAGAGCCGCCGAGGCTG (forward) and ATAGTTTACGCGCCGCCAGGCGCTCAATCCAT (reverse). These primers include 5’ Xho I and Not I restriction sites (in the forward and reverse primers, respectively). Survivin cDNA was purified, cloned...
into the vector, and sequenced. Twenty-four hours prior to transfection, cells were seeded in 6-well plates in antibiotic-free medium, at the appropriate density to achieve 80% to 90% confluency. Cells were transfected with 2 μg of pcDNA3.1-survivin or pcDNA3.1 (mock) per well using Lipofectamine-2000 (Invitrogen). Six hours after transfection, the medium was replaced, and 48 hours later, transfected cells were selected with geneticin (500 μg/mL).

Immunoprecipitation and RNA isolation

Endogenous SF2/ASF from A549 cells was immunoprecipitated and total RNA was isolated. Briefly, cells were lysed by scraping in lysis buffer [10 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 0.5% Nonidet P-40, 1% Triton X-100] supplemented with complete proteinase inhibitors (Roche). Ten micrograms of an anti-SF2/ASF antibody (Zymed) were incubated with protein A beads (GE, Healthcare) for 3 hours at 4°C. After three washes with lysis buffer, beads were mixed gently with cell lysates for 1 hour at 4°C. Immunoprecipitates were washed five times with lysis buffer and RNA was eluted with Trizol Reagent (Invitrogen). RNA was isolated following the manufacturer's protocol. Immunoprecipitation without antibodies, or with an isotype control antibody (IgG1 Isotype Control; Sigma, M-5284) were used as negative controls. RT-PCR was done as described above, except for the amount of RNA (half of the RNA obtained was used in each reaction).

Statistical analysis

Wilcoxon signed rank sum test was applied to analyze differences between normal and tumor tissue. Data obtained from MTT and soft agar assays were analyzed by Student’s t test or Mann-Whitney U. Pearson’s correlation test was used to evaluate the association between survivin and SF2/ASF expression detected by Western blotting. Fisher’s exact test was used to analyze the association between survivin and SF2/ASF expression detected by immunohistochemistry. Kaplan-Meier survival curves were generated to evaluate the significance of individual survivin and SF2/ASF expression, or of the combination of both, in the prognosis of NSCLC patients. Patients were stratified in two groups according to the median levels of the H-score for survivin and SF2/ASF, or of the survivin-SF2/ASF score (sum of survivin and SF2/ASF H-scores). A log-rank test was carried out to assess statistical differences. The influence of clinicopathologic variables on recurrence was assessed with the Cox proportional hazards model. A P value of <0.05 was considered as statistically significant. Statistical analysis was done using the SPSS software package, version 15.0.

Results

SF2/ASF is upregulated in lung cancer

To evaluate the role of SF2/ASF in human NSCLC, its expression was analyzed in a series of normal and tumor lung tissues from 81 NSCLC patients (Supplementary Table S1). Immunohistochemical analysis showed a significant increase in SF2/ASF expression in tumors compared with that in their normal counterparts (P < 0.0001; Fig. 1A and B). In normal lung, SF2/ASF expression was found in a number of alveolar macrophages, few type II pneumocytes, and some epithelial cells in the bronchioli. Furthermore, some endothelial cells were positive for SF2/ASF protein. Nevertheless, in each patient, the intensity of the staining in normal tissue was usually lower than that in the tumor counterpart. We also analyzed the SF2/ASF expression in a panel of 11 human lung cancer cell lines and 2 normal human lung primary cultures. Western blotting showed an upregulation of SF2/ASF in nearly all of the tumor cell lines, compared with primary cells (Supplementary Fig. S1).

SF2/ASF downregulation: effects on proliferation and apoptosis

To examine the contribution of SF2/ASF to the maintenance of the transformed phenotype in NSCLC cells, we carried out downregulation experiments in lung cancer cell lines to reduce SF2/ASF to a level comparable with that found on normal primary cells. We then analyzed the effect of SF2/ASF inhibition on cell proliferation. SF2/ASF knockdown was carried out in A549 and NCI-H157 cells using two different siRNAs. Downregulation was confirmed by Western blotting (Fig. 2A). Anchorage-dependent cell growth, determined by MTT assay, was

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**Fig. 1.** SF2/ASF is overexpressed in human NSCLC. Representative images of normal (A) and tumor (B) lung sections analyzed for SF2/ASF expression. Lung tissues were resected, formalin-fixed, paraffin-embedded, sectioned, and analyzed for SF2/ASF-immunoreactivity. Bar, 100 μm.
significantly inhibited in SF2/ASF downregulated cells compared with control cells transfected with a scrambled siRNA (P < 0.001). Both SF2/ASF siRNAs produced a decrease of 70% to 80% in the proliferation of lung cancer cells (Fig. 2B). Anchorage-independent cell growth in soft agar was also deeply affected by SF2/ASF knockdown. The number of colonies formed by NSCLC cells transiently downregulated for SF2/ASF was significantly lower than the number of colonies formed by control cells (Fig. 2C).

A cell cycle analysis by flow cytometry was done to determine whether the decrease in cell proliferation due to SF2/ASF knockdown was associated with cell cycle arrest. Downregulation of SF2/ASF produced a significant increase in the percentage of cells in G0-G1 phase (15.9% ± 1.05%; P < 0.05), along with a decrease in S phase (8.2% ± 0.78%, P < 0.05), indicating that SF2/ASF knockdown induced a cell cycle arrest in both cell lines (Fig. 2D). This effect was accompanied by an increase in the sub-G0 cell population (15.1% ± 1.21%; P < 0.05), suggesting an increase of apoptosis. Detection and quantification of apoptotic cells by flow cytometry showed that SF2/ASF downregulated cells had a significant increase in the percentage of apoptotic cells (P < 0.01), compared with control cells transfected with a scrambled siRNA (Fig. 2E). The induction of apoptosis in these cells was confirmed by the increase in caspase 3 activation (Fig. 2F). These data show that SF2/ASF plays a critical role in the survival of lung cancer cells.

**SF2/ASF knockdown decreases survivin expression levels in NSCLC cells**

We next examined the mechanism by which SF2/ASF downregulation causes apoptosis. Apoptosis-related genes are frequently controlled by changes in their mRNA isoform ratios; therefore, we evaluated the alternative mRNA splicing patterns of genes for which several isoforms with different apoptotic properties had been described in cancer. The analysis included Bcl-x, caspase 2, caspase 8, caspase 9, HDMX, and survivin (Supplementary Fig. S2). Interestingly, SF2/ASF knockdown caused a decrease in the major isoform of survivin, which encodes an important antiapoptotic protein (Supplementary Fig. S2). We did not detect a clear shift in survivin isoforms, but we observed a marked decrease in the main isoform of survivin. This reduction was confirmed at the protein level by Western blotting, showing a very pronounced effect (Fig. 3A). The antibody used for this analysis only detected the main isoform of survivin, not the other isoforms observed by RT-PCR (2B and ΔEx3). SF2/ASF knockdown also caused a shift in the splicing pattern of caspase 9 towards the antiapoptotic isoform 9b, suggesting a compensatory survival response to apoptosis (Supplementary Fig. S2).

To assess whether endogenous levels of SF2/ASF in lung cells were also correlated with those of survivin, we analyzed the panel of tumor and normal lung cells by Western blotting. We found a significant correlation between the expression of both proteins (P = 0.003; Fig. 3B). All these observations suggest that SF2/ASF regulates survivin levels in lung epithelial cells.

**Survivin is a mediator of the effect of SF2/ASF on apoptosis**

To analyze whether downregulation of survivin was responsible for the induction of apoptosis after SF2/ASF knockdown in NSCLC cells, we first inhibited survivin expression by siRNA technology (Fig. 4A) and analyzed the percentage of apoptotic cells by flow cytometry. Survivin inhibition in A549 and NCI-H157 cells caused a significant increase of apoptotic cells (P < 0.05; Fig. 4B), which showed that, in these cells, downregulation of survivin expression is sufficient to induce cell death. More importantly, we evaluated the effect of SF2/ASF downregulation on cells with constitutive high levels of survivin expression. To that end, we generated A549 cells that overexpressed the coding sequence of survivin (Fig. 4C). Overexpression of survivin in SF2/ASF downregulated cells caused a significant reduction of apoptosis (P < 0.01; Fig. 4E). These data show that survivin is an important mediator of the effect of SF2/ASF on apoptosis.

**SF2/ASF enhances survivin mRNA translation and stabilization via mTOR activation in lung cancer cells**

We next evaluated the possibility that SF2/ASF could regulate survivin mRNA translation initiation and/or stability. First we determined whether SF2/ASF binds to survivin mRNA. We immunoprecipitated SF2/ASF and extracted total RNA. RT-PCR showed the presence of survivin mRNA in the immunoprecipitate, but not of Bcl-x mRNA, used as a negative control (Fig. 5A). We hypothesized that SF2/ASF could enhance translation of survivin mRNA by phosphorylation and inactivation of the translation inhibitor 4E-BP1, via mTORC1 activation (18, 19). The addition of rapamycin, an mTOR kinase inhibitor, decreased survivin protein levels in a concentration-dependent manner (Fig. 5B). These results show that regulation of survivin expression by SF2/ASF in lung cancer cells is mediated through the activation of the mTOR pathway. Moreover, we found a decrease in phosphorylated 4E-BP1 in those cells in which SF2/ASF was downregulated (Fig. 5C). The stability of survivin mRNA has also been described to be controlled by the mTOR pathway in other cell types (25). Thus, we measured survivin mRNA levels in SF2/ASF knockdown cells after inhibiting transcription with actinomycin D, and observed a higher rate of survivin mRNA degradation (Fig. 5D). As a control, we also analyzed the expression of a short-lived mRNA, c-Jun, which did not show a higher rate of degradation after SF2/ASF inhibition (Fig. 5D), indicating the specificity of the effect of SF2/ASF on the stability of survivin mRNA. These data indicate that SF2/ASF also has a role in stabilizing survivin mRNA.
SF2/ASF and survivin expression correlate in human primary lung tumors

We examined whether there is an association between SF2/ASF and survivin expression in human primary NSCLC tumors. We determined the expression of survivin by immunohistochemistry in the same series of patients used previously for the analysis of SF2/ASF expression. Quantification of the immunostaining revealed that SF2/
Fig. 2. Continued. D, cell cycle distribution of A549 and NCI-H157 cells at different days after siRNA transfection, as indicated. Cells were transfected with a siRNA targeting SF2/ASF (siRNA SF2/ASF 2) or a control siRNA, and the medium was replaced after 6 hours. Then, at 24-hour intervals, attached and floating cells were collected and processed for cell cycle analysis. Representative cell cycle profiles of one independent experiment are shown. Gray, cell cycle profiles of control cells; black line, profiles of cells transiently downregulated for SF2/ASF. Percentages of cells in different phases of the cell cycle of this representative experiment are indicated. E, percentages of Annexin V– and PI-positive cells at 6 days after transfection. A549 and NCI-H157 cells were transfected with a siRNA targeting SF2/ASF or a control siRNA, and the medium was replaced after 6 hours. Then, at 6 days, attached and floating cells were collected and analyzed by flow cytometry for Annexin V-FITC and PI detection. Each bar, mean percentage of apoptotic cells; error bars, SD (n = 6). Statistical significance of SF2/ASF downregulated cells compared with control cells transfected with a control siRNA is indicated. F, representative immunoblottings of cleaved caspase 3 (17 and 19 kDa), pro-caspase 3, SF2/ASF, and β-actin in SF2/ASF downregulated cells, as indicated. Cells were transfected as above and total protein lysates from attached and floating cells were obtained at 24-hour intervals.
ASF expression strongly correlated with survivin expression in tumors \( (P < 0.0001; \text{Fig. 6A}) \). Immunoreactivity for survivin was also present in some cells of the mucosa of bronchi and bronchioli, a number of alveolar macrophages, few type II pneumocytes, and a small number of endothelial cells. To validate these data, we performed Western blotting in protein extracts from 20 tumors for which frozen material was available. Only the main isoform of survivin was detected in these tumors. The results were consistent with those obtained by immunohistochemistry, showing a significant correlation between the expression levels of SF2/ASF and survivin \( (P < 0.0001; \text{Fig. 6B}) \). Dot-plots of SF2/ASF and survivin expression determined by immunohistochemistry or Western blotting can be found in Supplementary Fig. S3. Taken together, our data show that SF2/ASF regulates the expression of the main isoform of survivin in human lung cancer by controlling postsplicing activities on survivin mRNA metabolism.

**SF2/ASF and survivin prognostic value**

Finally, we investigated whether the expression of survivin and SF2/ASF was associated with progression in the NSCLC series. High levels of either survivin or SF2/ASF expression tended to be associated with poor disease-free survival \( \text{Fig. 6C} \), although neither of them reached statistical significance \( \text{survivin, } P = 0.094; \text{SF2/ASF, } P = 0.118 \). We then assessed whether analysis of the combined expression of survivin and SF2/ASF in each patient improved their prognostic value. We defined the survivin+SF2/ASF score as the sum of the main isoform of survivin and SF2/ASF H-scores in each individual. Kaplan-Meier survival analysis revealed that the high survivin+SF2/ASF score was associated with a significant decrease in recurrence-free time \( P = 0.038; \text{Fig. 6C} \). Moreover, the independent prognostic value of survivin+SF2/ASF was confirmed in a multivariate analysis \( \text{Hazard ratio, 2.964; 95% confidence interval, 1.143-7.683; } P = 0.025 \) after adjusting for histology, smoking status, and stage.

**Discussion**

SF2/ASF has recently been described as an oncoprotein \( (12) \). We have studied in depth its potential role in lung cancer, a malignancy in which several pre-mRNA processing proteins have been found to be altered \( (9-11) \). We show here that SF2/ASF protein is upregulated in most lung cancer primary tumors as compared with normal lung epithelial cells. This is the first time that such protein analysis has been done on a large series of lung cancer patients, and it confirms previous results carried out at the mRNA level in a smaller number of lung tumors \( (12) \). Most samples analyzed in our study were early-stage tumors \( \text{stages I-II} \), which suggests that SF2/ASF overexpression is an early event in the progression of lung cancer. This observation is in agreement with previous results from our group showing overexpression of SF2/ASF in early-stage tumors in a mouse model of lung carcinogenesis \( (10) \).

SF2/ASF overexpression is sufficient to transform immortal fibroblasts and is necessary for the maintenance of tumors initially driven by SF2/ASF upregulation \( (12) \). We have now examined the contribution of SF2/ASF overexpression to the maintenance of the transformed phenotype in NSCLC cells. Downregulation of this factor induced cell cycle arrest in G0-G1 phase, followed by caspase 3-mediated apoptosis, showing an important role of SF2/ASF in the survival of lung cancer cells. Therefore, the effects observed in MTT and soft agar assays after SF2/ASF downregulation can be explained not only by a lower rate of proliferation but also by a decrease in lung cancer cell...
viability. Conversely, Li et al. have shown that depletion of SF2/ASF in a chicken B lymphocyte cell line results in G2 phase cell cycle arrest and subsequent apoptosis induction, which seems to be caspase 3 independent (26). The differences between our results and those of Li et al. suggest that the specific mechanisms of cell cycle arrest and induction of apoptosis upon SF2/ASF downregulation may be dependent on the cell type. Besides, these mechanisms may depend on the extent of SF2/ASF knockdown, as SF2/ASF expression was completely abrogated in the Li et al. cellular model, whereas in the present work it was reduced to a level comparable with that found on normal primary cells.

Studies on the oncogenic potential of SF2/ASF have focused on its pre-mRNA splicing activity (12, 27). Many regulators of apoptosis are encoded by immature mRNAs that can produce different alternatively spliced products, with opposing effects on programmed cell death (28). Therefore, we analyzed the alternative splicing pattern of some of these transcripts, searching for an alteration that could be responsible for the induction of apoptosis observed in SF2/ASF downregulated cells. The effect of SF2/ASF knockdown on the RNA splicing machinery was not generalized, as the alternative splicing of some transcripts, such as Bcl-x, was unaffected. We observed a change in the alternative splicing of caspase 9 pre-mRNA towards the antiapoptotic isoform 9b. This effect, previously described by Massiello et al. (29), may be a cell survival response prior to cell death. Interestingly, SF2/ASF knockdown also resulted in a remarkable decrease in the main isoform of survivin.

![Image](image_url)

**Fig. 4.** Expression of survivin prevents lung cancer cells from undergoing apoptosis. A, A549 cells were transfected with a control siRNA or a siRNA targeting survivin. Survivin and β-actin were analyzed by Western blotting in proteins extracted 3 days later. B, A549 and NCI-H157 cells were transfected with a control siRNA or a siRNA targeting survivin. Annexin V and PI staining were analyzed at day 6 after transfection. The figure is representative of three independent experiments. Statistical significance (compared with control cells) is indicated. C, A549 cells were transfected with pcDNA-mock or pcDNA-survivin. After selection of transfected cells with geneticin, proteins were extracted, and survivin and β-actin were analyzed by Western blotting. D, A549 cells transfected with pcDNA-mock or pcDNA-survivin were transfected with a control siRNA or a siRNA targeting SF2/ASF. Six hours after siRNA transfection, cells were harvested and seeded into 96-well plates. Proliferation was measured at 24-hour intervals by MTT conversion. A representative plot is shown. Each point, mean relative absorbance; error bars, SD (n = 6). E, A549 cells overexpressing survivin or control cells were transfected with a control siRNA or a siRNA targeting SF2/ASF, as described above. Annexin V and PI staining was analyzed at day 6 after transfection. Statistical significance between cells overexpressing survivin and control cells is indicated.
Survivin is a member of the inhibitor of apoptosis protein (IAP) family, which is linked to multiple pathways of cellular homeostasis. Survivin has an essential and active role in mitosis; it is critical to allow correct cell division, protecting cells from mitotic catastrophe. Moreover, survivin has an important role in suppressing apoptosis induced by multiple pathways, and it promotes cell survival against proapoptotic stimuli, including chemotherapy and radiation (30, 31). Survivin has attracted attention due to its particular expression pattern; it is undetectable in most adult tissues, but it is frequently overexpressed in human tumors, including lung cancer (32).

Due to this tumor-specific expression and to its antiapoptotic functions, survivin is considered a promising therapeutic target. In fact, a decrease of survivin levels has been consistently associated with arrest of cell proliferation and apoptosis (33). Moreover, a reduction of survivin levels enhances chemotherapy and radiation-induced cell death in lung cancer, indicating a key role of this protein in lung tumors (34). It is, therefore, logical to hypothesize that the inhibition of survivin showed in our study accounts for the observed cell cycle arrest and induction of apoptosis in SF2/ASF downregulated lung cancer cells. Nevertheless, the implication of other mRNAs cannot be ruled out.

The mechanisms that lead to an overexpression of survivin in lung cancer are not yet clearly understood (33). The regulation of survivin expression is complex, and it involves both transcriptional and posttranscriptional mechanisms. There is evidence that shows a link between mTOR and survivin in cancer. Jim et al. have reported that mTOR mediates survivin protein synthesis induced by tobacco carcinogens in lung cancer (35). Besides, activation of the mTOR pathway by insulin-like growth factor I has been shown to enhance the stability and translation of survivin mRNA in prostate cancer cells (25). In the present work, inhibition of mTOR by rapamycin led to a decrease in survivin expression in a concentration-dependent manner, indicating that levels of survivin are dependent on the activity of the mTOR pathway in lung cancer. More interestingly, we show that overexpression of SF2/ASF in lung cancer is a mediator of the overexpression of survivin through the activation of mTOR. It has been suggested that stimulation of mTOR by SF2/ASF would occur in an RNA-dependent manner, i.e., the required molecular machinery for mTOR activation would be recruited to the mRNAs bound by SF2/ASF (18).
SF2/ASF and survivin expression correlate in human primary lung tumors and is associated with prognosis. A, serial consecutive sections of each tumor were analyzed for SF2/ASF and for the main isoform of survivin by immunohistochemistry ($r = 0.5537; P < 0.0001$). Representative images from two cases showing different expression for both proteins are shown. Bar, 100 μm. B, total protein lysates from 20 tumors containing at least 70% tumor cells were analyzed by Western blotting to detect survivin and SF2/ASF ($r = 0.7788; P < 0.0001$). C, Kaplan-Meier progression-free survival curves for the main isoform of survivin and SF2/ASF protein expression. Survivin, $P = 0.094$. SF2/ASF, $P = 0.118$. Survivin+SF2/ASF score, $P = 0.038$. Patients were stratified in two groups according to the median levels of the H-score for survivin, SF2/ASF or survivin+SF2/ASF score [sum of survivin (main isoform) and SF2/ASF H-scores].
The complex eIF4F initiates translation of all capped mRNAs, but there is a pool of mRNAs that require elevated activity of eIF4F for their translation; thus, the translation of these mRNAs is highly sensitive to any alteration of the activity of the eIF4F complex. Survivin mRNA is part of this pool of highly sensitive mRNAs. We have found that SF2/ASF binds survivin mRNA, and that SF2/ASF downregulation decreases 4E-BP1 phosphorylation. In this situation, hypophosphorylated 4E-BP1 sequesters the translation initiation factor eIF4E, blocking cap-dependent translation of survivin and thus reducing survivin protein levels. Furthermore, we have shown that SF2/ASF knockdown accelerates survivin mRNA degradation, indicating an additional role of SF2/ASF in survivin mRNA stability.

Due to the functions of survivin in tumor cell maintenance, survivin has been proposed as a novel prognostic factor in several human neoplasms. In lung cancer, some studies have determined that survivin expression in tumors is associated with worse overall survival and neoplastic vessel invasion (32, 36). However, the potential prognostic value of survivin in NSCLC has been somewhat controversial. To throw light on this issue, a meta-analysis has recently been published, suggesting that survivin expression may be a prognostic factor for poor overall survival of NSCLC patients (37). In the case of SF2/ASF, its genomic region (17q23) is commonly amplified in breast cancer, and this amplification is associated with poor prognosis (38). In our study, either high SF2/ASF or high survivin expression tended to be associated with poor prognosis. Interestingly, those tumors with high levels of both survivin+SF2/ASF score showed a significantly worse outcome. These results indicate that both proteins are related to the progression of NSCLC. The fact that the combined expression of them is more informative than that of each protein alone suggests that the expression of survivin is regulated by other factors apart from SF2/ASF, and that survivin is an important but not exclusive mediator of the oncogenic effects of SF2/ASF, as it has been described (12).

In summary, in the present work we describe a mechanism by which lung cancer cells enhance survivin levels via the mTOR pathway; moreover, it is the first description of a causal relationship between the expression of a splicing factor and survivin. This observation is strongly reinforced by the tight correlation found between the expression of SF2/ASF and survivin in human lung carcinomas, which suggests that the expression of the oncoprotein SF2/ASF could be an important mediator of the upregulation of survivin expression in tumors. More interestingly, the data obtained in the present study strongly suggest that both proteins are involved in lung cancer progression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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The Oncoprotein SF2/ASF Promotes Non–Small Cell Lung Cancer Survival by Enhancing Survivin Expression

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