A Functional Role for KLF6-SV1 in Lung Adenocarcinoma Prognosis and Chemotherapy Response

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Introduction

Kruppel-like factor 6 (KLF6) is a tumor suppressor gene that is functionally inactivated in human cancer by loss of heterozygosity, somatic mutation, decreased expression, and increased alternative splicing into an oncogenic splice variant, KLF6-SV1. Here we show that increased expression of KLF6-SV1 is associated with decreased survival in patients with lung adenocarcinoma. In addition, KLF6-SV1 is a novel antiapoptotic protein in lung cancer cell lines, and targeted reduction of KLF6-SV1 using siRNA induces apoptosis both alone and in combination with the chemotherapeutic drug cisplatin. Together, these findings highlight a critical role for KLF6-SV1 in lung cancer, and show a potential novel therapeutic strategy for the treatment of lung cancer. [Cancer Res 2008;68(4):965–70]

Abstract

Kruppel-like factor 6 (KLF) is a tumor suppressor gene that is functionally inactivated in human cancer by loss of heterozygosity, somatic mutation, decreased expression, and increased alternative splicing into an oncogenic splice variant, KLF6-SV1. Here we show that increased expression of KLF6-SV1 is associated with decreased survival in patients with lung adenocarcinoma. In addition, KLF6-SV1 is a novel antiapoptotic protein in lung cancer cell lines, and targeted reduction of KLF6-SV1 using siRNA induces apoptosis both alone and in combination with the chemotherapeutic drug cisplatin. Together, these findings highlight a critical role for KLF6-SV1 in lung cancer, and show a potential novel therapeutic strategy for the treatment of lung cancer.
associated with poor clinical outcomes. Targeted down-regulation of KLF6-SV1 using RNA interference (RNAi) in several lung adenocarcinoma cell lines induced marked apoptosis associated with induction of known proapoptotic and inhibition of anti-apoptotic regulators. In addition, overexpression of KLF6-SV1 abrogated the proapoptotic effects of chemotherapy on lung cancer cell lines. Finally, combination therapy using siRNA to KLF6-SV1 and cisplatin induced a marked increase in apoptosis compared with either agent alone. Combined, these data suggest an important role for KLF6-SV1 in lung cancer development and points to a novel role for KLF6-SV1 as an antiapoptotic regulator of cell death both alone and in combination with chemotherapy.

Materials and Methods

Cell culture and cell line generation. All cell lines were obtained from the American Tissue Culture Collection. Retroviral infection with KLF6-SV1 was performed as previously described (10).5,6 Transient transfection of the American Tissue Culture Collection. Retroviral infection with KLF6-SV1 was performed as previously described (10).5,6 Transient transfection of the proprietary modification, which includes a silyl ethers used to protect the stability to over 120 h compared with 12 h for traditional siRNAs. This v2 modification system patented by Dharmacon, which increases serum

Table 1. Prognostic significance of KLF6-SV1 as continuous variable in a multivariate Cox proportional hazards model

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<th>Factor in the final model</th>
<th>Univariate</th>
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<td>Grade</td>
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</tbody>
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* A. DiFeo, G. Narla, F. Huang, et al. Inhibition of KLF6-1 increases ovarian cancer survival through the regulation of NOXA, submitted.
* G. Narla, A. DiFeo, Y. Fernandez, et al. KLF6-1 is a key regulator of metastasis and survival in prostate cancer, submitted.

Figure 1. Expression of KLF6-SV1 in normal tissues and lung cancer patient samples. A, qRT-pCR of a panel of normal tissues and 70 lung adenocarcinoma patient samples using KLF6-SV1 specific real-time primers (10). KLF6-SV1 expression is increased 7-fold in lung cancer specimens when compared with all normal tissue and normal lung specifically; **, P < 0.01; B, the relative amount of KLF6-SV1 to GAPDH expression was determined using qRT-PCR (10, 11) and treated it as a continuous variable in a Cox multivariate survival analysis. In a univariate analysis, KLF6-SV1 expression (P = 0.0091) and nodal stage (P = 0.0288) were associated with survival. WT, wild-type; HR, hazard ratio; 95% CI, 95% confidence interval.

Western blot analysis. Cell extracts for Western blotting were harvested in radioimmunoprecipitation assay buffer (standard protocols; Santa Cruz Biotechnology). Tumor tissue extracts were analyzed. RNA was isolated using Trisol reagent (Life Technologies) and purified using RNeasy columns (Qiagen, Inc.).

5 http://www.dharmacon.com
Analysis of proliferation. Proliferation was determined by estimating [3H]thymidine incorporation. A549 stable cell lines expressing pBABE and pBABE-SV1 were plated at a density of 50,000 cells per well in 12-well dishes. Forty-eight hours after plating, 1 μCi/mL [3H]thymidine (Amersham) were added. After 2 h, cells were washed four times with ice-cold PBS and fixed in methanol for 30 min at 4°C. After methanol removal and cell drying cells were solubilized in 0.25% sodium hydroxide/0.25% SDS. After neutralization with hydrochloric acid, dpm were estimated by liquid scintillation counting.

RNA and quantitative real-time PCR analysis. A panel of normal tissues was obtained from Clontech; lung cancer patient samples were collected and extracted as described above. Cell line and tumor RNA was extracted using the Rneasy Mini and Midi kit (Qiagen). All RNA was treated with DNase (Qiagen). A total of 1 μg of RNA was reverse transcribed per reaction using first-strand complementary DNA synthesis with random primers (Promega). Quantitative real-time PCR (qRT-PCR) was performed using the PCR primers previously described (10) on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). All experiments were done in triplicate and independently validated thrice. All values were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels. All primer sequences and methods for quantifying KLF6 alternative splicing were performed as previously described (10, 15).

FACS analysis. Cells were harvested from 12-well dishes and fixed in absolute ethanol (Sigma) for 24 h. On the day of analysis, cells were pelleted and the absolute ethanol was removed. Cells were then stained with a 1-mL solution containing propidium iodide, RNase A, and PBS. FACS analysis was performed on the FACSCalibur instrument (BD Biosciences).

Results

KLF6-SV1 expression is increased in lung cancer samples and correlates with poor survival. qRT-PCR analysis of a panel of normal tissues and 70 lung adenocarcinoma patient samples revealed a 7-fold increase in KLF6-SV1 expression in lung cancer samples when compared with a panel of normal tissue including four normal lung samples (Fig. 1A; refs. 10, 15, 17). Interestingly, wtKLF6 levels were decreased in lung cancer samples when compared with either all normal tissues or lung tissue specifically (data not shown). We next addressed the possibility that high KLF6-SV1 expression could be an adverse prognostic factor in this same cohort of patients with resected lung adenocarcinomas of mixed stages (IA–IIIB), for whom clinical follow-up for up to 10 years was available. Based on previously published studies in...
other tumor types (10, 15, 17), we calculated the amount of KLF6-SV1 expression relative to the housekeeping gene GAPDH and treated it as a continuous variable in a Cox multivariate survival analysis, using a model that included tumor size and nodal stage, two clinical prognostic factors that are well-established in NSCLC. In a univariate analysis, KLF6-SV1 expression (\(P = 0.0091\)) and nodal stage (\(P = 0.0288\)) were associated with survival (Fig. 1B).

A post hoc trichotomization of the sample set suggested a 6.5-year difference in median survival between patients in the lowest tertile of KLF-SV1 expression and in the highest. Together these data suggest that KLF6-SV1 is overexpressed in primary lung cancer when compared with normal lung tissue, and that increased KLF6-SV1 expression is associated with poor survival in lung adenocarcinoma patients.

**Targeted reduction of KLF6-SV1 expression results in increased apoptosis in lung cancer cell lines.** To determine the biological and functional relevance of these findings, we next sought to directly target KLF6-SV1 using RNAi. Using chemically modified siRNA, we explored the biological effect of targeted-reduction of KLF6-SV1 on the behavior of three independent lung cancer cell lines. Transient transfection of KLF6-SV1 specific siRNA resulted in efficient and equal silencing of SV1 expression at both the RNA and protein level at either 72 or 96 h posttransfection in all three lung cancer cell lines tested with no effect on the expression of other KLF6 isoforms (1, 2).

Targeted reduction of KLF6-SV1 resulted in a marked increase in spontaneous apoptosis as shown (Fig. 2B). The induction of apoptosis by targeted reduction of KLF6-SV1 was shown in all three lung cancer cell lines tested (Fig. 2C).

Based on our previous findings (1, 2), we analyzed the expression of specific proapoptotic and antiapoptotic genes at both the mRNA and protein level in one of the transfected cell lines with siRNA to SV1. Transfection of si-SV1 in A549 cells resulted in a significant increase in poly(ADP)ribose polymerase cleavage and increased expression of both active Caspase 3 and 8 (Fig. 2D). qRT-PCR for the proapoptotic NOXA and antiapoptotic Bcl-2 was then performed. NOXA mRNA expression was increased 6-fold in cell lines transfected with si-SV1 compared with control, whereas Bcl-2 expression was reduced 80% in this same experiment at both the mRNA and protein level (Fig. 2D). Together, these data suggest that targeted down-regulation of KLF6-SV1 using RNAi results in...
spontaneous apoptosis through activation of both the intrinsic and extrinsic signaling pathways in lung cancer cell lines.

Overexpression of KLF6-SV1 results in increased cellular proliferation and cell survival. To further characterize the role of KLF6-SV1 and to determine the biological relevance of these findings, we generated stable cell lines overexpressing KLF6-SV1 in the A549 lung cancer cell line. Retroviral infection of this cell line resulted in an 8-fold overexpression of KLF6-SV1 mRNA and protein when compared with pBABE-infected control cell lines (Fig. 3A). Overexpression of SV1 resulted in >2-fold increase in cellular proliferation when compared with control (Fig. 3B). Of significance, increased cellular proliferation was associated with increased expression of Bcl-2 with concomitant down-regulation in NOXA gene expression. Based on our previous findings that targeted reduction of KLF6-SV1 results in a marked increase in apoptosis, we sought to determine if overexpression of SV1 abrogated the ability of the chemotherapeutic agent cisplatin to induce apoptosis. The IC50 for the apoptotic response of the three lung cancer cell lines was determined (data not shown), and cells were treated with that concentration of cisplatin for all subsequent experiments. As predicted, overexpression of SV1 resulted in a significant decrease in apoptosis in response to cisplatin treatment (Fig. 3D). Similar findings were shown in the H388 and H1944 cell line (data not shown). This change was associated with an abrogation of Bcl-2 down-regulation and NOXA up-regulation at the mRNA level (Fig. 3E).

Targeted reduction of KLF6-SV1 increases lung cancer cell apoptosis in combination with cisplatin treatment. To further characterize the role of KLF6-SV1 in the regulation of cisplatin-mediated DNA damage and apoptosis, we measured the levels of KLF6-SV1 after treatment with cisplatin. Increasing doses of cisplatin resulted in up-regulation of KLF6-SV1 mRNA and protein in the chemotherapy-resistant adherent cells, whereas KLF6 levels remained unchanged (Fig. 4A and B). Based on these findings, we hypothesized that increased expression of KLF6-SV1 may be associated with cisplatin resistance. Combination therapy with siRNA specific to SV1 and cisplatin resulted in increased activation of the intrinsic pathway of apoptosis (Fig. 4C). The increased apoptosis seen with the combination therapy was associated with a marked increase in NOXA expression at both

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Targeted down-regulation of KLF6-SV1 increases the induction of apoptosis in combination with cisplatin. A, increasing doses of cisplatin result in increased KLF6-SV1 expression with no changes in wild-type KLF6 levels as measured by qRT-PCR, using wild-type KLF6- and SV1-specific primers (10). RNA and protein were harvested from adherent cells only after cisplatin treatment. B, Western blot of A549 cells treated with increasing doses of cisplatin probed with a KLF6 monoclonal antibody shows increased KLF6-SV1 expression with higher doses of cisplatin. C, targeted reduction of KLF6-SV1 in combination with subtherapeutic doses of cisplatin results in a significant increase in apoptosis in the A549 cell line (***, P < 0.001). Cells were first transfected with the indicated siRNA (nontargeting control (NTC) or SV1 (si-SV1)); 24 h after transfection, 10 μM of cisplatin or vehicle control was added to the cells. FACS analysis was performed at the indicated time points (48, 72, and 96 h; *, P < 0.01). D, qRT-PCR revealed an additive effect in NOXA up-regulation after treatment of A549 cells with si-SV1 and cisplatin at all time points assayed (48, 72, and 96 h; **, P < 0.001). Numbers above the bars, fold up-regulation in NOXA expression for each condition. All experiments were repeated three independent times. Column, mean of all three experiments; bars, SD.
the mRNA and protein level (Fig. 4D). Combined, these findings highlight a functional role for KLF6-SV1 in chemotherapy resistance and provide a potential biological basis for our finding that increased levels of KLF6-SV1 in lung adenocarcinomas is associated with poor survival.

Discussion
Lung cancer is the leading cause of cancer-related death in the United States. Patients with early stage disease can be treated with surgery and have a 5-year survival exceeding 70%; however, the prognosis of patients with metastatic lung cancer remains dismal (4). A better understanding of the molecular mechanisms underlying lung cancer progression will allow for both markers for risk prognostication to better guide treatment decisions and for the development of targeted therapies. Here, we show that an alternative splice variant of the KLF6 tumor suppressor gene, KLF6-SV1, is specifically up-regulated in lung adenocarcinoma, and that increased KLF6-SV1 expression is associated with poor survival.

Reference
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