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Targeted reduction of KLF6-SV1 restores chemotherapy sensitivity in resistant lung adenocarcinoma

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ABSTRACT

Kruppel-like factor 6 splice variant 1 (KLF6-SV1) is an oncogenic splice variant of the KLF6 tumor suppressor gene that is specifically overexpressed in a number of human cancers. Previously, we have demonstrated that increased expression of KLF6-SV1 is associated with decreased survival in lung adenocarcinoma patient samples and that targeted reduction of KLF6-SV1 using siRNA induced apoptosis both alone and in combination with the chemotherapeutic drug cisplatin. Here, we demonstrate that chemoresistant lung cancer cells express increased levels of KLF6-SV1. Furthermore, targeted reduction of KLF6-SV1 using RNA interference restores chemotherapy sensitivity to lung cancer cells both in culture and *in vivo* through induction of apoptosis. Conversely, overexpression of KLF6-SV1 resulted in a marked reduction in chemotherapy sensitivity in a tumor xenograft model. Combined, these findings highlight a functional role for the KLF6-SV1 splice variant in the regulation of chemotherapy response in lung cancer and could provide novel insight into lung cancer therapy.

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1. Introduction

Lung cancer is a leading cause of caner death in the United States, with over 162,460 deaths from lung cancer in the US exceeding cancer mortality from colorectal, breast, prostate, and pancreatic cancer combined [1]. The aggregate survival benefit from use of chemotherapy in the treatment of lung cancer is supported by evidence from dozens of randomized controlled trials [2]. Though the data supports a benefit in large groups of patients, variability in individual response to chemotherapy and the development of resistance after either discontinuation of treatment or during treatment itself is a major cause of patient morbidity and mortality [2,3]. Treatment failure results from the development of resistance to chemotherapy and is characterized by the selection for cancer cells with either defects in the apoptotic cascade, increased expression of nucleotide excision repair pathway members, and/or increased activity of drug efflux transporters [4,5]. Targeted therapy to the specific molecular alterations underlying the development of chemotherapy resistance represents an appealing therapeutic strategy.

KLF6 (Zf9/CPBP) (GeneBank accession number AF001461) is a member of the Krüppel-like factor (KLF) family, that was originally shown to be functionally inactivated by loss of heterozygosity (LOH) and somatic mutation in sporadic prostate adenocarcinomas [6]. More recent reports have extended the range of human tumors and the mechanisms by which KLF6 can be inactivated to include deletion of the KLF6 locus and mutation in colorectal cancers [7], hepatocellular and gastric carcinomas [8,9], LOH in ovarian carcinoma and gliobastoma [10,11], decreased KLF6 expression in non-small cell lung cancer [12,13], hypermethylation of the promoter region in esophageal SCC cell lines and hepatocellular carcinoma patient samples [14,15]. In addition, three alternatively spliced KLF6 isoforms have been identified [16] and at least one of them, KLF6-SV1, has been shown to be biologically active, antagonizing the tumor suppressor function of KLF6 and promoting tumor growth and dissemination [10,16,17].

A role of KLF6 in non-small cell lung cancer (NSCLC) was first suggested by microarray studies comparing gene expression between normal and lung cancer specimens [18]. COPEB/KLF6 was decreased in malignant compared to benign lung tissue but high KLF6 expression levels in tumor specimens were associated with advanced disease stages and contributed to a prognostic gene signature of poor survival [18]. Furthermore, recent reports suggest that decreased KLF6 expression is a common event in lung adenocarcinoma and overexpression of KLF6 in lung cancer cell lines

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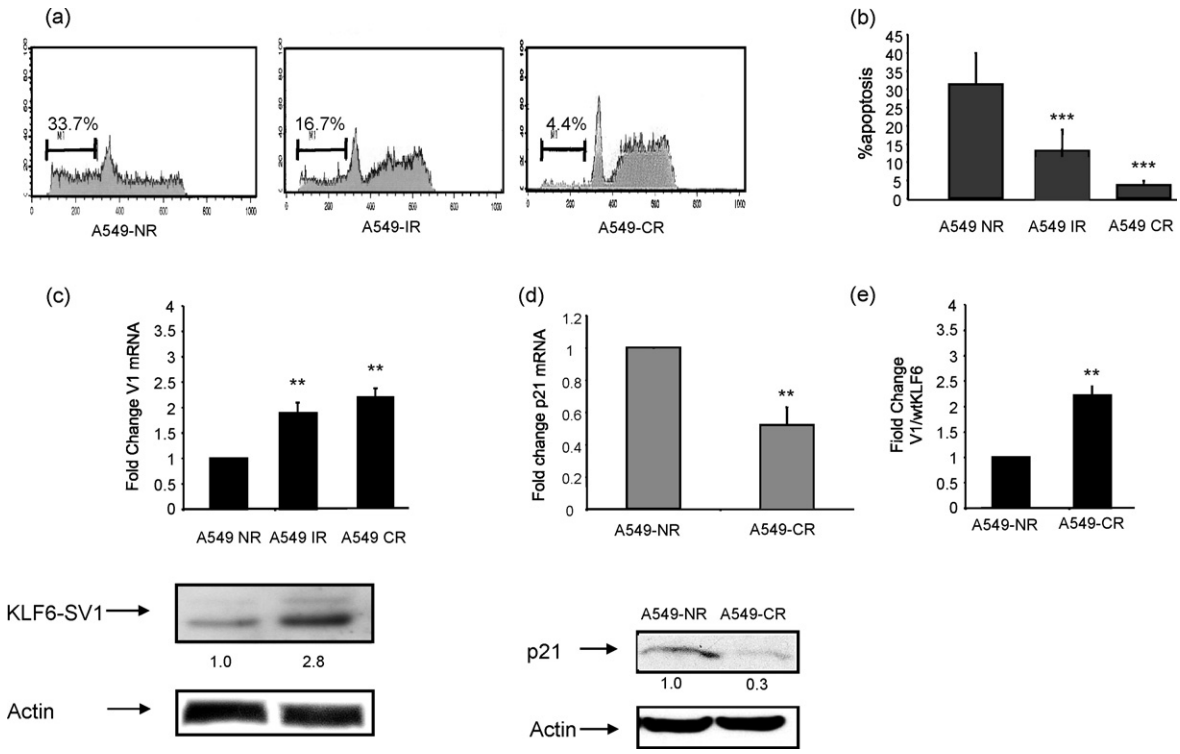

Fig. 1. Expression of KLF6-SV1 in cisplatin resistant lung cancer cell lines. a, Generation and validation of cisplatin resistance in the A549 lung adenocarcinoma cell line. The A549-IR and A549-CR cells were generated by serial selection of the parental A549 cell line in 20 μM cisplatin for 2 and 8 passages respectively. All cell lines were plated at equal densities and treated with 20 μM of cisplatin, 72 h after treatment cells were harvested. A, Representative FACS analysis of the A549-NR, A549-IR and A549-CR cell line treated with 20 μM cisplatin. FACS analysis of the treated cell lines revealed a marked reduction in the induction of apoptosis in parental A549 cell line (A549-NR) (33.7%) vs. A549-IR (16.7%) vs. A549-CR (4.4%) cell lines. A marked induction of apoptosis is seen in the control A549 cell line while addition of cisplatin results in a G2/M arrest with little induction of apoptosis in the A549-CR cell line. The A549-IR cell line displayed an intermediate sensitivity to cisplatin. b, A significant decrease in apoptosis in the A549-IR and A549-CR compared to the control A549 cell line ($^{***}p < 0.001$). This was repeated three independent times. C, qtRT-PCR and western blot analysis of the A549-NR, A549-IR, and A549-CR demonstrate a marked upregulation of KLF6-SV1 at both the mRNA and protein level ($^{**}p < 0.001$). d, p21 expression at both the mRNA and protein level in the A549-CR cell line is significantly lower than the parental A549-NR cell line ($^{**}p < 0.001$). e, the ration of KLF6-SV1 to wtKLF6 was determined by quantitative real time PCR (qRT-PCR) using KLF6-SV1 and wtKLF6 specific primers. There is a significant increase in the ratio of KLF6-SV1/wtKLF6 in the cisplatin resistant cell line.

induced spontaneous apoptosis [13]. Previously, we have demonstrated that increased expression of KLF6-SV1 is associated with decreased survival in lung adenocarcinoma patient samples and that targeted reduction of KLF6-SV1 using siRNA induced apoptosis both alone and in combination with the chemotherapeutic drug cisplatin [19]. Given the evidence for a role for the KLF6 tumor suppressor gene in lung cancer [12,13,19], we sought to define the role of the oncogenic splice variant, KLF6-SV1 in the development of chemotherapy resistance and whether targeted reduction of KLF6-SV1 could restore chemotherapy sensitivity in chemoresistant lung cancer cell lines.

2. Materials and methods

2.1. Cell culture and cell line generation

All cell lines were obtained from the American Tissue Culture Collection (ATCC). Retroviral infection with KLF6-SV1 was performed as previously described [10,20,21]. The A549 resistant cell lines were generated by serial selection in cisplatin (final concentration 20 μM) for 2 passages for the A549-IR cell line and 8 passages for the A549-CR cell line. Transient transfection of a non-targeting control and SV1 siRNA was performed with Lipofectamine 2000 (Invitrogen) in the A549 lung cancer cell lines as previously described [20,21]. Briefly, 50,000 cells per 12 well dish were plated for each cell line. Cells were then transfected with equal amount of the siRNA and harvested at 72hr for RNA, protein, and FACS analysis. For chemotherapy experiments, cisplatin (Sicor Laboratories) was added to a final concentration of 20 μM 24 h after either plating of the A549-P, A549-IR, or A549-CR stable cell lines or transfection with si-NTC or si-SV1 in all lung cancer cell line studied.

2.2. Western blot analysis

Cell extracts for Western blotting were harvested in radioimmunoprecipitation assay buffer (standard protocols, Santa Cruz Biotechnology). Tumor tissue extracts were harvested and prepared in the T-PER reagent (Pierce, Rockford, IL). Equal amounts of protein (50 μg) as determined by the Bio-Rad (Richmond, CA) DC Protein quantification assay were loaded and separated by PAGE and transferred to nitrocellulose membranes. Western blotting was done using a goat polyclonal antibody to Actin, rabbit polyclonal antibodies to NOXA, MCL1, Caspase 3, 8, and 9 (Cell Signalling Technology), and monoclonal antibodies to KLF6 2A2 (Zymed).

2.3. RNA and qRT-PCR analysis

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). qRT-PCR was performed using the PCR primers previously described [10,11,20] on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). All experiments were done in triplicate and independently validated three times. All values