

Krüppel-Like Factor 6 Is Frequently Down-Regulated and Induces Apoptosis in Non-Small Cell Lung Cancer Cells

Genshi Ito,^{1,2} Mika Uchiyama,^{1,3} Masashi Kondo,² Shoichi Mori,^{1,3} Noriyasu Usami,^{1,3} Osamu Maeda,¹ Tsutomu Kawabe,² Yoshinori Hasegawa,² Kaoru Shimokata,² and Yoshitaka Sekido¹

Departments of ¹Clinical Preventive Medicine, ²Respiratory Medicine, and ³Thoracic Surgery, Nagoya University School of Medicine, Nagoya, Japan

ABSTRACT

Krüppel-like factor 6 (KLF6) is a ubiquitously expressed zinc finger transcriptional factor, which has been suggested to be a candidate tumor suppressor gene in prostate cancer and astrocytic glioma. Because *KLF6* is located at chromosome 10p15, where non-small cell lung cancers (NSCLCs) also exhibit frequent allelic loss, we hypothesized that the inactivation of *KLF6* is also involved in the development of NSCLC. To determine this, we performed mutational analysis for 105 NSCLCs, including 9 cell lines and 96 primary tumors, and Northern blot analysis for 74 NSCLCs, including the 9 cell lines and 65 primary tumors. Although somatic mutations were not detected in the coding sequence of *KLF6*, expression of *KLF6* mRNA was down-regulated in the 9 cell lines and in 55 (85%) of the 65 primary tumors compared with normal lung tissue. Treatment of two cell lines expressing *KLF6* at low levels with 5-azacytidine did not induce *KLF6* expression, suggesting that *KLF6* down-regulation is not due to promoter hypermethylation. We also performed loss of heterozygosity (LOH) analysis using the laser capture microdissection technique, and found that 21 of 62 (34%) informative samples had LOH in the *KLF6* gene locus. Comparing the LOH status with mRNA expression of *KLF6*, we found that 14 of the 14 (100%) samples with LOH showed *KLF6* down-regulation, and that even 23 of 31 (74%) samples without LOH also showed this down-regulation. We also studied the expression of the *WAF1* gene, a possible downstream gene of *KLF6*, and detected simultaneous down-regulation of *WAF1* and *KLF6* mRNA in 6 of 9 (67%) cell lines and 48 of the 55 (87%) primary tumors, although there was not a significant association between loss of *KLF6* and *WAF1* expression. Furthermore, colony formation assay of two NSCLC cell lines (NCI-H1299 and NCI-H2009) induced a markedly reduced colony formation by *KLF6* transfection, and Annexin V staining and terminal deoxynucleotidyl transferase-mediated nick end labeling assays revealed that *KLF6* induced apoptosis. Our present studies demonstrated that *KLF6* is frequently down-regulated in NSCLC and suppresses tumor growth via induction of apoptosis in NSCLC, which may suggest that *KLF6* is a tumor suppressor for NSCLC.

INTRODUCTION

Non-small cell lung cancer (NSCLC) is a leading cause of cancer death in Western countries and Japan, and genetic and epigenetic alterations of proto-oncogenes and tumor suppressor genes (TSGs), which are thought to be primarily induced by tobacco-carcinogens, are implicated in the development of lung tumors. Cytogenetic and allelotyping studies have revealed many chromosomal regions showing loss of heterozygosity (LOH) in lung cancer, with each targeted TSG identified subsequently, including 17p13 for *p53*, 9p21 for *p14^{ARF}* and *p16^{INK4a}*, 13q14 for *RB*, and multiple loci of 3p for *FHIT*, *RASSF1A*, and/or other unidentified genes (1). In addition to these chromosomal loci, new regions of frequent allelic loss in lung cancer

have been reported from studies using the comparative genomic hybridization technique and single-nucleotide arrays (2–6). An allelotyping analysis comparing lung cancer cell lines and matched B-lymphoblastoid cell lines using multiple microsatellite markers also demonstrated new regions showing frequent LOH, including 1p22, 4q21–23, 6q22, 10p11, 10p15, 13q11, 19p13, 20p12, and Xq22 (7). However, none of these sites have been clearly identified in regard to their target genes.

Krüppel-like factor (KLF) family members are transcriptional factors, which bind GC box and up-regulate and/or down-regulate the expression of target genes. Functionally, the KLF family members are shown to be involved in cell differentiation, development, growth related signal transduction, cell proliferation, and apoptosis (8). *KLF6* contains a proline- and serine-rich NH₂-terminal activation domain, and like other KLFs, three COOH-terminal C₂H₂ zinc fingers. Whereas several members of the KLF family have been shown to be involved in carcinogenesis, such as down-regulation of *KLF4* found in colon cancer (9), and *KLF5* and *KLF10* in breast cancer (10, 11), *KLF6* has also been suggested to be a candidate TSG at 10p15, with frequent mutations observed in prostate adenocarcinoma (12). Furthermore, *KLF6* was also shown to transactivate *WAF1*, which encodes a cyclin-dependent kinase inhibitor of the cell cycle via a p53-independent pathway (12).

Because the *KLF6* gene is located on chromosome 10p15, which has been indicated to be a site showing frequent allelic loss in NSCLC (7), we hypothesized that the *KLF6* gene might be the target TSG for NSCLC as well. Moreover, a cDNA microarray analysis that showed frequent *KLF6* gene down-regulation in lung adenocarcinoma also seemed to support our hypothesis (13). To determine this, we analyzed 105 NSCLCs for genetic mutation and 65 NSCLCs for mRNA expression. Although somatic mutations were not detected in the coding sequence of *KLF6*, expression of *KLF6* mRNA was down-regulated in 9 of 9 (100%) cell lines and 55 of 65 (85%) surgical specimens compared with normal lung tissues. We detected LOH at the *KLF6* locus in 21 of 62 (34%) informative cases. Comparing the LOH status with *KLF6* expression, we found that 14 of 14 (100%) samples with LOH showed *KLF6* down-regulation, and that 23 of 31 (74%) samples that retained both alleles also showed *KLF6* down-regulation. Furthermore, we demonstrated that the exogenously transfected *KLF6* suppressed tumor growth of lung cancer cells and revealed that this was due to apoptosis. Our studies suggested that the frequent down-regulation of *KLF6* in NSCLC might be involved in lung cancer development.

MATERIALS AND METHODS

Cell Lines and Tumor Samples. Nine lung cancer cell lines, including four adenocarcinomas (NCI-H358, -H920, -H1666, and -H2009), 2 squamous cell carcinomas (NCI-H157 and -H226), and 3 large cell carcinomas (NCI-H460, -H1155, and -H1299), were received as gifts from Dr. Adi F. Gazdar. Ninety-six surgical specimens containing both tumor and nearby noncancerous tissues were collected from the Nagoya University Hospital, Nagoya 1st Japan Red Cross Hospital, Nagoya 2nd Japan Red Cross Hospital, Kasugai City Hospital, and Chukyo Hospital in Nagoya, Japan (Table 1). Ethical approval was obtained from each of the five hospitals, and fully informed consent

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Requests for reprints: Yoshitaka Sekido, Department of Clinical Preventive Medicine, Nagoya University School of Medicine, Tsurumai 65, Showa-Ku, Nagoya 466-8560, Japan. Phone: 81-52-744-1974; Fax: 81-52-744-1975; E-mail: ysekido@med.nagoya-u.ac.jp.

Table 1 Patient characteristics

Characteristics	Number
Total patients	96
Age (mean \pm SD) (yr)	64.6 \pm 6.4
Gender	
Male	67
Female	29
Histologic type	
Adenocarcinoma	62
Squamous	27
Adenosquamous	2
Large	4
Undifferentiated	1
Stage	
IA	31
IB	18
IIA	5
IIB	8
IIIA	27
IIIB	3
IV	4

was obtained from all of the patients before tissue collection. The material comprised 62 adenocarcinomas, 27 squamous cell carcinomas, 4 large cell carcinomas, 2 adenosquamous cell carcinomas, and 1 undifferentiated carcinoma.

DNA and RNA were prepared from these samples by standard technique (14). Random-primed, first-strand cDNAs were synthesized from 2 μ g of total RNAs using Superscript II according to the manufacturer's instructions (Life Technologies Inc., Rockville, MD).

Mutational Analysis. We developed 7 primer sets from the *KLF6* genomic sequence (GenBank accession no. AF001461) to cover the entire coding region of exon 1–4. Primer sets used were as follows (numbers after "ex" indicate exon number, and "S" and "AS" indicate sense and antisense, respectively): Exon 1 and 2 were analyzed with two and three primer sets, respectively): *KLF6*-ex1-1S, 5'-TGGCAGCGGAGCTTTGAATA-3', and *KLF6*-ex1-1AS, 5'-TCCTGGAAGATGCTGCACAT-3'; *KLF6*-ex1-2S, 5'-GAGCTGGAGT-TTGCATGAA-3', and *KLF6*-ex1-2AS, 5'-TGCGTTACCTGTTGCCAGT-3'; *KLF6*-ex2-1S, 5'-TTCATTTTGCACAGACCTGC-3', and *KLF6*-ex2-1AS, 5'-TAAACTTGGCCGTGGGAGAAAAGTT-3'; *KLF6*-ex2-2S, 5'-TTACA-ACCTAGAGACCAACAGCCT-3', and *KLF6*-ex2-2AS, 5'-CATTTCCCTTGTACCTGGCTT-3'; *KLF6*-ex2-3S, 5'-GTTTTGGTTCAGCTCGGGA-AAATTG-3', and *KLF6*-ex2-3AS, 5'-AACTGAGCAGGGAACCTTCT-3'; *KLF6*-ex3-1S, 5'-TGAAGTCATGGGCTGCTGT-3', and *KLF6*-ex3-1AS, 5'-CCTGGTCATCACATTCCCAA-3'; and *KLF6*-ex4-1S, 5'-GGAACCTA-ACTTCTTCTGTG-3', and *KLF6*-ex4-1AS, 5'-GGTGCTATGCCGTTCT-TACAGGA-3'. Twenty ng of genomic DNA was used as a template for PCR. In PCR for *KLF6*, we used a modified "Touchdown PCR" (15).

For cell lines, PCR products were directly sequenced using an Applied Biosystems model 377 DNA sequencer (Perkin-Elmer, Norwalk, CT) with a PCR primer and a BigDye terminator 3.0 Cycle sequencing FS Ready Reaction kit (PE Applied Biosystems, Foster City, CA). For surgical specimens, PCR and single-strand conformation polymorphism analysis was performed as described previously (16).

Northern Blot Analysis. Northern blot was performed as described previously (17). The cDNA probes synthesized with reverse transcription-PCR were a 197-bp fragment covering from nucleotide 140 to nucleotide 337 of the *KLF6* gene and a 494-bp fragment covering the entire coding sequence of the *WAF1* gene. The mRNA levels of *KLF6*, *WAF1*, and β -actin were quantified with an imaging analyzer (BASStation; Fujifilm, Tokyo, Japan), and the relative amounts of *KLF6* and *WAF1* were normalized with the amounts of β -actin reprobated on the same membrane. Equal or less than 0.6 (*KLF6*) or 0.4 (*WAF1*) expression of a normal lung was defined as down-regulation.

Laser Capture Microdissection. Laser capture microdissection was performed as described previously using a PixCell laser capture microscope (Arcturus Engineering, Mountain View, CA; Ref. 15). In general, 2000 hits with a laser pulse were used to obtain >2000 cells. The cells were immersed in 40 μ g of digestion buffer, containing 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.4 mg/ml proteinase K, and 1% Tween 20, and digested at 37°C overnight. After digestion, the enzyme was heat inactivated (95°C for 10 min),

and the extract was directly used for allelotyping PCR. Genomic DNA extracted from the noncancerous lung tissues served as the normal control for LOH analysis.

LOH Analysis. Genomic DNAs extracted from the microdissected tumor specimens were amplified by PCR, which was carried out in a 20- μ l volume containing extracted DNA from at least 100 microdissected nuclei, 4 pmol of each primer for *KLF6M1* and *KLF6M2* (12), 200 μ M of each deoxynucleoside triphosphate, and TaqDNA polymerase (Takara Bio, Otsu, Japan) containing 10 mM Tris (pH 8.3), 50 mM KCl, and 1.5 mM MgCl₂. The analysis was performed using the ABI Genescan and Genotyper software packages (Perkin-Elmer). Relative allele ratio <0.65 was determined as LOH.

Transient Transfection and Colony Formation Assay. Full-length *KLF6*-cDNA was amplified with reverse transcription-PCR using cDNA synthesized from normal lung RNA and a primer set of *KLF6*-forward, 5'-AGCGAATTCGACATGGACGTGCTCCCATG-3' (italicized nucleotides indicate artificial *EcoRI* site), and *KLF6*-reverse, 5'-AGCTCTAGATCAGAG-GTGCCTTTCATGTG-3' (italicized nucleotides indicate artificial *XbaI* site). The PCR product was double-digested with *EcoRI* and *XbaI* and subcloned into the *EcoRI* and *XbaI* sites of pcDNA3.1 (+) (Invitrogen, Carlsbad, CA). Preparation of *p53*-construct is described elsewhere (18). The entire insert was confirmed by sequencing from both directions. For colony formation assay, 4×10^5 NCI-H1299 cells or 3×10^5 NCI-H2009 cells were seeded into six-well plates a day before transfection. Transient transfection of the *KLF6* and *p53* genes was performed with Lipofectamine PLUS reagent according to the manufacturer's protocol (Invitrogen). Lung cancer cells in six-well dishes were transfected with 1 μ g per well of the expression vector. After transfection, cells were trypsinized, replated, and cultured in 600 μ g/ml G418 (G7034; Sigma, St. Louis, MO) supplemented medium (RPMI 1640 and 10% fetal bovine serum) for 2 weeks. The numbers of the G418-resistant colonies were counted after staining with methylene blue in ethanol/H₂O (50/50%). The surviving colonies of the vector control were set at 100%. The data represent the mean \pm SD of three independent experiments, each carried out in triplicate plates.

Western Blot Analysis. Preparations of total cell lysates and Western blotting were performed as described previously (19). First antibodies used were anti-zf9 (*KLF6*) antibody (R-173; Santa-Cruz Biotechnology, Santa Cruz, CA), anti-p53 antibody (NCL-p53-DO7; Novocastra Laboratories, Newcastle, United Kingdom), anti-p21 (*WAF1*) antibody (H-164; Santa-Cruz Biotechnology), anti-caspase-3 antibody (Cell Signaling Technology, Beverly, MA), antipoly(ADP-ribose) polymerase antibody (Cell Signaling Technology, Inc.), and anti- β -actin antibody (AC-15; Sigma, St. Louis, MO).

Apoptosis Assay. For the Annexin V staining, the Annexin V-Biotin Apoptosis Detection kit (BV-K 109-3; MBL, Nagoya, Japan) was used as described previously (18). In brief, cells (0.5×10^5) were plated on cover glasses on 24-well plates the day before transfection. Twenty-four h after cotransfection with a construct and pEGFP-F vector (Clontech Laboratories Inc., Palo Alto, CA) as a cotransfection marker, cells were incubated with Annexin V-biotin for 5 min at room temperature. They were then incubated with streptavidin-Alexa 568 (Molecular Probes, Eugene, OR). The cover glasses were inverted on a drop of Mounting Medium (Shandon, Pittsburgh, PA) on slide glasses and observed with a confocal laser-scanning microscope (MRC1024, Bio-Rad Laboratories, Hercules, CA). For the terminal deoxynucleotidyl transferase-mediated nick end labeling assay, an *in situ* cell death detection kit (Roche, Mannheim, Germany) was used as described previously (18). Briefly, 36 h after cotransfection with pEGFP-F vector, the cells were fixed with 4% paraformaldehyde in PBS and terminal deoxynucleotidyl transferase-mediated nick end labeling reaction was performed according to the manufacturer's protocol. For quantification, stained cells were counted, and the results were presented as percentage of green fluorescent protein-positive cell number. Three independent $\times 200$ fields containing a minimum of 300 green fluorescent protein-positive cells on three replicate slides were evaluated for each condition.

Statistical Analysis. Fisher's exact tests were used for the correlation analysis, and paired *t* tests were used for significance. Statistical calculations were performed using a computer statistical package (StatView version 5.0; SAS Institute Inc., Cary, NC).

RESULTS

Mutation Analysis of the *KLF6* Gene. To determine whether the *KLF6* gene is genetically altered in NSCLC, we performed sequencing analysis for 9 NSCLC cell lines and single-strand conformation polymorphism analysis for 96 surgical specimens. Among the 9 cell lines, we detected a heterozygous mutation (C to A) at 4 bp upstream from the starting codon in NCI-H920 (data not shown) but found no other mutations. In the 96 surgical specimens, we detected that 1 primary tumor sample (KD623) had an aberrant band with single-strand conformation polymorphism using a primer set covering exon 2 (data not shown). Sequence analysis of this tumor DNA and the constitutional DNA from this patient revealed that this change was a silent polymorphism at codon 168 (data not shown).

Expression of the *KLF6* Gene. To determine whether the *KLF6* gene was down-regulated in NSCLCs, we performed Northern blot analysis for the 9 cell lines and 65 surgical specimens that were available for RNA. Whereas all of the 9 cell lines showed down-regulation of *KLF6* mRNA compared with normal lung tissue, 55 of 65 (84%) showed down-regulation of *KLF6* mRNA (Fig. 1). We compared the *KLF6* expression with the patient characteristics but did not find any association between the *KLF6* expression and the histology or surgical-pathological stages of lung tumors (data not shown). Next, to determine whether the *KLF6* gene is silenced by promoter hypermethylation, we tested 2 NSCLC cell lines, NCI-H1299 and

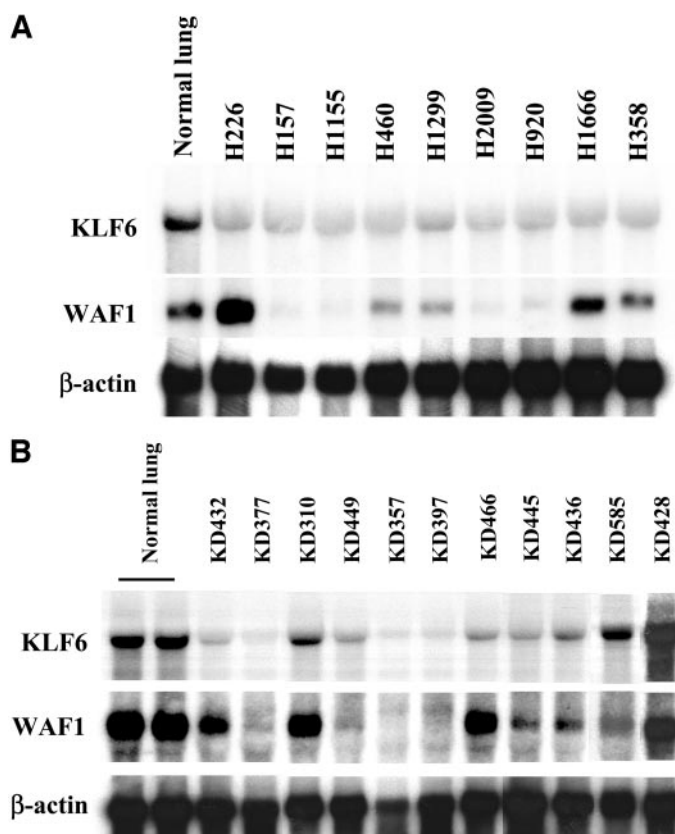


Fig. 1. Representative Northern blot analysis of *KLF6*, *WAF1*, and β -actin. A, non-small cell lung cancer cell lines. Whereas *KLF6* mRNA is down-regulated in all 9 cell lines, *WAF1* mRNA was down-regulated in NCI-H157, -H1155, -H460, -H1299, -H2009, and -H920. The histological types were adenocarcinoma: NCI-H358, -H920, -H1666, and -H2009; squamous cell carcinoma: NCI-H157 and -H226; and large cell carcinoma: NCI-H460, -H1155, and -H1299. B, surgical specimens. *KLF6* mRNA was down-regulated in KD432, KD377, KD449, KD357, KD397, KD466, KD445, and KD436. *WAF1* mRNA was down-regulated in KD377, KD449, KD357, KD397, KD445, KD436, and KD585. The histological types were adenocarcinoma: KD428, KD436, KD445, KD449, and KD466; squamous cell carcinoma: KD310, KD377, KD397, and KD585; adenocarcinoma: KD432; and large cell carcinoma: KD357.

Table 2 Correlation between expression status of *KLF6* and *WAF1* in 65 NSCLCs^a

<i>KLF6</i> Expression	<i>WAF1</i> Expression		<i>P</i>
	High	Low	
High	3	7	0.18 ^b
Low	7	48	

^a NSCLC, non-small cell lung cancer.

^b Fisher's exact test

Table 3 Association between LOH^a status and expression status of *KLF6* in 63 NSCLCs

	<i>KLF6</i> expression		<i>P</i>
	High	Low	
Informative (45 cases)			0.04 ^b
Heterozygous	8	23	
LOH	0	14	
Not informative (18 cases)	2	16	

^a LOH, loss of heterozygosity; NSCLC, non-small cell lung cancer.

^b Fisher's exact test.

NCI-H2009, which were shown to be down-regulated for *KLF6* with a demethylation reagent, 5-azacytidine. However, we did not detect an induction of *KLF6* expression in these cell lines after treatment of 5-azacytidine (data not shown).

No Correlation Was Found between *KLF6* and *WAF1* Expression. The *WAF1* gene has been suggested to be a downstream target of *KLF6* as well as of wild-type *p53* (12). To determine whether the down-regulation of *KLF6* causes down-regulation of *WAF1*, we performed Northern blot analysis for *WAF1* (Fig. 1B). We found that 55 of the 65 (85%) primary tumors had down-regulation of *WAF1*. Among the 55 tumors with *WAF1* down-regulation, 48 (87%) showed simultaneous *KLF6* down-regulation, although it was not statistically significant ($P = 0.18$; Table 2). Furthermore, we analyzed the 65 tumors for *p53* alteration and found that 27 cases had missense mutation, 12 had nonsense or frameshift mutation, and 26 had wild-type *p53* (20). However, we did not find any significant correlation between *p53* mutation and *WAF1* expression status ($P > 0.99$; data not shown). Meanwhile, among the 9 cell lines, 6 cell lines (NCI-H157, -H460, -H920, -H1155, -H1299, and -H2009) showed down-regulation of *WAF1* mRNA, whereas 3 did not (NCI-H226, -H358, and -H1666; Fig. 1A).

LOH Analysis of Chromosome 10p15. To determine whether the *KLF6* gene locus at chromosome 10p15 shows frequent allelic loss in NSCLCs, we performed LOH study using the laser capture microdissection method for all but 4 of the 96 samples. Among the 92 surgical specimens, 30 samples were not informative of either the *KLF6M1* or *KLF6M2* microsatellite markers, which are located 42 kb and 12 kb away from the *KLF6* gene. Among the 62 informative cases for either marker, 16 samples showed allelic loss of *KLF6M1*, 3 samples showed allelic loss of *KLF6M2*, and 2 samples showed allelic loss of both microsatellite markers. Overall, 21 (34%) samples showed LOH, and 41 (66%) samples showed heterozygosity at the *KLF6* locus.

Next, we compared the LOH status with the expression status of *KLF6* mRNA in the 63 samples for which we were able to perform both analyses, with 45 cases being informative and 18 cases not informative (Table 3). Of the 45 informative cases, 31 (69%) showed heterozygosity, and 14 (31%) cases showed allelic loss. Whereas all 14 cases with LOH showed down-regulation, 23 of the 31 (75%) cases with heterozygosity indicated down-regulation of the *KLF6* gene. We detected a significant correlation between LOH status and *KLF6* down-regulation in the informative cases ($P = 0.04$).

Inhibition of Colony Formation by *KLF6* in NSCLC Cells. Frequent down-regulation of *KLF6* strongly suggested that it is also a candidate of TSG for NSCLC. To test whether *KLF6* has a growth-

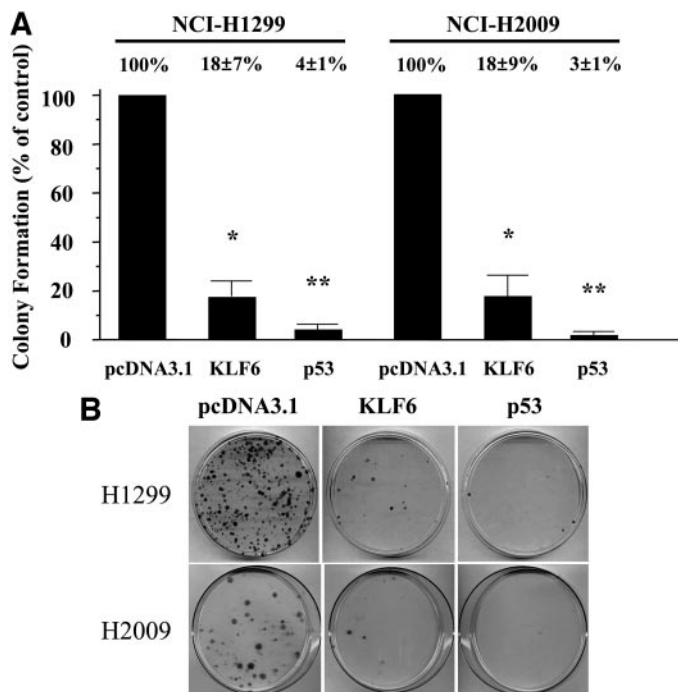


Fig. 2. Colony formation assay of the NCI-H1299 cells transfected with the *KLF6* and *p53* expression constructs. **A**, the graph indicates the number of colonies relative to the number of colonies formed by control vector transfection, which were set to 100%. *KLF6* and *p53* suppressed colony formation of NCI-H1299 relative to vector control pcDNA3.1(+) (*, $P < 0.05$; **, $P < 0.001$). The results were from three separate transfection experiments. **B**, representative cases are shown. NCI-H1299 and NCI-H2009 cells were transfected with pcDNA3.1(+) (left), *KLF6* (middle), or *p53* (right), respectively; bars, \pm SD.

inhibitory activity for NSCLC cells, we performed a colony formation assay selecting for the *neo* gene carried by our expression constructs. We transfected the *KLF6* or *p53* constructs into the NCI-H1299 (large cell carcinoma) and NCI-H2009 (adenocarcinoma) cells, selected them with G418 for 2 weeks, and counted the number of surviving colonies with the vector control set at 100%. As expected, *p53* showed strong suppression for NCI-H1299 ($4 \pm 1\%$) and NCI-H2009 ($3 \pm 1\%$; $P < 0.001$; Fig. 2). *KLF6* also showed strong suppression for colony formation in both the NCI-H1299 ($18 \pm 7\%$) and NCI-H2009 ($18 \pm 9\%$) cells ($P < 0.05$).

KLF6 Induced Apoptosis in NSCLC. To determine whether *KLF6* induces *WAF1* expression in NSCLC cells like prostate adenocarcinoma cells (12), we performed transient transfection of the *KLF6* gene into NCI-H920, -H1299, and -H2009, in all of which we found down-regulation of both *KLF6* and *WAF1* (Fig. 1A). First, we tested whether *p53* induces *WAF1* expression in NSCLCs. Of the 3 cell lines, *WAF1* up-regulation was found in NCI-H1299 and NCI-H2009 but not in NCI-H920 (Fig. 3). Next, we transfected *KLF6* into these 3 cell lines but did not find the induction of *WAF1* in any of these cells (Fig. 3).

These data suggested that growth inhibition of NSCLCs by *KLF6* might result from apoptosis but not from G_1 -arrest of the cell cycle. To determine this, we performed Annexin V staining, which detects the early stage of apoptosis, for the *KLF6*-transfected cells with cotransfection of pEGFP-F vector as an indicator of transfected cells. As shown in Fig. 4, A and B, *KLF6* transfection into the NCI-H1299 cells showed significantly higher percentages of Annexin V-positive cells than the empty pcDNA3.1 (+) vector. The percentage of Annexin V-positive cells in *KLF6*-transfected cells was equal to that in the *p53*-transfected cells (Fig. 4, A and B). Next, we performed terminal deoxynucleotidyl transferase-mediated nick end labeling as-

say, which detects the end stage of apoptosis. Transfection of the NCI-H1299 cells with *KLF6* resulted in significantly higher percentages of terminal deoxynucleotidyl transferase-mediated nick end labeling-positive cells than the empty pcDNA3.1 (+) vector (Fig. 4, C and D).

Furthermore, to determine whether the caspase signal is activated during the apoptosis induced by *KLF6*, we performed Western blotting for caspase-3 and poly(ADP-ribose) polymerase. We detected the 19-kDa fragment, which indicates caspase-3 activation, and the 24-kDa fragment of poly(ADP-ribose) polymerase, which also indicates its activation, in the transfectants by *KLF6* as well as *p53* (Fig. 5).

DISCUSSION

In the present study, we have shown that the expression of the *KLF6* gene is frequently down-regulated in NSCLCs and that *KLF6* inhibits cell growth of NSCLC cells by induction of apoptosis, suggesting that *KLF6* may be a tumor suppressor for NSCLC.

All 9 of the NSCLC cell lines examined and 55 of 65 (85%) primary NSCLCs showed down-regulation of the *KLF6* with Northern blot analysis, including 37 of 44 (84%) adenocarcinomas. The frequency of NSCLCs with *KLF6* down-regulation seems to be consistent with a previous study using a cDNA microarray and reverse transcription-PCR assays, which demonstrated that 8 of 14 (57%) primary adenocarcinomas showed down-regulation of *KLF6* (13). Meanwhile, with respect to allelic loss at 10p15, Girard *et al.* (7) reported that 40% of NSCLC cell lines showed LOH at 10p15 using D10S591 and D10S189 microsatellite markers by comparing them with the corresponding EBV-transformed B lymphoblastoid cell lines. We found that 21 of 62 (34%) informative cases showed LOH at the *KLF6* gene locus. Thus, the present study also showed that NSCLCs have LOH at 10p15 with a frequency similar to other studies.

Comparing the mRNA expression level with LOH status of *KLF6*, all 14 cases with LOH showed down-regulation of *KLF6*. Moreover, 23 of 31 (75%) surgical specimens with heterozygosity also showed down-regulation of *KLF6*. Despite the frequent down-regulation of *KLF6*, no somatic mutation was detected in the coding region in NSCLCs compared with sporadic pituitary tumors (21), astrocytic gliomas (22), and prostate adenocarcinomas (12, 23), with the frequencies of mutation reported to be 5%, 5–11%, and 15–55%, respectively. Only a rare polymorphism was seen in 1 surgical specimen (KD623), which has not been identified before. Thus, a point mutation

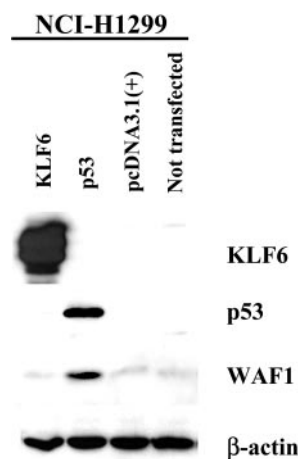


Fig. 3. Western blot analysis of *KLF6*, *p53*, *WAF1*, and β -actin. Each lane was loaded with 30 μ g of total cell lysates from the NCI-H1299 cells transfected with *KLF6*-cDNA, *p53*-cDNA, or pcDNA3.1(+), and no transfectants. *KLF6* transfection did not induce *WAF1* expression. In contrast, *p53* transfection induced *WAF1* expression.

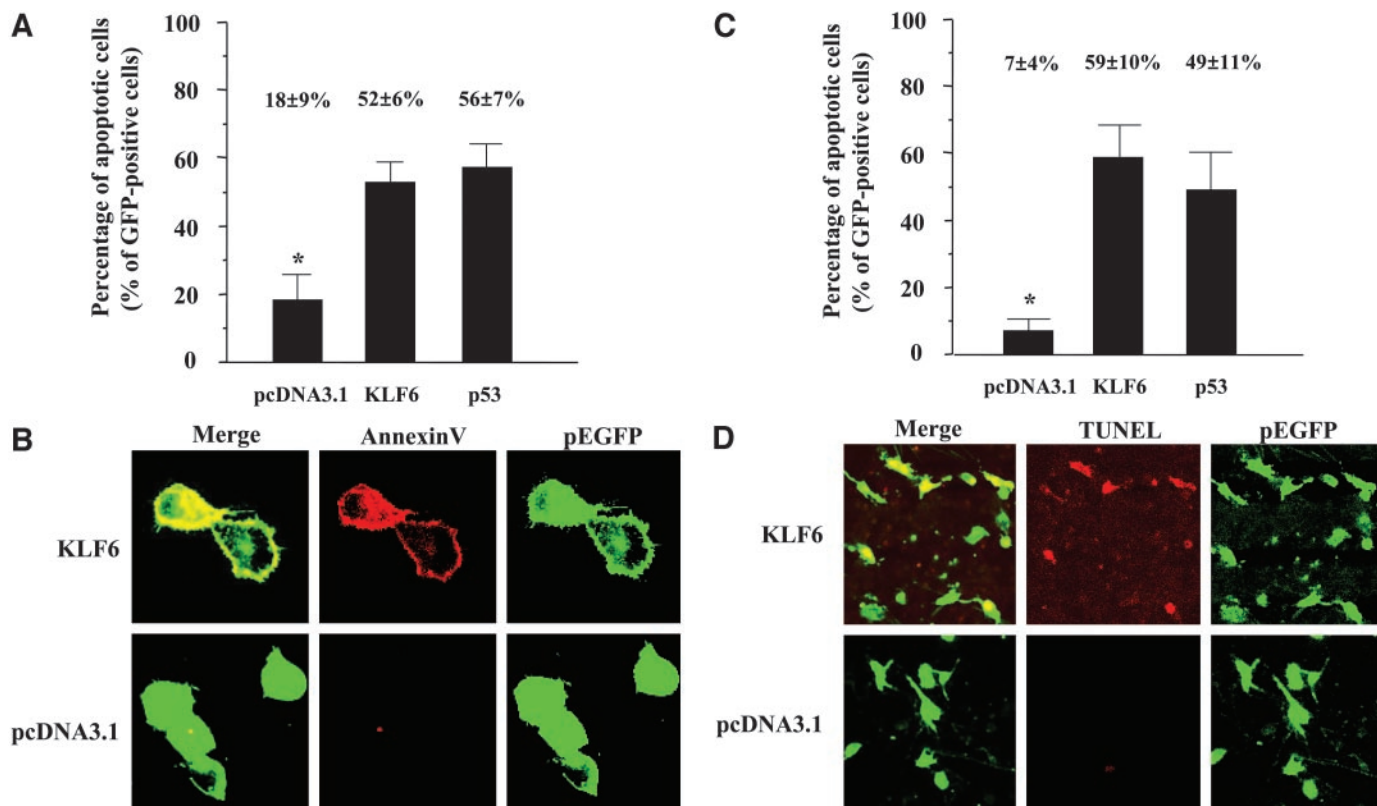


Fig. 4. Detection of apoptotic cells of the NCI-H1299 cells. **A**, the percentages of apoptotic cells detected with Annexin V-conjugated Alexa 568 compared with the positive staining cells of enhanced green fluorescent protein are shown (*, $P < 0.05$). **B**, cells transfected with *KLF6*, which were marked by pEGFP-F, showed positive staining in Annexin V staining. **C**, the terminal deoxynucleotidyl transferase-mediated nick end labeling assay of the NCI-H1299 cells. The percentages of apoptotic cells detected with terminal deoxynucleotidyl transferase-mediated nick end labeling compared with the positive staining cells of enhanced green fluorescent protein are shown (*, $P < 0.05$). **D**, cells transfected with *KLF6*, which were marked by pEGFP-F, showed positive staining; bars, \pm SD.

does not seem to be a common mechanism to inactivate *KLF6* in NSCLCs.

NCI-H920 had a mutation (C to A) at 4 bp upstream from the starting codon and showed down-regulation of *KLF6*, which was identical to a mutation that was found in a prostate adenocarcinoma (23). Whether this mutation could alter *KLF6* expression or translation remains unclear; this mutation might be associated with down-regulation of *KLF6*. Conversely, *KLF6* was also suggested to be a methylation-silenced gene in esophageal squamous cell carcinoma, because 5-aza-deoxycytidine treatment for the KYSE30 and KYSE410 cells induced up-regulation of *KLF6* (24). However, in our study, *KLF6* expression was not induced by another demethylation reagent, 5-azacytidine, in the NCI-H1299 or -H2009 cells. Thus, although our results suggest that LOH may partly contribute to its down-regulation, additional studies seem to be necessary to identify the mechanism for the strong down-regulation of *KLF6* expression in NSCLCs.

Transfection of the *KLF6* gene expression construct markedly inhibited colony formation of NCI-H1299 and -H2009. Because NCI-H1299 is a p53-null cell line (25), our data also indicate that *KLF6* inhibits tumor cell growth through a p53-independent pathway. In prostate adenocarcinoma cells, *KLF6* was also shown to suppress tumor growth by demonstrating reduced [3 H]thymidine incorporation into DNA, possibly due to up-regulation of WAF1 (12). However, we did not detect WAF1 up-regulation by *KLF6* transient transfection in NCI-H920, -H1299, or -H2009, although the latter 2 cell lines were strongly suppressed in colony formation assay. These data suggest that *KLF6* does not induce WAF1 expression in NSCLC cells, indicating that growth inhibition may not be due to G_1 -S arrest of the cell

cycle. Indeed, we found that the transfection of *KLF6* induced a marked apoptotic cell death in p53-null NSCLC cell line, NCI-H1299. To our knowledge, the present study is the first report demonstrating that *KLF6* induces apoptosis in a human cancer cell line.

Regarding other KLF family members, apoptosis has been implicated in bladder cancer cells by *KLF4* (26), and in pancreatic epithelial cells by *KLF10*, which mediates apoptotic effects of transforming growth factor β 1 (27). Because *KLF6* has been shown to up-regulate transforming growth factor β 1 and its type I and II receptors (28), the

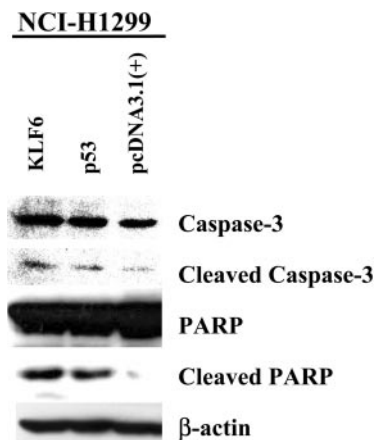


Fig. 5. Western blot analysis of caspase-3, poly(ADP-ribose) polymerase (PARP), and β -actin. Each lane was loaded with 60 μ g of total cell lysates from NCI-H1299 transfected *KLF6*-cDNA, *p53*-cDNA, or pcDNA3.1(+).

induction of apoptosis by KLF6 in NSCLC cells may also be via the transactivation of transforming growth factor β 1. Other putative transcriptional targets of KLF6 may include the genes encoding a placental glycoprotein (29), collagen α 1 (30), urokinase type plasminogen activator (31), inducible nitric oxide synthase (32), and corneal keratin-12 (33). Additional studies need to determine the target gene of KLF6 for induction of apoptosis in NSCLC cells.

In conclusion, *KLF6* is frequently down-regulated in NSCLCs, and exogenously induced KLF6 induces apoptosis and inhibits tumor cell growth, indicating that *KLF6* might be a tumor suppressor gene like other genes in the KLF family. Our present study provides the basis for additional analysis of KLF6 and other KLFs in carcinogenesis.

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