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

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ABSTRACT

Kruppel-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 down-regulation of KLF6 in 9 NSCLC 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-aza-cytidine 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 (85%) 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-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 p14ARF and p16INK4a, 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.

Kruppel-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 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...
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 μg 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): KLF6-ex1–1S, 5′-TTGGACGGCAGCTTGAATA-3′, and KLF6-ex1–1AS, 5′-TCTCTGGAATGTGCTGACAT-3′; KLF6-ex1–2S, 5′-GAGGCTGAGTTTGTGCTGAA-3′, and KLF6-ex1–2AS, 5′-TGCGTTTTACGTTGCGAGT-3′; KLF6-ex2–1S, 5′-TTCTTGTCAGGCCAGCTGC-3′, and KLF6-ex2–1AS, 5′-TAAACATTGGCTGGTTAAGGATCTG-3′; KLF6-ex2–2S, 5′-TTACAACCTTAGACCAACAAGCCT-3′, and KLF6-ex2–2AS, 5′-CATTCTCCCTTGTACCTGACTCC-3′; KLF6-ex3–1S, 5′-TGTGCTATGCCGCTTCT-3′, and KLF6-ex3–1AS, 5′-GGAACCTA-CTCTCTGTCTTG-3′; KLF6-ex4–1S, 5′-GTTTTGGTCAGCTCGGAGATTGTCACCTGGCTT-3′, and KLF6-ex4–1AS, 5′-TCCTGGAAGATGCTGCACAT-3′; and KLF6-ex4–2S, 5′-CATTTCCCATTAGAGACCAACAGCCT-3′.

**Western Blot Analysis.** Preparations of total cell lysates and Western blotting were performed as described previously (19). First antibodies used were anti-z9 (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 for 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 ×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-H209 (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 hypermethylation, we tested 2 NSCLC cell lines, NCI-H1299 and 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. Using Northern blot analysis for KLF6 mRNAs in these cell lines after treatment of 5-azacytidine, we found that 55 of 65 (84%) primary tumors had decreased expression of KLF6. 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. However, we did not find any significant correlation between p53 mutation and KLF6 expression status (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-H1157, -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 suggests that it is also a candidate of TSG for NSCLC. To test whether KLF6 has a growth-
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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, ±SD.

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 D10S91 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 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 ± 1%) and NCI-H2009 (3 ± 1%; P < 0.001; Fig. 2). KLF6 also showed strong suppression for colony formation in both the NCI-H1299 (18 ± 7%) and NCI-H2009 (18 ± 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 G1-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 assay, 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).
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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 NSCLC.

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 [3H]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 G1-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 β1 (27). Because KLF6 has been shown to up-regulate transforming growth factor β1 and its type I and II receptors (28), the

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, ±SD.

Fig. 5. Western blot analysis of caspase-3, poly(ADP-ribose) polymerase (PARP), and β-actin. Each lane was loaded with 60 µg 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 β1. Other putative transcriptional targets of KLF6 may include the genes encoding a placental glycoprotein (29), collagen α1 (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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