

## Original Article

# Methylation of kruppel-like factor 2 (KLF2) associates with its expression and non-small cell lung cancer progression

Wenbin Jiang<sup>1</sup>, Xueqing Xu<sup>2</sup>, Shaoli Deng<sup>1</sup>, Jie Luo<sup>1</sup>, Huan Xu<sup>1</sup>, Chao Wang<sup>1</sup>, Tingting Sun<sup>1</sup>, Guoqin Lei<sup>1</sup>, Fengling Zhang<sup>1</sup>, Cheng Yang<sup>1</sup>, Lin Zhou<sup>1</sup>, Feng Wang<sup>1</sup>, Ming Chen<sup>1</sup>

<sup>1</sup>Department of Clinical Laboratory, Daping Hospital, The Third Military Medical University, Chongqing 400042, China; <sup>2</sup>Molecular Biology Center, The State Key Laboratory of Trauma, Burn, and Combined Injury, Daping Hospital, The Third Military Medical University, Chongqing 400042, China

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**Abstract:** Kruppel-like factor 2 (KLF2) is a putative tumor suppressor gene. This study investigated its role and epigenetic mechanisms in human non-small cell lung cancer (NSCLC) in ex vivo and in vitro. A total of 47 paired NSCLC and normal tissues and six cell lines were analyzed using qRT-PCR for KLF2 expression. KLF2 methylation was assessed using the methylation specific PCR (MSP) or bisulfite sequencing PCR (BSP). Functional KLF2 region 4 (+567 to +906) was confirmed using the dual-luciferase reporter assay, while CCK-8 cell viability and flow cytometric assays were used to assess changes in cell viability, cell cycle distribution, and apoptosis after knockdown or re-expression of KLF2. Western blot was performed to analyze the effects of KLF2 shRNA on knockdown of KLF2 expression and p15 and p21 expression in cells. We found that KLF2 expression was significantly reduced in NSCLC cells and tissues via KLF2 methylation. Reduction of KLF2 expression was associated with KLF2 region 4 hypermethylation in 27 of 47 (57.45%) NSCLC tissues. Furthermore, methylation at KLF2 region 4 was significantly associated with lymph node metastasis and advanced TNM stage. Re-expression of KLF2 suppressed NSCLC cell viability, arrested cells at G0/G1 cell cycle by induction of p15 and p21 expression, and promoted apoptosis, whereas knockdown of KLF2 expression had the opposite effects on cells. Taken together, KLF2 possesses tumor suppressor functions in NSCLC and detection of KLF2 methylation should be further evaluated as a tumor or prognostic biomarker for NSCLC.

**Keywords:** Non-small cell lung cancer, KLF2, gene methylation, clinicopathological data, biomarker

## Introduction

Lung cancer is still one of the most commonly diagnosed and lethal malignancies in the world and histologically, lung cancer can be divided into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) [1-5]. NSCLC contributed to approximately 80% of all lung cancer cases [1]. Although lung cancer incidence shows reduction in the United States of America [2], incidence and mortality of lung cancer was significantly increased in China, which could be due to country-wide industrialization, environmental pollution, tobacco smoking, population aging and other unknown factors [1]. Thus, lung cancer in China has become a serious threat to human health and indeed, lung cancer is the most common cancer with

an incidence rate of 733.3 per 100000 and the leading cause of cancer death at 610.2 per 100000 in 2015 according to statistic data from Chinese Bureau of Disease Control [1]. To date, although tumor-targeted therapy significantly advanced NSCLC treatment and prolonged the life of lung cancer patients [3-5], most of advanced lung cancer still cannot be cured after NSCLC diagnosis. Therefore, search for novel biomarkers for lung cancer and better understanding of NSCLC tumorigenesis could be significant in improved prevention, early diagnosis, treatment options, and clinical prognosis of lung cancer.

Kruppel-like factor 2 (KLF2), also known as lung Krüppel-like factor (LKLF), a member of the KLF family, can modulate expression of many down-

stream genes by binding to the GC enriched regions of gene promoters as a zinc-finger transcription factor [6]. The KLF family has at least 17 mammalian family members [7], most of which act as potential tumor suppressor genes and involved in induction of cell apoptosis and inhibition of cell proliferation, migration, and angiogenesis [6, 7]. KLF2 was reported to be highly expressed in embryo and adult normal lung tissues and be essential for embryonic development and lung later development [8]. However, KLF2 expression was frequently low in various cancer tissues and thus, it could act as a potential tumor suppressor gene to induce cell quiescence [8] and DNA damage-associated apoptosis [9], but to inhibit cell growth [10] and endothelial growth factor-mediated angiogenesis [11].

Gene silence through DNA methylation is an epigenetic regulation mode in cells and tissues. The methyl group is enzymatically introduced to the 5' carbon of cytosine in cytosine-guanine (CpG) dinucleotide, which is often enriched to be an island [12, 13]. As a result, DNA methylation will change the chromatin structure, DNA stability, and the interaction with its transcription factor in order to silence gene expression [13]. Thus, change in DNA methylation status will epigenetically regulate gene expression, which is therefore used to monitor gene regulation and serve as a biomarker for early cancer detection, prognosis, and treatment responses [14, 15]. Thus, in this study, we first analyzed KLF2 expression in NSCLC tissue samples and cells and then detected KLF2 methylation status to explore the mechanism on KLF2 down-regulation in NSCLC. We also associated KLF2 methylation with NSCLC progression and investigated KLF2 function in regulation of NSCLC cell viability, apoptosis, and gene expression. We expect to provide useful information regarding KLF2 as biomarker for prediction of NSCLC development and progression and modulation of KLF2 expression as a novel strategy for future control of NSCLC clinically.

## Materials and methods

### *Cell lines and culture*

Five human non-small cell cancer cell lines, A549, HCC827, SK-MES-1, NCI-H1299 and NCI-H1975, and human embryonic kidney HEK293T cell line were obtained from the Cell

Bank, Chinese Academy of Sciences (Shanghai, China), while a normal bronchial epithelial cell line BEAS-2B was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). HCC827, NCI-H1299, NCI-H1975, and HEK 293T cells were maintained in RPMI-1640 (Gibco, Gaithersburg, MD, USA), while SK-MES-1 was maintained in Dulbecco's modified Eagle's medium (DMEM) from Gibco and A549 was maintained in Ham's F12K (Gibco), all of which were also supplemented with 10% fetal bovine serum (FBS; Gibco) and penicillin/streptomycin (100 units/ml) and cultured in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> at 37°C. Moreover, BEAS-2B was maintained in serum-free bronchial epithelial cell growth medium (BEGM; Lonza, Basel, Switzerland) supplemented with additives provided by the company in the BEGM kit and cultured in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> at 37°C.

### *Tissue specimens*

A total of forty-seven pairs of primary NSCLC and matched adjacent non-tumor lung tissues were collected from Daping Hospital in 2016. These patients included thirty male and seventeen female with age ranged from 43 to 75 years (median age of 60 years). These patients were diagnosed histologically and the TNM staging system was used to classify and stage their diseases according to the 7th edition of the AJCC Cancer Staging Manual [16], resulting in 20 patients with stage I, 14 with stage II, 12 with stage III, and one with stage IV. The Institutional Review Board of Daping Hospital approved this study and written informed consent was obtained from all participants.

### *5-aza-2'-deoxycytidine treatment*

HCC827 and A549 cell lines at a density of  $5 \times 10^5$  were seeded into 25 cm<sup>2</sup> flasks overnight and then treated with or without 100 μM 5-aza-2'-deoxycytidine (5-Aza-Dc, Sigma, St Louis, MO, USA) dissolved in 50% acetic acid for 3 days. The cell growth medium was changed daily and 5-Aza-Dc concentration was also maintained.

### *RNA isolation and qRT-PCR*

Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA) from cell lines and tissue samples according to the manufacturer's

**Table 1.** Primers for BSP

KLF2 regions	Name of Primer	Primer Sequences
Region 1 (-411 to -117)	KLF2I1P1	5'-TTTTTGAGAGTTTTTGAGAGG-3' 5'-AACACAACCTAAATTTAACC-3'
Region 2 (-114 to +111)	KLF2I1P2	5'-TATATTGTGGTTAAATTTAGGT-3' 5'-CAACAAAATAAATCACTCAAC-3'
Region 3 (+447 to +728)	KLF2I2P1	5'-TGTTTATGGGGTTGGATGG-3' 5'-CACGAATCAACCCGAAAAAC-3'
Region 4 (+567 to +906)	KLF2I2P2	5'-GTGGTTTGGTGTGTGAGTTGTT-3' 5'-AAACCAAAACCACCGAAAAA-3'
Region 5 (+1919 to +2287)	KLF2I3	5'-TAAGGTTTTGGTTAGGGATAGG-3' 5'-TAAATCCAATAAATACCAATCACA-3'

instructions and then reversely transcribed into cDNA in a 20 µl volume containing 1 µg RNA sample of each with the FastQuant RT Kit (Tiangen, Beijing, China). qPCR amplification was performed with PowerUp SYBR Green Master Mix (Invitrogen) following the manufacturer's protocol. To amplify KLF2 and β-actin mRNA, we designed and used specific primers (β-actin, 5'-CATGTACGTTGCTATCCAGGC-3' and 5'-CTCCTTAATGTACACGACGAT; KLF2, 5'-CGGC-AAGACCTACCAAGAGT-3' and 5'-CGCACAGATGGCACTGGAATG-3') and quantified using the 2<sup>-ΔΔCt</sup> method.

*Bisulfite modification, methylation specific PCR (MSP), and bisulfite sequencing PCR (BSP)*

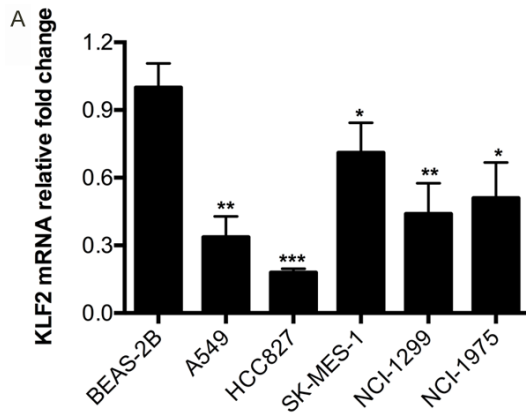
Genomic DNA was extracted from tissues and cell lines using the Genomic DNA Purification Kit (Promega, Madison, WI, USA). Genomic DNA samples of 500 ng each were then modified by sodium bisulfite treatment obtained from the EZ DNA Methylation-Gold kit (ZymoLaboratories, South San Francisco, CA, USA) according to the manufacturer's protocol. The modified DNA samples were then subjected to PCR amplification of specific KLF2 CpG island regions using ZymoTaq Premix kit with an ABI 2720 (Applied Biosystems, Foster city, CA, USA). The primers used to detect methylation regions of KLF2 CpG islands were designed by Methyl Primer software (Thermo-Fisher, Waltham, MA, USA) and their sequences are listed in **Table 1**. PCR conditions were as followings: 95°C for 10 min followed by 35 cycles of 95°C for 30 s, 62°C for 30 s and 72°C for 30 s, and final extension at 72°C for 7 min. In the end of PCR amplification, PCR products were subjected to gel purification and DNA sequenced with BGI Sequencing

(Guangzhou, Guangdong, China) with the corresponding reverse primer directly or cloning of PCR products into the pUCm-T vector (Sangon Biotech, Shanghai, China) according to the manufacturer's protocol. In particular, after PCR products were ligated into pUCm-T vector, transfected into *E. coli* and screened in the agar plates, plasmids were then isolated and purified using E.Z.N.A Plasmid Mini Kit (Omega, Norcross, GA, USA). Eight

randomly selected PCR fragments in each sample were sequenced by BGI Sequencing with the M13 reverse primer. For MSP, the primers were designed according to differentially methylated region proved by BSP (see **Table 1**). These MSP primers were M primer, 5'-GGT-TACGGTTGCGTTTTTC-3' and 5'-AAACGACGATAT-ATCGAACG-3'; U primer, 5'-GTGGTTATGGTTGTG-TTTTT-3' and 5'-CTAAACAACAATATATCAAACAA-CA-3'. PCR amplification conditions were 95°C for 10 min, 35 cycles of 95°C for 30 s, 60°C for M primer or 56°C for U primer for 30 s, and 72°C for 30 s, and 72°C for 7 min. PCR products were analyzed using 2% agarose gel electrophoresis.

*Plasmid construction and transfection*

The KS fragment was amplified from BEAS-2B cell line and then cloned into pGL3-promoter vector (Promega). The pGL3-promoter-mKS was obtained by digestion with M. SssI-methyltransferase (Thermo-Fisher) to methylate pGL3-promoter-KS enzymatically. KLF2 cDNA was cloned into the GV230 vector (Genechem, Shanghai, China) between KpnI and XhoI sites. KLF2 expression was knocked down using KLF2 shRNA that was cloned into GV248 (Genechem). The target sequences were shRNA#1, 5'-CCGCCATTCCAGTGCCAT-3'; shRNA#2, 5'-TTCGCATCTGAAGGCGCAT-3'; shRNA#3, 5'-CCTTTCGGTGGCCCTGGTT-3'; shRNA#4, 5'-GCACCGACGACGACCTCAA-3'; negative shRNA control, 5'-TTCTCCGAACGTGTCACGT-3'. After successfully construction and DAN sequencing confirmation, these plasmids were transfected into cells using Lipofectamine 3000 (Invitrogen).



**Figure 1.** Downregulation of KLF2 mRNA level in NSCLC cell lines. A. KLF2 expression was downregulated in five NSCLC cell lines (A549, HCC827, SK-MES-1, NCI-H1299, and NCI-H1975) compared to BEAS-2B. Results represent the mean fold changes after normalized to KLF2 expression in BEAS-2B from 3 independent experiments (\* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  vs. that of BEAS-2B cells).

#### Dual-luciferase reporter assay

A549, HCC827 and HEK293T cell lines were seeded into 96-well plates at a density of  $2 \times 10^4$ /well and cultured for 24 h. Subsequently, cells were co-transfected with 10 ng/well pRL-TK and 100 ng/well of pGL3-promoter, pGL3-promoter-KS or pGL3-promoter-mKS. For 48 h and then subjected to protein extraction and the luciferase reporter assay using Dual-Glo Luciferase Assay System (Promega) according to the kit instructions. The data were expressed as mean  $\pm$  SD and the experiments were in triplicate and repeated at least once.

#### Cell viability assay

Cells were seeded into 96-well plates at a density of  $3 \times 10^3$ /well and cultured overnight and then transfected with different plasmids (see results section for detail). After that, cell viability was measured daily for 4 days using 10  $\mu$ l/well Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Japan) according to the manufacturer's instructions. The absorbance rate was then measured at 450 nm using Multiskan MK-3 (Thermo-Fisher). The experiments were in triplicate and repeated at least three times.

#### Flow cytometry assay

For cell cycle distribution analysis, the cells were seeded and cultured overnight and then starved for 12 h to synchronize cell cycle in G0/G1 phase. Cells were then transfected with

plasmids using Lipofectamine 3000 (Invitrogen) and harvested after 48 h by trypsinization. After fixed in 70% ethanol for 12 h, cells were stained with propidium iodide (PI, Keygen Biotech, Jiangsu, China). For cell apoptosis analysis, transfected cells were cultured for 72 h and harvested by trypsinization without EDTA and stained with PI and Annexin V-FITC from the Annexin V-FITC Apoptosis Detection Kit (Keygen Biotech). After that, cells were analyzed using Navios Flow Cytometry (Beckman Coulter, Indianapolis, IN, USA). The data were expressed as mean  $\pm$  SD and the experiments were in triplicate and repeated at least once.

#### Protein extraction and western blot

Cells were lysed in the RIPA Buffer (Beyotime) containing a protease inhibitor cocktail (Sigma). After quantitation, these protein samples were resolved in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred onto 0.22  $\mu$ m polyvinylidene fluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA). These PVDF membranes were then blocked in 5% dry skim milk solution in phosphate buffered saline (PBS) for 2 h at room temperature and incubated with specific primary antibodies with gentle agitation at 4°C overnight. After incubating with secondary antibodies for 1 h at room temperature, immunoreactive protein bands were visualized by using the enhanced chemiluminescence (ECL) solution (Thermo-Fisher). The anti- $\beta$ -actin and anti-p21 antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA), while the anti-KLF2 and anti-p15 antibodies were from Abcam (Cambridge, MA, USA).

#### Statistical analysis

GraphPad Prism 6 software was used to analyze data statistically and data were summarized as the mean  $\pm$  standard deviation (SD) of three independent experiments. The  $\chi^2$  and Fisher's exact tests were performed for the counting data comparisons, whereas Student's *t* test and one-way ANOVA were used for measurement data.  $P < 0.05$  was considered as statistically significant.

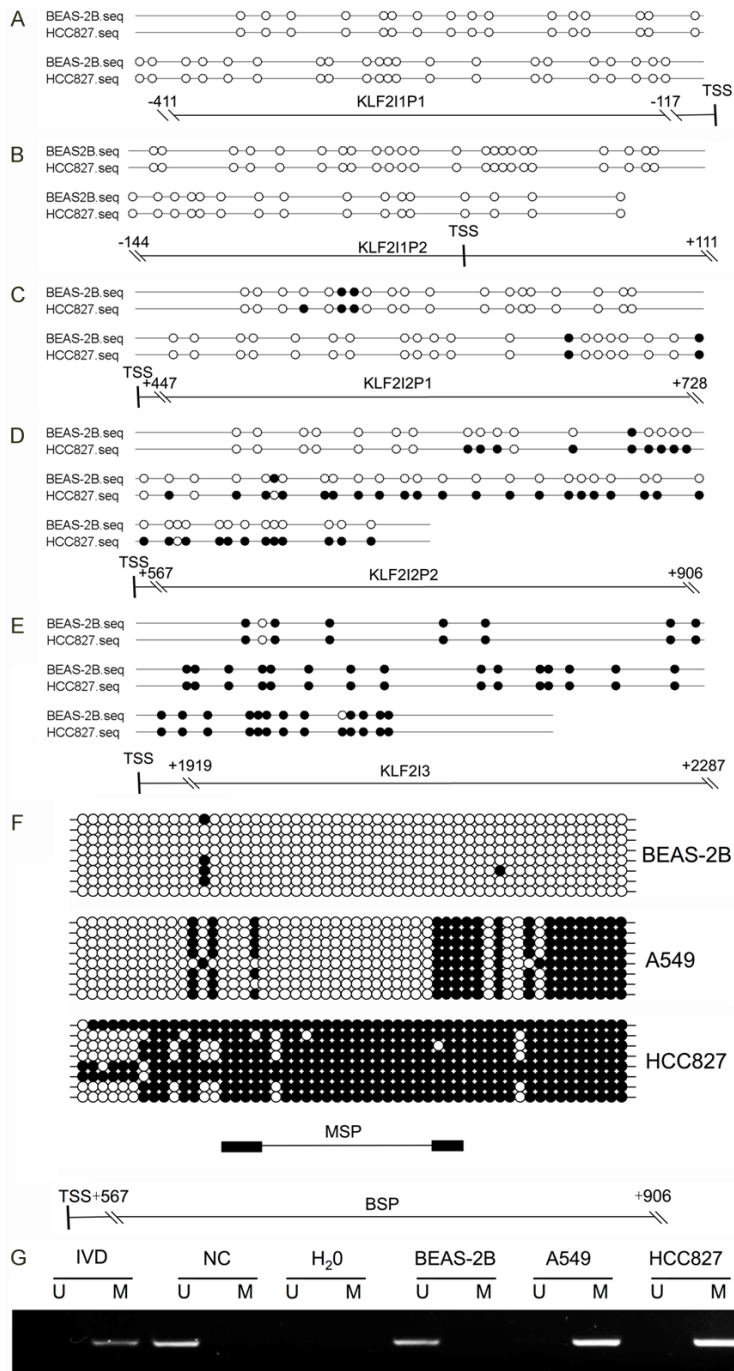
## Results

### Downregulation of KLF2 expression in NSCLC cells and tissues

We performed qRT-PCR to detect KLF2 expression in both NSCLC cells and tissues and found



## KLF2 hypermethylation promotes NSCLC progression



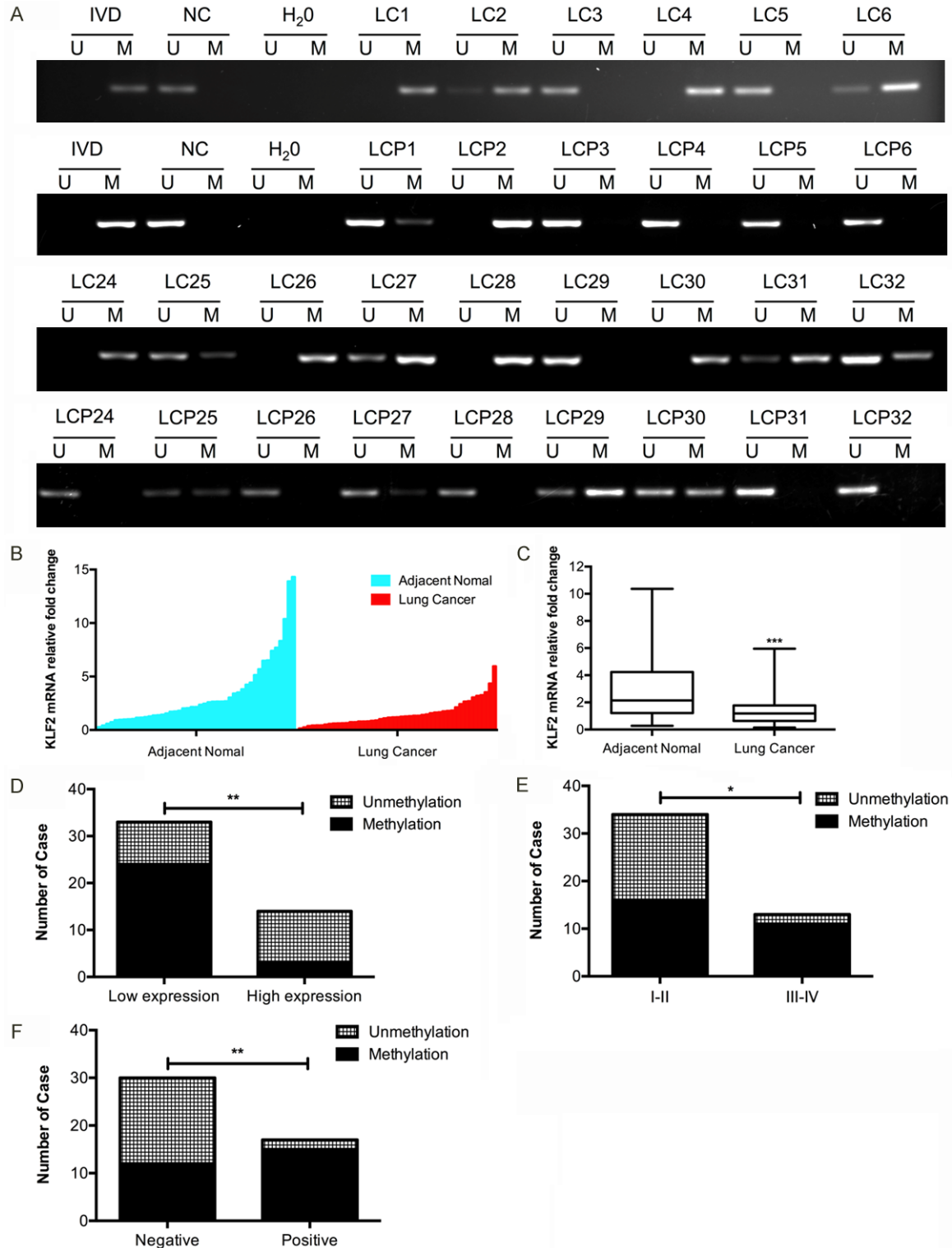
**Figure 2.** Comparison of KLF2 methylation in five regions between BEAS-2B and HCC827 cells detected by using direct bisulfite PCR sequencing. (A-E) Data on the direct bisulfite PCR sequencing of region 1 (A), region 2 (B), region 3 (C), region 4 (D) and Region 5 (E) was analyzed in BEAS-2B and A549 cells. Differential methylation sites were observed in region 4. The solid circles represent methylated CpG site, while hollow circles represent unmethylated CpG site. TSS represents the transcription start site of KLF2. (F) Monoclonal bisulfite PCR sequencing of KLF2 region 4 in BEAS-2B, A549 and HCC827 cell lines (A total of eight clones for each cell line). Double-headed rectangle line indicates MSP PCR product spanned 132 bp in KLF2 and rectangles represent the location and size of MSP primers. (G) MSP data on KLF2 in BEAS-2B and NSCLC cell lines. IVD and NC represent in vitro methylated DNA and negative control, respectively, while U and M are for unmethylated and methylated alleles, respectively.

KLF2 expression was low in all five NSCLC cell lines compared with that in human normal bronchial epithelial cell line BEAS-2B. Specifically, KLF2 expression was higher in SK-MES-1 than in other four NSCLC cell lines and was lowest in HCC827 and A549 cell lines (**Figure 1A**). Moreover, we also found that KLF2 expression was significantly lower in 47 pairs of NSCLC tissues than in adjacent normal tissues (**Figure 3B and 3C**).

### *KLF2 methylation in NSCLC cells and tissues and its association with clinicopathological data from patients*

We then investigated the potential cause of KLF2 reduction in NSCLC cells and tissue samples using 5 pairs of bisulfite sequencing PCR primers (KLF2I1P1, KLF2I2P2, KLF2I2P1, KLF2I2P2, and KLF2I3) to assess the methylation status of KLF2 CpG islands. We found that KLF2 region (-411 to +111) amplified by KLF2I1P1 (region 1) and KLF2I1P2 (region 2) was unmethylated in both HCC827 and BEAS-2B cell lines (**Figure 2A and 2B**), while there were few differential methylation sites in region 3 (+447 to +728) amplified by KLF2I2P1 in these two cell lines (**Figure 2C**). Nevertheless, the region 4 (+567 to +906) amplified by KLF2I2P2 was hypermethylation in HCC827 cells but hypomethylation in BEAS-2B cells (**Figure 2D**). The region 5 (+1919 to +2287) amplified by KLF2I3 was methylated in both HCC827 and BEAS-2B cell lines (**Figure 2E**). To further approve the differential methylation status in region 4 (+567 to +906) between normal and cancer cell lines, we cloned KLF2I2P2 fragment into pUCm-T vector

# KLF2 hypermethylation promotes NSCLC progression



**Figure 3.** Comparison of KLF2 methylation with KLF2 mRNA levels in NSCLC tissues. (A) MSP results of KLF2 methylation show that KLF2 was methylated in NSCLC tissues but unmethylated in adjacent normal tissues. LC and LP denote lung cancer and adjacent normal lung tissues, respectively. (B, C) Relative expression of KLF2 mRNA level were analyzed in 47 pairs of NSCLC and adjacent normal tissues, shown by individual levels (B) and mean  $\pm$  SD (C, D) The bar graph shows that KLF2 methylation associated with mRNA low expression in NSCLC tissues. (E) KLF2 methylation data associated with NSCLC stages. (F) KLF2 methylation data associated with NSCLC lymph node metastasis. (\*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$  represent significant difference).

**Table 2.** Association of KLF2 methylation with clinicopathological data from 47 NSCLC patients

Variables	Number of Cases	KLF2 Methylation Status		P value
		Methylated, n=27 (57.45%)	Unmethylated, n=20 (42.55%)	
Age (years)				
<60	22	15	7	0.23
≥60	25	12	13	
Gender				
Male	30	18	12	0.76
Female	17	9	8	
Tumor size (cm)				
<5	29	15	14	0.37
≥5	18	12	6	
Tumor stage				
I/II	34	16	18	0.024
III/IV	13	11	2	
Lymph node metastasis				
No	30	12	18	0.0018
Yes	17	15	2	

for DNA sequencing and found that KLF2 methylation sites in A549 and HCC827 cells were more than in BEAS-2B cells (**Figure 2F**), which further confirmed by using MSP assay (**Figure 2G**). These findings suggest that KLF2 expression may be silenced via hypermethylation of region 4 (+567 to +906) in NSCLC cell lines.

Furthermore, we also detected KLF2 methylation status in 47 pairs of cancer and adjacent normal tissues using MSP assay (**Figure 3A**) and found that KLF2 was methylated in 27 of 47 (57.45%) cancer tissues (**Table 2**). We associated KLF2 methylation status with KLF2 expression and found that 24 of 31 (77.42%) KLF2 low expression group were methylated (**Figure 3D**), which indicated that KLF2 methylation was significantly associated with KLF2 low expression ( $P<0.01$ ). We then associated KLF2 methylation status with clinical data from NSCLC patients and found that KLF2 methylation was significantly associated with the TNM stage ( $P<0.05$ , **Figure 3E**) and lymph node metastasis ( $P<0.01$ , **Figure 3F**), although there was no association of KLF2 methylation with age, gender, and tumor size (**Table 2**). These findings suggest that KLF2 expression was silenced by KLF2 methylation in NSCLC and that KLF2 methylation associated with NSCLC development. Methylation of KLF2 region 4 (+567 to +906) could be further evaluated as

an early detection or prognostic marker for NSCLC patients.

#### *Silence of KLF2 expression via hypermethylation of KLF2 region 4 (+567 to +906)*

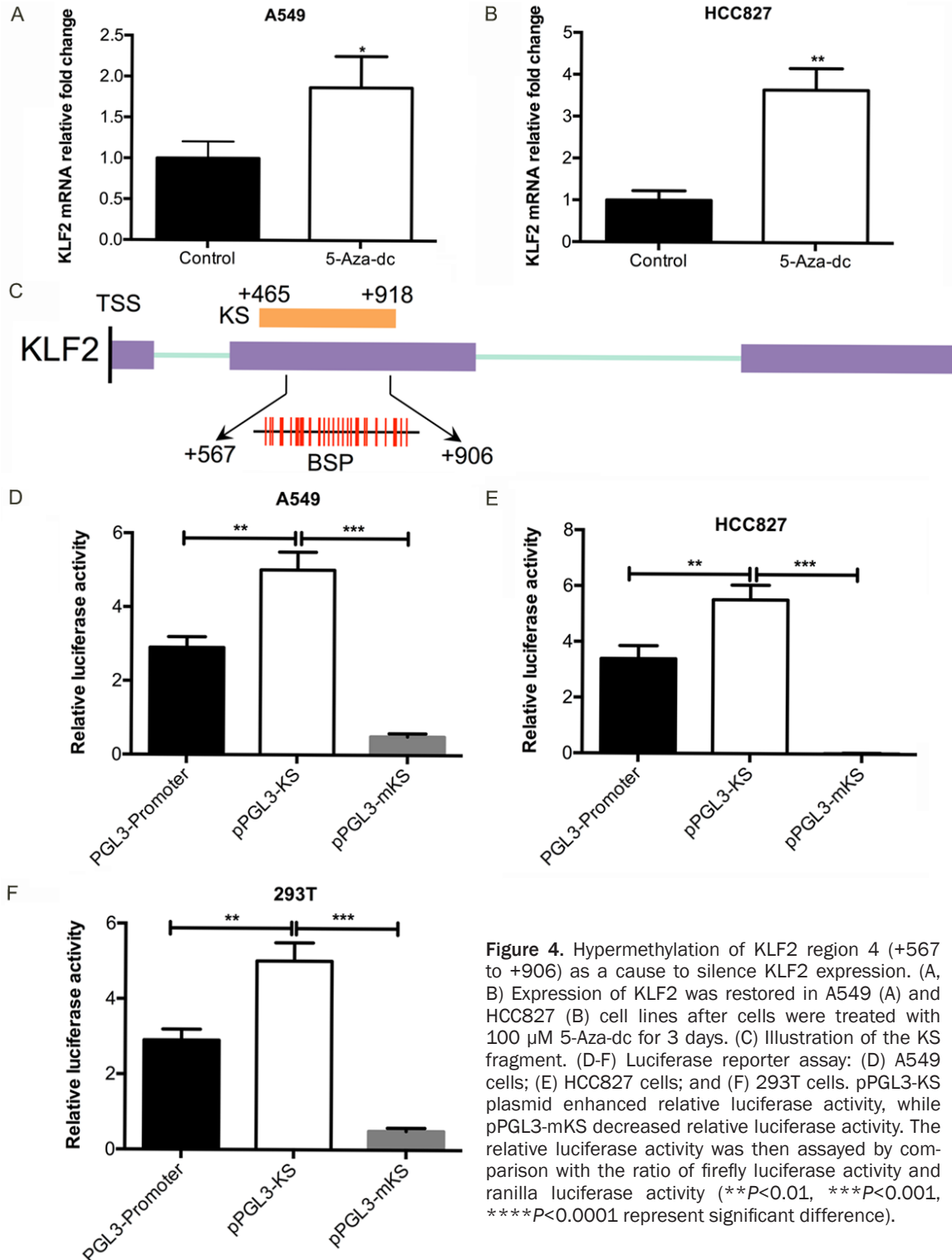
To further assess the cause of low KLF2 expression, we treated A549 and HCC827 cell lines with 5-aza-2'-deoxycytidine (5-Aza-dc) and found that significant re-expression of KLF2 was observed both in A549 and HCC827 cell lines (**Figure 4A** and **4B**). To further investigate whether hypermethylation of KLF2 region 4 (+567 to +906) could be responsible for lost KLF2 expression, we cloned KS fragment containing this region into the pGL3-promoter vector (**Figure 4C**) and obtained the pGL3-

promoter-mKS using M.SssI methyltransferase treatment. We then analyzed function of KS and methylated KS (mKS) fragments using the dual-luciferase reporter assay of A549, HCC827 and 293T cell lines. Our data showed that the relative luciferase activity was significantly enhanced by the KS fragment but inhibited by the mKS fragment in all three cell lines (**Figure 4D-F**). These data indicate that the KS fragment may serve as a regulation element of KLF2 and low KLF2 expression was due to methylation of the KS fragment.

#### *Manipulation of KLF2 expression for association with changed NSCLC cell viability, cell cycle distribution, and apoptosis*

KLF2 was re-expressed in A549 and HCC827 cell lines after KLF2 cDNA transfection (**Figure 5A** and **5B**). Since KLF2 was relative highly expressed in SK-MES-1 than in other four NSCLC cell lines, we transferred four different shRNA constructs into SK-MES-1 cells to knockdown KLF2 expression (**Figure 5C**) and found that KLF2 shRNA#3 and shRNA#4 had better effects on KLF2 knockdown and thus, used in our following experiments. We therefore performed the CCK-8 cell viability assay to explore the effect of KLF2 knockdown or re-expression on regulation of A549 and HCC827 cell viability. We found that the optical density (OD) value

## KLF2 hypermethylation promotes NSCLC progression



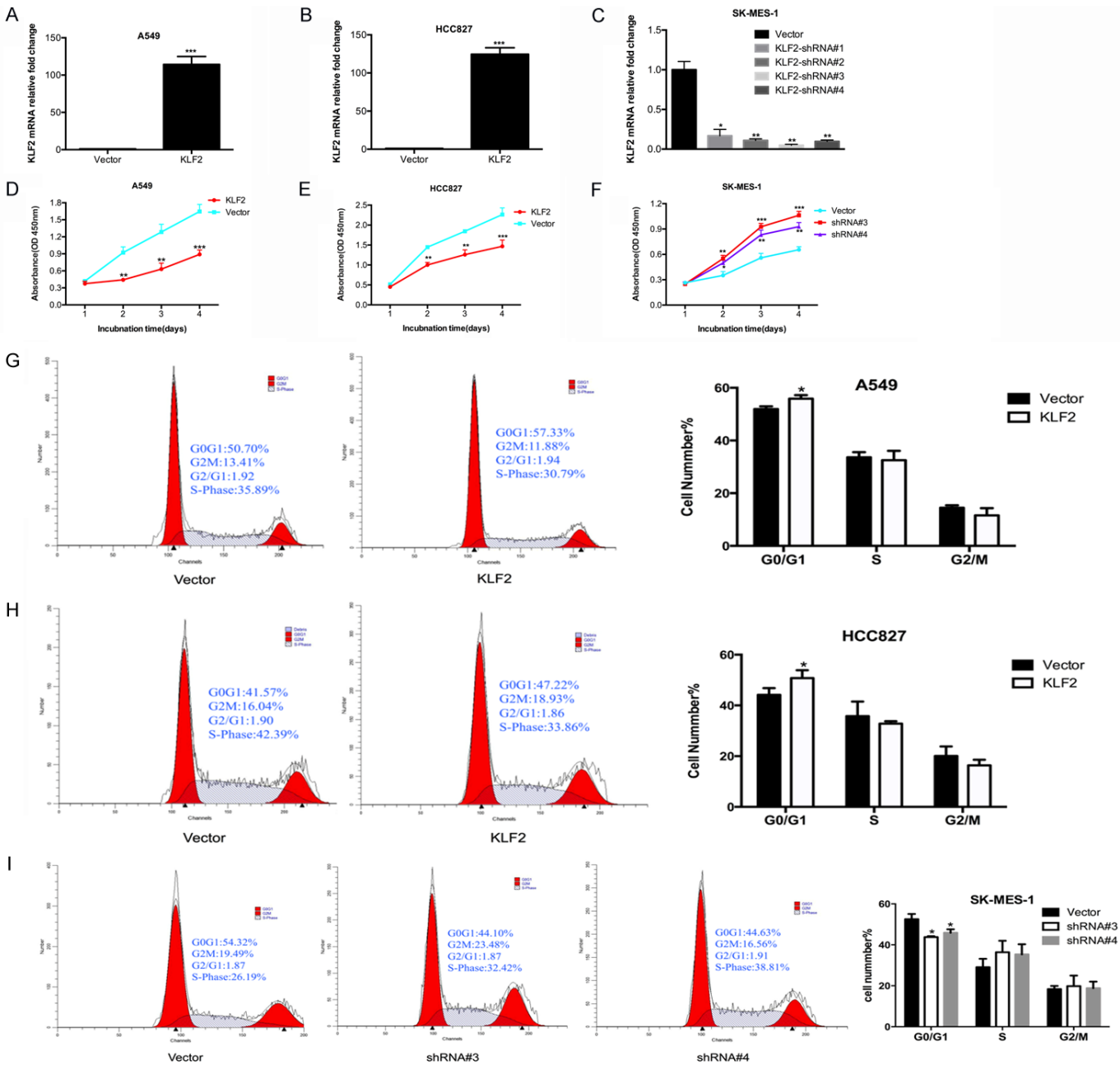
**Figure 4.** Hypermethylation of KLF2 region 4 (+567 to +906) as a cause to silence KLF2 expression. (A, B) Expression of KLF2 was restored in A549 (A) and HCC827 (B) cell lines after cells were treated with 100  $\mu$ M 5-Aza-dc for 3 days. (C) Illustration of the KS fragment. (D-F) Luciferase reporter assay: (D) A549 cells; (E) HCC827 cells; and (F) 293T cells. pPGL3-KS plasmid enhanced relative luciferase activity, while pPGL3-mKS decreased relative luciferase activity. The relative luciferase activity was then assayed by comparison with the ratio of firefly luciferase activity and renilla luciferase activity (\*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$  represent significant difference).

was decreased after KLF2 re-expression (Figure 5D and 5E), whereas tumor cell proliferation was significantly enhanced after knock-down of KLF2 expression in SK-MES-1 cells (Figure 5F). Flow cytometry was used to ana-

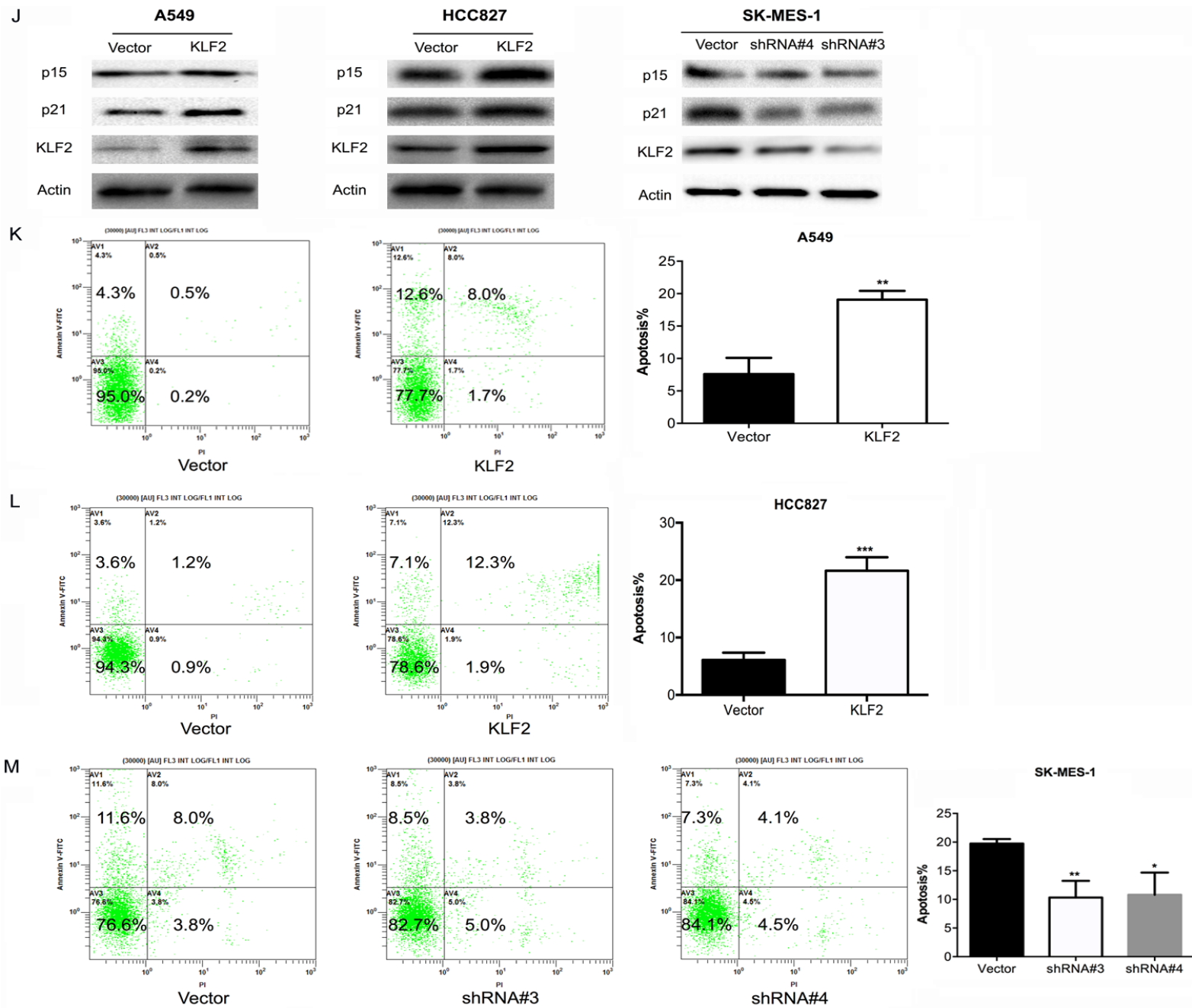
lyze the effects of KLF2 on cell cycle and the data showed that cells number in the G0/G1 phase was significantly increased after KLF2 re-expression in both A549 and HCC827 cell lines ( $P < 0.05$ , Figure 5G and 5H). However,



KLF2 hypermethylation promotes NSCLC progression



KLF2 hypermethylation promotes NSCLC progression



**Figure 5.** Effect of KLF2 re-expression and knockdown on regulation of NSCLC cell viability, cell cycle distribution and apoptosis. (A, B) Level of KLF2 mRNA was increased after KLF2 cDNA transfection in A549 (A) and HCC827 (B) cells. (C) The level of KLF2 mRNA was decreased in SK-MES-1 cells after transfected with four different constructs of KLF2 shRNA (shRNA#1, shRNA#2, shRNA#3, and shRNA#4). (D-F) CCK-8 assay of cell viability in A549 (D), HCC827 (E), and SK-MES-1 (F) cells. Re-expression of KLF2 inhibited tumor cell viability, whereas knockdown of KLF2 expression promoted tumor cell viability. (G-I) Flow cytometric analysis of cell cycle distribution in A549 (G), HCC827 (H), and SK-MES-1 (I) cells. Re-expression of KLF2 arrested cells at the G0/G1 cell cycle, whereas knockdown of KLF2 expression inhibited tumor cell apoptosis. (J) Western blot assay shows that KLF2 up-regulated p15 and p21 expression. (K-M) Flow cytometric analysis of apoptosis in A549 (K), HCC827 (L), and SK-MES-1 (M) cells shows that KLF2 promoted tumor cell apoptosis. The bar graphs were summarized data on mean  $\pm$  SD of three independent experiments (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs. negative control vectors).

SK-MES-1 cells transfected with KLF2 shRNA#3 or shRNA#4 had a significant decrease in the G0/G1 phase but the cell cycle progressed into the S and G2/M phase (**Figure 5I**). To further explore the underlying mechanisms of KLF2 re-expression involved in cell cycle arrest at the G0/G1 phase we performed western bolt analysis of p15 and p21 levels in cells. We found that levels of p15 and p21 proteins were increased up on KLF2 re-expression in both A549 and HCC827 cell lines but decreased while knockdown of KLF2 expression in SK-MES-1 cells (**Figure 5J**).

The percentage of apoptotic cells was  $7.6 \pm 1.44\%$  vs.  $19.07 \pm 0.79\%$  in A549 cells (**Figure 5K**) and  $6.1 \pm 0.72\%$  vs.  $21.63 \pm 1.36\%$  in HCC827 cells (**Figure 5L**) before and after KLF2 re-expression. Apoptosis cells were significantly decreased in cells transfected with shRNA#3 or shRNA#4 compared with negative control vector (**Figure 5M**). These results indicate that KLF2 suppressed NSCLC cell viability by arresting cells at the G0/G1 cell cycle and promoted tumor cells to undergo apoptosis.

## Discussion

In this study, we first assessed KLF2 mRNA level and methylation in NSCLC tissues and cell lines. We then associated KLF2 methylation with clinicopathological data from NSCLC patients. After that, we focused on the region of KLF2 methylation as a cause of KLF2 silence in NSCLC cells and tissue samples and then manipulated KLF2 expression to determine the role of KLF2 in NSCLC cells in vitro. We found that KLF2 level was significantly decreased in NSCLC cells and tissue samples, while KLF2 was frequently methylated in NSCLC cells and tissue samples. Moreover, reduced KLF2 expression was associated with KLF2 region 4 hypermethylation in majority of NSCLC tissues,

while KLF2 methylation at the region 4 was associated with NSCLC lymph node metastasis and advanced TNM stage. In addition, re-expression of KLF2 in NSCLC cell lines significantly suppressed tumor cell viability, arrested tumor cells at G0/G1 cell cycle via p15 and p21 expression, and promoted tumor cells to apoptosis. In contrast, knockdown of KLF2 expression had the opposite effects on SK-MES-1 cell line. Our current data indicate that KLF2 protein could function as a tumor suppressor in NSCLC and that detection of KLF2 methylation should be further evaluated as a tumor or prognostic biomarker for NSCLC.

As we know, KLF2 plays a key role in suppression of cancer development and progression because KLF2 expression was reduced or lost in a number of different human cancers, such as gastric [17], hepatocellular [18], pancreatic glands [19], mammary [20] and lung cancer [21]. Our current data further supported and confirmed the notion showing that KLF2 expression was significantly reduced in NSCLC cell lines and tissues. We then explored the underlying mechanism responsible for KLF2 down-regulation in NSCLC by detected methylation status of KLF2 in different NSCLC and BEAS-2B cells as well as NSCLC tissues. Intriguingly, the region 4 (+567 to +906) of KLF2 gene was hypermethylated in HCC827 cells but hypomethylated in BEAS-2B cells. Indeed, half of the CpG islands in human genome are localized outside gene promoter regions [22] and the whole genome bisulfite PCR sequencing recently revealed that methylation of tumor suppressor genes in cancer could occur outside the gene promoter regions [23]. Thus, our early speculation of hypermethylation of tumor suppressor gene promoter as a common cause of cancer development [24] needs to be updated since methylation of the CpG islands in intergenic or intragenic regions also significantly

contributed to cancer development as well [25, 26]. Our current study surely supported such a notion by showing that methylation of KLF2 region 4 (KLF2 internal region) was associated with reduction of KLF2 expression. Treatment with 5-aza-2'-deoxycytidine (5-Aza-dc), a DNA methyltransferase inhibitor and commonly used to demethylate DNA [27], could restore KLF2 expression in NSCLC cells, further supporting DNA methylation as an epigenetic mechanism to inhibit gene expression. In addition, we further analyzed methylation of KLF2 region 4 (+567 to +906) using the KS fragment (+465 to +918) by cloned it into pGL3-promoter vector. Our data showed that the KS fragment was able to promote luciferase activity, whereas the methylated KS fragment inhibited luciferase activity. Our current data demonstrated that KLF2 region 4 (+567 to +906) acted as an enhancer to positively regulate KLF2 expression, whereas hypermethylation of this region could associate with NSCLC development.

Furthermore, our current data showed that hypermethylation of KLF2 region 4 associated with KLF2 reduction. Bae et al. recently reported that the CpG islands in gene enhancers relative to the promoters had more dynamic changes of DNA methylation in cancer [28] and suggested that methylation of such enhancers could be more suitable as a biomarker than gene promoters do. Indeed, we associated methylation of KLF2 enhancer with clinicopathological data from NSCLC patients and found that methylation of KLF2 enhancer did associate with NSCLC lymph node metastasis and advanced tumor stages; however, further study using a larger sample size could verify our current data before detection of KLF2 enhancer methylation as a potential biomarker for early detection or prognosis of NSCLC.

Overall, KLF2 was reported to be a putative tumor suppressor in several types of human cancer [19-21, 29, 30]. To further confirm it in NSCLC, we re-expressed and knocked down KLF2 in NSCLC cell lines and found that KLF2 re-expression of KLF2 after transfection of KLF2 cDNA into NSCLC cells significantly inhibited tumor cell viability, arrested cells at the G0/G1 cell cycle, promoted tumor cell apoptosis, whereas knockdown of KLF2 expression promoted tumor cell viability, but inhibited tumor cell apoptosis. We also found that KLF2 re-expression induced G0/G1 phase arrest by

up-regulation of p15 and p21 expression. However, much more is needed to do before conclusion of KLF2 as a tumor suppressor gene in NSCLC because our current study is just proof-of-principle and there are numbers of limitations (e.g., a small sample size and association data). In conclusion, KLF2 expression was reduced in NSCLC cell lines and tissues and KLF2 methylation was responsible for these reductions. KLF2 methylation was associated with NSCLC lymph node metastasis and advanced tumor stages. Re-expression of KLF2 associated with reduced malignant tumor behaviors, whereas knockdown of KLF2 expression promoted malignant tumor behaviors.

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## Disclosure of conflict of interest

None.

**Address correspondence to:** Drs. Ming Chen and Feng Wang, Department of Clinical Laboratory, Daping Hospital, The Third Military Medical University, Chongqing 400042, China. Tel: +86-23-68757601; Fax: +86-23-68757601; E-mail: chming1971@126.com (MC); Tel: +86-23-68757551; E-mail: wangfnew@gmail.com (FW)

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