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ANKRD18A as a novel epigenetic regulation gene in lung cancer

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ABSTRACT

Lung cancer is one of the most common causes of cancer-related mortality worldwide. Effective early diagnosis and targeted therapies for lung cancer to reduce incidence and mortality would benefit from a better understanding of the key molecular changes that occur from normal to malignant tumor cells during lung cancer initiation and development, but these are largely unknown. Previous studies have shown that DNA methylation, an important mechanism for the regulation of gene expression, plays a key role in lung carcinogenesis. In this study, we screened a novel methylation gene, *ANKRD18A*, encoding ankyrin repeat domain 18A, to determine whether it is regulated by DNA methylation in lung cancer. Methylation-specific PCR and bisulfite sequencing PCR were used to analyze gene methylation status, and real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) examined mRNA levels. Promoter hypermethylation of *ANKRD18A* was detected in 68.4% (26/38) of lung cancer tissues but not (0/20) in normal lung tissues ($P < 0.01$), whereas *ANKRD18A* mRNA expression was significantly decreased in lung cancer tissues compared with adjacent normal tissues. In addition, we found that *ANKRD18A* expression was significantly decreased in 9 of 10 lung cancer cell lines. This was associated with hypermethylation of the *ANKRD18A* promoter region. Moreover, weak expression of *ANKRD18A* in methylated lung cancer cell lines increased markedly after treatment with the DNA methylation inhibitor 5-aza-2′-deoxycytidine. These results suggest that *ANKRD18A* hypermethylation and consequent mRNA alterations might be a vital molecular mechanism in lung cancer.

1. Introduction

The incidence and mortality of lung cancer have shown a rising trend and appear to have stabilized the leading cause of cancer-related mortality among men in China, Western Europe and the United States [1]. Although lung cancer can be treated by surgery, radiation, drugs and other means, the 5-year survival rate remains under 15%, mainly because of the lack of effective early diagnosis. Thus, an in-depth study on the molecular mechanisms of lung cancer may help to find novel and effective molecular biomarkers for early screening of high-risk groups to reduce lung cancer incidence and mortality. However, the molecular events responsible for its initiation and development are still largely unknown.

Emerging evidence indicates that epigenetic modification, especially DNA methylation, plays an important role in the development of lung carcinogenesis [2,3]. DNA methylation is considered to be the third mechanism of tumor suppressor gene inactivation and tumorigenesis following the loss of heterozygosity and mutations [4]. In addition to the methylation analysis of known tumor-associated genes, a growing body of studies has applied high-throughput screening technology to identify novel genes regulated by DNA methylation in many types of cancer [5–7]. Moreover, the functions of these genes are closely related to tumor cell growth, apoptosis and migration [5–7]. The identification of such genes may further our understanding of molecular mechanisms, and aid in the development of alternative approaches for diagnostic and therapeutic evaluation.

Through methylation-sensitive arbitrarily primed PCR, we have identified a novel preferentially methylated gene, ankyrin repeat domain 18A (*ANKRD18A*), in human lung cancer. *ANKRD18A* is located on chromosome 9p13.1, contains 16 exons [8,9], and its

Abbreviations: 5-aza-dC, 5-aza-2′-deoxycytidine; *ANKRD18A*, ankyrin repeat domain 18A; BSP, bisulfite sequencing polymerase chain reaction; MSP, methylation-specific polymerase chain reaction; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction.

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encoded protein sequence has a typical encoding domain with an ankyrin repeat. These repeat sequences usually contain 33 residues, and are commonly found in a protein sequence motif; they participate in eukaryotic, prokaryotic and viral protein–protein interactions in cell growth, cell cycle regulation, signal transduction and other functions closely related to a variety of diseases [10]. Previous studies have shown that genes encoding proteins with ankyrin repeats, such as *p16*, kidney ankyrin repeat-containing protein (*Kank*), *HACE1*, *ASPP1* and *ASPP2*, are often inactivated by methylation in many types of cancers [11–14]. However, limited research has been carried out on the regulation of *ANKRD18A* expression. In this study, we compared the methylation status of the *ANKRD18A* promoter region and its expression levels in normal and tumor tissue and cell lines. We also performed a demethylation experiment to identify the biological function of *ANKRD18A* methylation.

2. Materials and methods

2.1. Tissue samples and cell lines

After surgical resection, fresh lung cancer and normal lung tissues from patients were collected at the Affiliated Xi'nan Hospital of Third Military Medical University and immediately snap-frozen in liquid nitrogen, then kept at −80 °C before use. All experimental protocols were approved by the human ethics committee of the Medical Center of Third Military Medical University and written informed consent was obtained from all subjects.

The immortalized human bronchial epithelial cell line HBE and 10 lung cancer cell lines (A549, SPC-A-1, H1975, H358, H1650, LTEP, and H1395 (adenocarcinoma), H446 (small cell lung carcinoma), H460 (large cell carcinoma), 95D (highly metastatic giant cell carcinoma)) were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and the Cell Biology Institute of Chinese Academy of Science (Shanghai, China). Cell lines were cultured in RPMI 1640 medium (Gibco BRL, Rockville, MD) supplemented with 10% fetal bovine serum (Gibco BRL) at 37 °C in a humid incubator with 5% CO_2.

2.2. DNA extraction and methylation analysis

Total genomic DNA was isolated from normal and lung cancer tissues and cell lines using the DNA extraction kit (Promega, Madison, WI) according to the manufacturer's instructions. Genomic DNA was then modified using the EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA) following the manufacturer's protocol.

The primer sequences used for methylation-specific polymerase chain reaction (MSP) and bisulfite sequencing PCR (BSP) are shown in Table 1. MSP and BSP conditions for *ANKRD18A* have been standardized in our laboratory and previously reported [15,16]. For MSP, PCR were performed using a primer-specific annealing temperature (Table 1) for unmethylated and methylated reactions. Normal untreated or *Sss*I-methylase-treated (New England Biolabs, Beverly, MA) genomic DNA samples from blood provided fully unmethylated and fully methylated positive MSP controls and were used in all PCR experiments. Sterile water with no DNA template was included as a negative control. Following amplification, each PCR product was electrophoresed on a 3% agarose gel, stained with ethidium bromide and visualized under UV illumination. The investigators who performed the assays were blinded to all sample information. For BSP, amplified products were purified and cloned into the pGEM-T vector (Promega). Ten colonies of each specimen were randomly chosen for plasmid DNA extraction with the Promega Spin Mini kit (Promega), and were sequenced by an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA) to identify the CpG methylation status.

Table 1
Primer sequences used in this study.

Gene	Primer sequence (5′–3′)	Length (bp), position	Annealing temperature (°C)
For MSP			
ANKRD18A(M)	Forward: TTAGGGAGGGTAATATTTACGAGAC Reverse: ATAAAAAACGACCTACTAACACGAC	101 −1744 to −1644	56
ANKRD18A(U)	Forward: TTTTAGGGAGGGTAATATTTATGAGAT Reverse: ATAAAAAACAACCTACTAACACAAC	103 −1746 to −1644	54
For BSP			
ANKRD18A	Forward: GGGATTTTGAGTTTTGTATG Reverse: AACCTCCTACACCTATCATCCTAAC	280 −1815 to −1536	57
For RT-PCR			
ANKRD18A	Forward: GAGGCTTGTGCCATCGTT Reverse: AAGCAGGAGGGTGACGAT	321 418 to 738	52
β-actin	Forward: GGCATGGAGTCCTGTGG Reverse: AGAAGCATTTGCGGTGG	325 886 to 1210	58
For qRT-PCR			
ANKRD18A	Forward: AAGCAAGAAAGGCTCCAAAGAAGT Reverse: GAGCATGGCAATGTCTTTCTTCA	117 973 to 1089	60
β-actin	Forward: CCACGAAACTACCTTCAACTCC Reverse: GTGATCTCCTTCTGCATCCTGT	132 906 to 1037	60

2.3. RNA extraction and quantitative reverse transcription-PCR (qRT-PCR)

Total RNA was extracted from normal and lung cancer tissues and cell lines using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. cDNA was synthesized from 1 μg of total RNA using the PrimeScript® RT reagent Kit with gDNA Eraser (Takara, Shiga, Japan). RT-PCR and real-time qRT-PCR analyses were performed using primers and annealing temperatures shown in Table 1. The housekeeping gene β-actin was amplified as an internal control. Negative controls (distilled water) were also run for each sample. RT-PCR products were analyzed using 2% agarose gel electrophoresis stained with ethidium bromide. Real-time qRT-PCR detection was carried out using an iQ5 real-time detection system (Bio-Rad Laboratories, Hercules, CA) and SYBR® Premix Ex Taq™ II (Takara). *ANKRD18A* mRNA levels were normalized to β-actin and the $2^{-\Delta\Delta Ct}$ method was used to analyze the relative levels of *ANKRD18A* expression.

2.4. Treatment of lung cancer cell lines with 5-aza-2′-deoxycytidine (5-aza-dC)

To assess whether the mRNA expression of *ANKRD18A* was restored by 5-aza-dC treatment, demethylation experiments were performed as previously described [15,16]. Briefly, lung cancer cells with *ANKRD18A* hypermethylation were exposed to 10 μM of 5-aza-dC (Sigma, St Louis, MO) for three days, with cell media and drugs being replaced daily. The control culture was treated