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Transcriptional silencing of the *RUNX3* gene by CpG hypermethylation is associated with lung cancer

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Abstract

RUNX family transcription factors are integral components of TGF-β signaling pathways and have been implicated in cell cycle regulation, differentiation, apoptosis, and malignant transformation. It was noted previously that allele loss and loss of expression of *RUNX3* are causally involved in gastric carcinogenesis. Our results demonstrate that *RUNX3* is inactivated by aberrant DNA methylation in approximately 19% of lung cancer cell lines and 24% of primary lung cancer specimens. *RUNX3* methylation is tumor-specific, since it is not observed in surrounding normal lung tissues. Our results suggest that loss of *RUNX3* expression by DNA hypermethylation is frequently associated with the evolution of lung cancer.

Keywords: RUNX3; Methylation; Transcription; Lung cancer; TGF-β

Lung cancer is the leading cause of cancer-related death worldwide. Cytogenetic and molecular analyses of lung cancers have revealed the frequent occurrence of multiple chromosomal deletions at 1p, 3p, 5q, 8p, 9p, 11p, 13q, 17p, 18q, and 22q [1]. The short arm of chromosome 1 is among the most frequently affected regions in various types of common adult cancers, including gastric, pulmonary, breast, colon, and hepatocellular carcinomas [2]. Introduction of a normal human chromosome 1 into a cancer cell line was seen to markedly suppress its tumorigenicity [3]. Furthermore, the distal portion of mouse chromosome 4, which is syntenic with human chromosome band 1p36, is observed to undergo frequent deletion during experimentally induced pulmonary carcinogenesis [4]. Therefore, the 1p36 region is believed to harbor one or more tumor suppressor genes responsible for lung cancer. The region of 1p commonly observed to be deleted in lung cancer was mapped between 1p36.1 and 1p36.2 [1].

The Runt family of transcription factors consists of three members, RUNX1 (PEBP2αB/CBFA2/AML1), RUNX2 (PEBP2αA/CBFA1/AML3), and RUNX3 (PEBP2αC/CBFA3/AML2). All three RUNX family members play important roles in normal developmental processes and in carcinogenesis [5]. The *RUNX1* locus, required for definitive hematopoiesis, is the most frequent target of chromosome translocation in leukemia and is responsible for about 30% of the cases of human acute leukemia [6]. *RUNX2*, essential for osteogenesis, is involved in the human disease cleidocranial dysplasia (CCD), an autosomal dominant bone disorder [7]. *RUNX3*, located at 1p36.11–1p36.13, has been identified

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as a gastric tumor suppressor [8,9]. The gastric epithelium of *RUNX3* knockout mice displays hyperplasia and a reduced rate of apoptosis, accompanied by a reduced sensitivity to TGF-β. In primary human gastric cancer specimens, frequent (30%) loss of heterozygosity of *RUNX3* has been observed. Furthermore, 45–60% of gastric cancer cell lines and tumor tissues exhibit loss of *RUNX3* expression due to hypermethylation of the CpG island located in the P2 promoter region [9,10]. Collectively, these results led us to examine whether inactivation of *RUNX3* is involved in the evolution of lung cancer. In this study, we report that 19–24% of lung cancer cell lines and primary cancer tissues tested exhibited loss of *RUNX3* expression due to hypermethylation of the P2-CpG island.

Materials and methods

Cell lines and tissue samples. The human lung cancer cell lines NCI-H522, 86-2, A549, LK79, LK87, LCSC#1, LCSC#2, NCI-H23, NCI-H226, NCI-H460, NCI-H322, Sq-19, NCI-H1915, HS888 Lu, LX-1, Sq-1, Lu61, S2, Lu99, O2, SHP-77, QG-56, NCI-H125, Lu1 16, A-427, Calu-1, Calu-3, Calu-6, ChaGo-K-1, DMS114, and MRC9, and the human gastric cancer cell lines SUN16 and MKN28, were obtained from the Genetic Resources Center, Korea Research Institute of Bioscience and Biotechnology (GRC KRIBB, Taejeon, South Korea). All media (Invitrogen, Carlsbad, CA) were supplemented with 10% fetal bovine serum (Invitrogen) and 1% antibiotic–antimycotic (Invitrogen) and grown at 37 °C in a 5% CO_2 atmosphere. Primary lung tissues were obtained from Yonsei University, South Korea. Among the 25 cases confirmed as primary lung carcinomas, 11 cases were squamous cell carcinomas, 11 cases were adenocarcinomas, and 3 cases were large cell carcinomas. In each case, grossly normal lung tissue remote from the tumor was included as a control. All of the cases were identified consecutively for the Lung Tumor Working Group Tissue Bank at Yonsei University Medical Center (Seoul, Korea) between December 1996 and November 1999. Tumor specimens were microdissected on a cryostat and fractionated to enrich the tumor cell population and DNAs were extracted from fresh frozen tissues.

RNA isolation and RT-PCR. Cells were washed and lysed with guanidine isothiocyanate solution. Total RNA was extracted from cell lines with QIAamp RNA Kit (Qiagen, Valencia, CA). The RT reaction was performed on 5 μg total RNA with SuperScript II First-Strand Synthesis using the oligo(dT) primer system (Invitrogen). PCR amplification was performed in a Perkin–Elmer-Cetus 9700 Gene-Amp PCR system under the following conditions: preheating of the mixture at 95 °C for 5 min, followed by 35 cycles of denaturation for 20 s at 95 °C, annealing for 1 min at 55 °C, and extension for 1 min 30 s at 72 °C, with a final extension for 10 min at 72 °C. The primers used for the amplification of *RUNX3* were Ps–NA, 5′-CGC CAC TTG ATT CTG GAG GAT TTG T-3′; Ps–NB, 5′-TGA AGT GGC TTG TGG TGC TGA GTG A-3′ and Ps–CA, 5′-GAG TTT CAC CCT GAC CAT CAC TGT G-3′; and Ps–CB, 5′-GCC CAT CAC TGG TCT TGA AGG TTG T-3′. The primer sequence for *GAPDH* sense strand was 5′-ACC ACA GTC CAT GCC ATC AC-3′ and the antisense strand was 5′-TCC ACC ACC CTG TTG CTG TA-3′. PCR products were electrophoresed on a 1.2% agarose gels and visualized by ethidium bromide staining.

Methylation-specific PCR and DNA sequencing. DNA was prepared from tissue samples and cell lines by standard methods, and bisulfite modification of genomic DNA was performed as reported by Herman et al. [11]. Treatment of genomic DNA with sodium bisulfite converts unmethylated but not methylated cytosines to uracil, which is then converted to thymidine during the subsequent polymerase chain reaction (PCR), producing sequence differences between methylated and unmethylated DNA. Amplification was carried out in a Perkin–Elmer–Cetus 9700 Gene-Amp PCR system. Primer sequences for the methylated *RUNX3* reaction were 5′-TTA CGA GGG GCG GTC GTA CGC GGG-3′ (forward) and 5′-AAA ACG ACC GAC GCG AAC GCC TCC-3′ (reverse), and primer sequences for the unmethylated *RUNX3* reaction were 5′-TTA TGA GGG GTG GTT GTA TGT GGG-3′ (forward) and 5′-AAA ACA ACC AAC ACA AAC ACC TCC-3′ (reverse). Methylation-specific PCR (MSP) products were purified with a QIAquick PCR Purification Kit (Qiagen) and subjected to direct DNA sequencing (model ABI 3100) using sequencing primers (for methylated DNA: 5′-CGC GTC GAG GAT GCG GGA; for unmethylated DNA: 5′-TGT GTT GAG GAT GTG GGA). Statistical analysis for the relationship between *RUNX3* methylation and cancer stage was done by SAS version 8.01.

Treatment of cells with 5-aza-2′-deoxycytidine and trichostatin A. Cell lines were maintained in appropriate media and treated with 1 μM of 5-aza-2′-deoxycytidine (Sigma Chemical) for 5 days (MKN28, NCI-H226, NCI-H460, and Sq-1 cells) or with 300 nM Trichostatin A (TSA, Sigma Chemical) for 5 days.

Results

Frequent loss of RUNX3 expression in lung cancer

We examined the expression of *RUNX3* in lung cancer cell lines by the reverse transcriptase-polymerase chain reaction (RT-PCR). As shown in Fig. 1, the expression of *RUNX3* was undetectable in NCI-H226, NCI-H460, Sq-1, SHP-77, Calu-3, and Calu-6 cell lines, while control reaction products (*GAPDH*) were detected in all cell lines. Amplifications with two independent PCR primer sets yielded essentially the same results. Overall, of the 31 cell lines tested, 6 (19%) did not express *RUNX3* at detectable levels.

DNA methylation of the RUNX3 promoter region in lung cancer

Patterns of DNA methylation are often altered in cancer cells. Growing evidence suggests that like coding region mutations, aberrant methylation of CpG islands around promoter regions can inactivate tumor suppressor genes [12]. Since the P2 promoter region of *RUNX3* contains a typical CpG island [10], we examined this region by methylation-specific PCR using the bisulfite method. We found that only *RUNX3* non-expressing cells exhibit CpG island methylation in the *RUNX3* promoter (Fig. 2A). This result indicates that the methylation status of the *RUNX3* P2 promoter region correlates inversely with the expression of *RUNX3*.

We further examined 25 pairs of primary lung cancer specimens and surrounding normal lung tissues by methylation-specific PCR. Six cancer specimens (2230, L6, L7, L8, L9, and L10) were found to exhibit methylation within the promoter region of *RUNX3* (Fig. 2B).

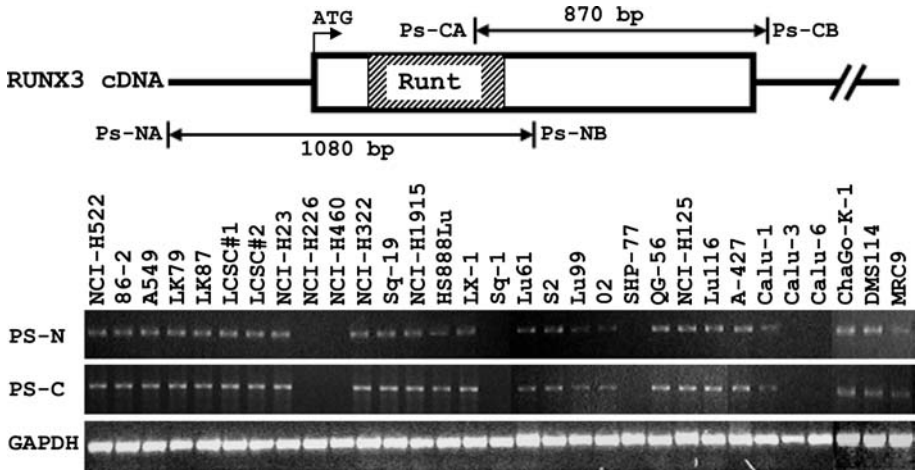

Fig. 1. Expression patterns of *RUNX3* gene in lung cancer cell lines. Upper panel: schematic diagram of *RUNX3* cDNA. The positions of the PCR primers are shown. The Runt domain is indicated by a hatched box (Runt). Lower panel: RT-PCRs with the primer pairs Ps–N and Ps–C, which cover the regions [Ps–NA and Ps–NB] and [Ps–CA and Ps–CB], yielded the expected 1080 and 870 bp DNA products, respectively. *GAPDH* is used as a positive control for RNA quality and loading.

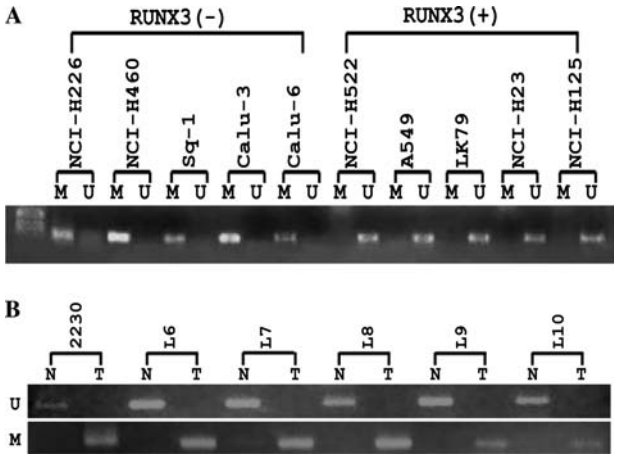

Fig. 2. Correlation of methylation in the promoter region with silencing of the *RUNX3* gene in lung cancer. The methylation status of the cytosine residues between −278 and −59 relative to the translation initiation site of *RUNX3* was determined by methylation-specific PCR using the bisulfite method. (A) The status of *RUNX3* expression in each cell line is indicated. (B) The status of *RUNX3* methylation of the primary lung cancer and surrounding normal tissues of six patients (2230, L6, L7, L8, L9, and L10) is shown. Lane U, product amplified with primers recognizing unmethylated sequences (220 bp); lane M, product amplified with primers recognizing methylated sequences (220 bp). Lane T, primary lung cancer tissue; lane N, surrounding normal lung tissue. *RUNX3* is unmethylated in all the surrounding normal lung tissues.

Methylation-specific PCR products were not detected from any surrounding normal tissues. Of 10 stage III cases, 5 (50%) showed *RUNX3* methylation. For stage I and II cases, 1 out of 8 (13%) and 0 out of 7 (0%) exhibited *RUNX3* methylation, respectively. Of 6 *RUNX3* methylated cases, 2 (2/11, 18%) cases were squamous cell carcinomas, 3 (3/11, 27%) cases were adenocarcinomas, and 1 (1/3, 33%) were large cell carcinomas. These results indicate that *RUNX3* is silenced in approximately 24% (6/25) of primary lung cancers due to hypermethylation in the P2 promoter region, and *RUNX3* methylation was detected mostly in advanced stage III cancer specimens (5/6).

We confirmed the status of DNA methylation of representative cell lines that express or do not express *RUNX3*. In three non-expressing lines, NCI-H226, Calu-3, and Sq-1, the cytosine residues of the CpG dinucleotide motifs in the *RUNX3* (−207 and −68 relative to the translation initiation site) were fully methylated, whereas in three *RUNX3*-expressing lines, NCI-H522, A549, and LK79, it was entirely methylation-free (Fig. 3). The state of DNA methylation of three primary lung cancer specimens which exhibit methylation of the *RUNX3* and surrounding normal lung tissues from the same patients was also verified by DNA sequencing. Most of the cytosine residues of the CpG dinucleotide motifs in the same region were methylated in tumors, whereas it was methylation-free in surrounding normal tissues (Fig. 3).

Reactivation of RUNX3 expression after treatment with 5-aza-2′-deoxycytidine and trichostatin A

Transcriptional repression can be mediated by the recruitment of histone deacetylase to methylated DNA [13]. Therefore, if methylation is the cause of gene silencing, DNA methyltransferase inhibitors, and histone deacetylase inhibitors may reactivate gene expression [14,15]. To examine whether the silencing of *RUNX3* is due to the observed methylation, lung cancer cell lines that normally do not express *RUNX3* (NCI-H226, NCI-H460, and Sq-1) were cultivated in the presence of 5-aza-2′-deoxycytidine (AZA), a DNA methyltransferase inhibitor, trichostatin A (TSA), a histone deacetylase inhibitor, or both inhibitors. As shown in Fig. 4A,

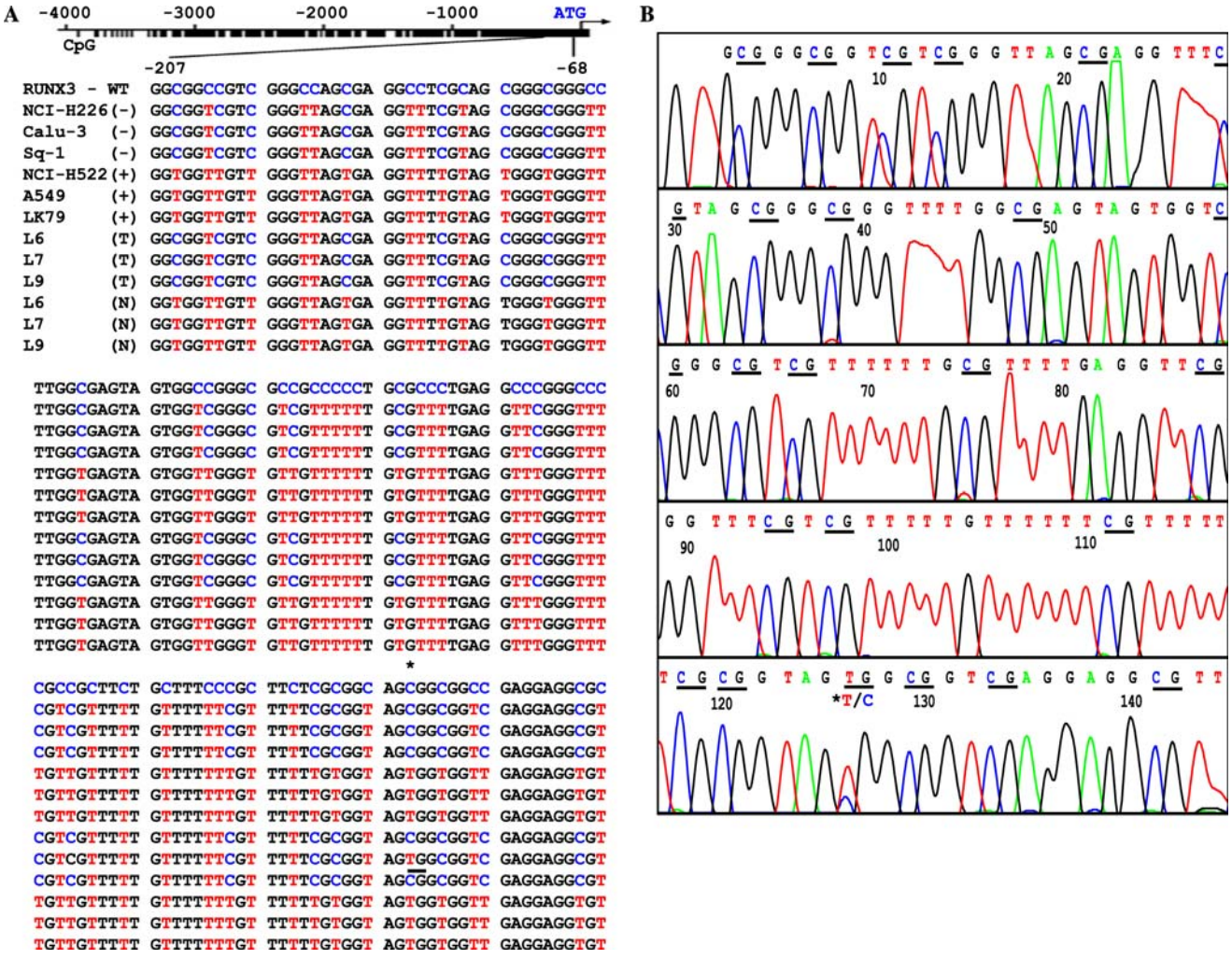

Fig. 3. Sequence analysis of methylation-specific PCR products for the *RUNX3* promoter region. Methylation-specific PCR products amplified from lung cancer cell lines, primary lung cancer specimens, and surrounding normal lung tissues from the same patients were directly sequenced. (A) Sequences derived from the three cell lines expressing *RUNX3* positive (+) and not expressing *RUNX3* negative (−) and three primary lung cancer specimens (L6, L7, and L9) which exhibit methylation of the *RUNX3* (T) and surrounding normal lung tissues (N) are shown. The blue C indicates a cytosine residue resistant to bisulfite treatment due to methylation. The red T indicates a thymidine that was derived from cytosine by bisulfite treatment, suggesting that this residue was not methylated. The major transcription initiation site is located at 411 bp upstream of the initiation codon [10]. (B) A representative sequence histogram of the methylated MSP product for the *RUNX3* promoter region of L6 (T). The methylcytosine at the CpG sites (underlined) remained as cytosines after sodium bisulfite modification, while other cytosines (non-CpG sites) were converted to uracil. C residue which is partially methylated is indicated by asterisk. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

RUNX3 expression was restored in all three lung cancer cell lines. As expected, demethylation of *RUNX3* was observed by AZA treatment, although it was not fully demethylated (Fig. 4B). These results confirm that the transcriptional inactivation of *RUNX3* is due to hypermethylation of the CpG island in the *RUNX3* P2 promoter region.

Discussion

DNA methylation is a powerful mechanism for suppression of gene activity. The reciprocal relationship between the density of methylated cytosine residues in CpG islands and local transcriptional activity has been widely documented [12]. Transcriptional repression by DNA methylation is mediated by changes in chromatin structure. Specific proteins bound to methylated DNA recruit a complex containing transcriptional co-repressors and histone deacetylases [13]. The deacetylation of histones results in chromatin compaction and thereby in the inhibition of transcription. Many tumor suppressor genes contain CpG islands and show evidence of methylation-specific silencing. The *RB* gene was the first tumor suppressor gene for which hypermethylation was reported to cause cancer, and about 9% of retinoblastomas exhibit hypermethylation of the *RB* 5′ region [16]. So far, several genes, including *RB*, *p16*, *RAR*-β2,

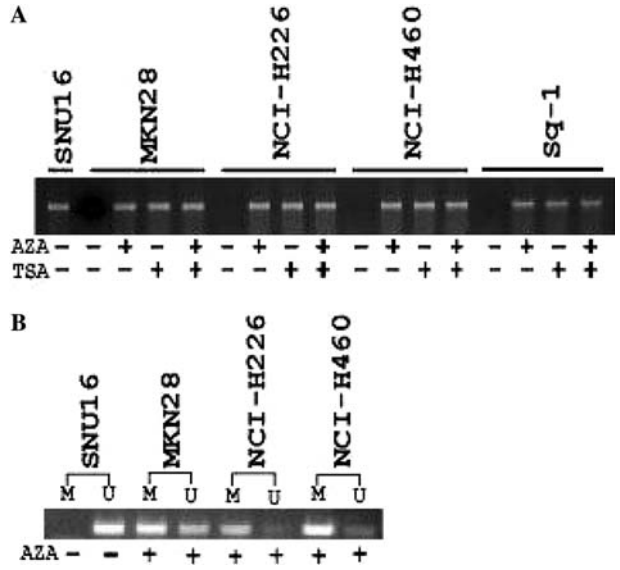

Fig. 4. Reactivation of *RUNX3* expression by 5-aza-2′-deoxycytidine and trichostatin A. (A) Cells were cultured for 5 days in the presence of AZA (1 μM) or TSA (300 nM) or both inhibitors. RT-PCR was performed using the Ps–N primer set shown in Fig. 1, and the products were visualized by ethidium bromide staining. (B) Cells were cultured for 5 days in the presence of AZA (1 μM) and the status of *RUNX3* methylation was analyzed by MSP. NCI-H226, NCI-H460, and Sq-1 are lung cancer cell lines which do not express *RUNX3* at normal condition. Two gastric cancer cell lines, SNU16, which expresses *RUNX3* normally, and MKN28, which does not express *RUNX3* due to DNA methylation, were used for positive controls. Reactivation of *RUNX3* by AZA and TSA in MKN28 cells has been reported [9].

H-cadherin, *DAPK*, and *RASSF1A*, have been reported to undergo methylation in lung cancers [12]. In some of these tumors, hypermethylation is associated with loss of heterozygosity and in others hypermethylation affects both alleles.

TGF-β exerts cellular effects by binding to transmembrane receptors that possess serine/threonine kinase activity [17]. Upon ligand binding, the receptor kinase phosphorylates the Smad2 and Smad3 signal transducers (R-Smads). These receptor-activated Smad proteins associate with Smad4 (Co-Smad) and move into the nucleus. Biochemical analysis has revealed that RUNX family transcription factors physically interact with TGF-β-activated Smads and mediate TGF-β signaling [18]. Consistent with this scheme, members of the RUNX family share numerous important biological functions with members of the TGF-β superfamily [5], and RUNX proteins are therefore considered to be important for TGF-β signaling [19]. It is noteworthy that TGF-β signaling pathways are interrupted in many types of cancers. The type II TGF-β receptor is altered in colon and gastric cancers, and *Smad2* mutations have been found in colon cancers. *Smad4*, which is identical to deleted in pancreatic cancer (DPC), is inactivated in colon cancer in addition to pancreatic cancers [17,20].

Mice heterozygous for a disruption of TGF-β1 display decreased liver and lung apoptosis. When these mice are challenged with carcinogens, they develop hepatic and pulmonary tumors of larger size, in greater number, and of enhanced malignancy [21]. Interestingly, chromosomal locus 1p36, in which *RUNX3* is located, is frequently deleted in various cancers, including lung cancer as well as gastric cancer. These results suggest the possibility that *RUNX3* is a tumor suppressor gene responsible for lung cancer.

Our findings demonstrate several points about the relationship between *RUNX3* promoter methylation and lung cancer. First, methylation of the *RUNX3* promoter region occurs commonly both in cell lines and in primary lung cancers. Although the number of patients in our study is relatively small, most cases of *RUNX3* methylation appeared at advanced stages of lung cancer (P value $= 0.0388$). Second, the aberrant methylation of *RUNX3* is tumor-specific, since it was not observed in surrounding normal lung tissues. Third, methylation is correlated with the silencing of *RUNX3* expression. Fourth, treatment with DNA methylase inhibitors and histone deacetylase inhibitors restores expression of *RUNX3* in each of the three cell lines tested. These results suggest that the loss of *RUNX3* expression caused by hypermethylation is frequently associated with the evolution of lung cancer.

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