

## Demethylation of *RUNX3* by Vincristine in Colorectal Adenocarcinoma Cells

JI WOOK MOON<sup>1</sup>, SOO KYUNG LEE<sup>1</sup>, JUNG OK LEE<sup>1</sup>, JI HAE KIM<sup>1</sup>,  
NAMI KIM<sup>1</sup>, JIN KIM<sup>2</sup>, HYEON SOO KIM<sup>1</sup> and SUN-HWA PARK<sup>1</sup>

<sup>1</sup>Institute of Human Genetics, Department of Anatomy, Brain Korea 21 Project for Biomedical Sciences and

<sup>2</sup>Department of General Surgery, Korea University Anam Hospital,  
Korea University College of Medicine, Seoul, Republic of Korea

**Abstract.** *Background: Methylation-mediated inactivation of tumor-suppressor genes is a critical event during the pathogenesis of many malignancies. Vincristine is a conventional anticancer drug used to treat various types of cancers. However, few studies describe the epigenetic-based effects of vincristine. In this study, changes in the methylation of runt-related transcription factor-3 (RUNX3) were investigated in CCD18Co normal colon cells and DLD-1 colorectal adenocarcinoma cells. Materials and Methods: CCD18Co and DLD-1 cells were treated with vincristine, and the methylation status was assessed using quantitative methylation-specific polymerase chain reaction (QMSP). Eleven normal colon tissues and 105 colorectal cancer tissues were investigated by methylation and mRNA expression of RUNX3 using QMSP and real-time reverse transcription polymerase chain reaction (real time-PCR). Results: RUNX3 was demethylated after vincristine treatment in DLD-1 cells. The expression of RUNX3 mRNA was down-regulated in DLD-1 cells because of DNA hypermethylation, but was restored after vincristine treatment. In addition, hypermethylation of RUNX3 was detected in 70 out of 105 colorectal carcinomas (66.7%). RUNX3 hypermethylation was greater in colon cancer tissues than in rectal cancer tissues. The expression of RUNX3 mRNA was reduced in 68 out of 105 colorectal cancer tissues (64.8%). Conclusion: These results demonstrate that vincristine demethylates RUNX3 in colorectal adenocarcinoma cells, and restores its expression.*

Correspondence to: Sun-Hwa Park, MD, Ph.D., Institute of Human Genetics, Department of Anatomy, Korea University College of Medicine, 126-1, Anam-dong 5-ga, Seongbuk-gu, Seoul 136-705, Korea. Tel: +82 222861152, Fax: +82 29295696, e-mail: parksh@korea.ac.kr

**Key Words:** DNA methylation, vincristine, colonic neoplasms, demethylation, methylation-specific polymerase chain reaction, *RUNX3*.

Aberrant DNA methylation can lead to inactivation of tumor-suppressor gene expression (1). In mammals, DNA methylation occurs only at the CpG dinucleotide pair, in which a 5'-cytosine residue is situated adjacent to a guanine residue. Hypermethylation of CpG-rich or intermediate promoters inactivates downstream gene expression. Promoter hypermethylation of tumor-suppressor genes is frequently observed during the pathogenesis of many human malignancies (2-5).

Epigenetic therapy is a rapidly developing area of clinical medicine. Azacytidine may induce cell differentiation by demethylating genes silenced by hypermethylation, resulting in their re-expression (6). The DNA methyltransferase inhibitor 5-azacytidine was approved in 2004 by the US Food and Drug Administration for treating myelodysplastic syndrome (7) and 5-aza-2'-deoxycytidine (5-Aza-dC) as 5-azacytidine analogue was widely used in DNA methylation studies (8). Several anticancer drugs, including the antitumor antibiotic doxorubicin, the microtubule inhibitor colchicine, the ribonucleotide reductase inhibitor hydroxyurea, and the antimetabolite 5-fluorouracil are associated with drug-induced changes in DNA methylation in human lung adenocarcinoma and rhabdomyosarcoma cells (9).

Vincristine is a vinca alkaloid from the plant *Catharanthus roseus*. Vincristine mainly arrests mitosis in metaphase by binding to tubulin dimers (10). It is used as a chemotherapy drug for various types of cancers, including non-Hodgkin's lymphoma (11), acute lymphoblastic leukemia, lung cancer, breast cancer, and colorectal cancer (CRC) (12-15). Recently, cyclophosphamide, vincristine, and prednisone (COP) chemotherapy was used to significantly improve overall survival and progression-free survival in patients with primary colonic lymphoma (16). However, few studies have described the epigenetic-based effects of vincristine.

Runt-related transcription factor-3 (*RUNX3*) is located on chromosome 1p36, where high frequency loss of heterozygosity has been detected in various types of cancers. Aberrant methylation of *RUNX3* DNA occurs in CRC (17,

18). In addition, the expression of *RUNX3* is reduced or lost in many gastric cancer tissues (19). *RUNX3* is an important target of transforming growth factor-beta (TGF- $\beta$ ) signaling in the gastrointestinal tract. A conserved domain within the C-terminus of *RUNX3* interacts directly with the MH2 domains of TGF- $\beta$  receptor-regulated SMAD family member 3 (SMAD3) and SMAD1. Inactivation of *RUNX3* in cancer disrupts the TGF- $\beta$  signal transduction pathway and induces cell-cycle arrest (20). In *Runx3*<sup>-/-</sup> mice, increased  $\beta$ -catenin activity enhanced proliferation and wingless-related MMTV integration (Wnt) signaling in intestinal epithelia (21). And *RUNX3* attenuated  $\beta$ -catenin activity in human colorectal adenocarcinomas (22). In addition, *RUNX3* is one member of a panel of markers for the CpG island methylator phenotype (CIMP), a distinct phenotype of CRC, identified in clinical trials (23). A panel of CIMP markers that included *RUNX3* was investigated as a predictive marker for response to 5-fluorouracil-based chemotherapy (24). However, demethylation of *RUNX3* by vincristine treatment has not been reported.

In the present study, we investigated the effect of vincristine on the methylation state of the hypermethylated *RUNX3* gene in colorectal adenocarcinoma cells. The methylation status of *RUNX3* was assessed in colorectal adenocarcinoma cells and normal colon cells using quantitative methylation-specific polymerase chain reaction (QMSP). After vincristine treatment, changes in DNA methylation and expression of *RUNX3* were examined by methylation-specific polymerase chain reaction (MSP) and real-time reverse transcription polymerase chain reaction (real-time PCR), respectively. In addition, we examined the DNA methylation status and expression levels of *RUNX3* in colorectal cancer tissues using QMSP and real-time PCR.

## Materials and Methods

**Tissue specimens.** Eleven normal colon tissues and 105 CRC tissues were collected from patients treated at the Department of Colorectal Surgery, Korea University Medical Center. The specimens were collected after obtaining consent from the patients, and the study was approved by the Institutional Review Board of Korea University College of Medicine (IRB no. KU-IRB-10-08-A-1). The study included 68 males and 48 females, ranging in age from 22-88 years (mean $\pm$ standard deviation: 63.7 $\pm$ 13.8 years).

**Cell lines.** A colon cancer cell line (DLD-1) and a normal colon cell line (CCD18Co) were obtained from the American Type Culture Collection (Manassas, VA, USA). The CCD18Co cell line was maintained in Eagle's minimum essential medium. The DLD-1 cell line was maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA) and grown at 37°C in an atmosphere with 5% CO<sub>2</sub>.

**3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay and pharmacological treatment.** To determine the optimal

concentration of vincristine for the treatment of DLD-1 and CCD18Co cells, the viability of each cell line after 1–1,000 nM vincristine treatment for two days was measured by the MTT assay. The cells were seeded at a density of 5 $\times$ 10<sup>3</sup> cells/well in a 96-well plate, and after 24 h, the cells were treated with 1 nM, 3 nM, 10 nM, 30 nM, 100 nM, 300 nM, or 1  $\mu$ M vincristine (Sigma-Aldrich, St. Louis, MO, USA) for 48 h. Subsequently, MTT reagents (10  $\mu$ l/well, 7.5 mg/ml in phosphate-buffered saline) were added, and the culture was incubated for 2 h at 37°C. The reaction was stopped by removing the media and reagents. Dimethyl sulfoxide (50  $\mu$ l/well; Sigma-Aldrich) was then added and cells incubated at room temperature for 20 min. The absorbance of the samples was measured using a SpectraMax M5e Multi-Mode Microplate Reader (Molecular Devices, LLC, Sunnyvale, CA, USA) at 540 nm. The cells were seeded at a density of 5 $\times$ 10<sup>3</sup> cells/well in a 96-well plate, and after 24 h, the cells were treated with 30  $\mu$ M 5-Aza-2'-deoxycytidine (5-Aza-dC) as demethylation agent for 48 h. The results are representative of experiments repeated at least three times.

**Genomic DNA extraction.** Genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's recommendations. Tissue samples were ground with 3-mm diameter punches and then mixed with 700  $\mu$ l of lysis buffer containing 20  $\mu$ g/ml Labo Pass proteinase K (Cosmo Genetech, Seoul, Korea), 20 mM Tris-HCl (pH 8.0), 5 mM EDTA (pH 8.0), 400 mM NaCl, and 1% sodium dodecyl sulfate (SDS) solution (Sigma, St. Louis, MO, USA). The mixed samples were incubated overnight at 42°C. After incubation, genomic DNA was purified via phenol/chloroform extraction. Genomic DNA was eluted in 100  $\mu$ l of water and quantified with a NanoDrop ND-100 device (Thermo Fisher Scientific, Waltham, MA, USA).

**Sodium bisulfite DNA modification.** Two micrograms of genomic DNA in 20  $\mu$ l of RNase-free water was bisulfite converted using the EpiTect® Fast DNA bisulfite kit (Qiagen) according to the manufacturer's recommendations. Briefly, 20  $\mu$ l of genomic DNA solution, 85  $\mu$ l of bisulfite mix solution and 35  $\mu$ l of DNA protect buffer were combined in 200- $\mu$ l PCR tubes at room temperature. The bisulfite-converted genomic DNA was eluted from the column with 100  $\mu$ l of distilled water and stored at -80°C until use.

**QMSP.** Briefly, the methylation status of the bisulfite-converted genomic DNA was quantified by quantitative real-time PCR using a 7000 HT Real-Time PCR System (Applied Biosystems, San Francisco, CA, USA) according to the manufacturer's recommendations. Methylation primers were designed using MethPrimer software (<http://www.urogene.org/methprimer/>). The QMSP primer sequences were: methylated sequence of *RUNX3* (-102 to +64, position from translational start site +1): 5'-TGT TCG CGA TGG GGG TTT CGT CGA TTG-3' (sense), 5'-CGA AAC TCG CCC GCG ACC GCC CCG ACT C-3' (antisense), and 6FAM5'-TTT CGC GCG GGC GGT CGC GGT TT-3'-TAMRA (probe); reference sequence of beta-actin (*ACTB*) (-1645 to -1513): 5'-TGG TGA TGG AGG AGG TTT AGT AAG T-3' (sense), 5'-AAC CAA TAA AAC CTA CTC CTC CCT TAA-3' (antisense), and 6FAM5'-ACC ACC ACC CAA CAC ACA ATA ACA AAC ACA-3'-TAMRA (probe). The product sizes were 166 bp and 132 bp, respectively. PCR reactions were performed in a final volume of 20  $\mu$ l using an optical 96-well tray. The reaction mixture consisted of 5  $\mu$ l of 2 $\times$  EpiTect® MethyLight Master Mix

(Qiagen), 250 nM of each primer, 200 nM probe, and 20 ng of bisulfite-converted DNA template. The QMSP program was initiated at 50°C for 2 min and 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. Each DNA sample was analyzed in duplicate, and the mean quantity was used for further analysis. The relative amplified gene level from the bisulfite-converted genomic DNA sample was quantified by measuring the threshold cycle ( $C_T$ ) values of target genes and *ACTB*. The mean quantity of each gene was divided by the mean quantity of *ACTB* and was used for the normalization of input DNA. The negative controls for *ACTB* were excluded from the methylation analysis. Bisulfite-converted genomic DNA of a known concentration was prepared at 1, 1/4, 1/16, and 1/64 *via* serial dilutions and then used as the standard curve for quantification. Genomic DNA, modified by the CpG methyltransferase M.SssI (New England BioLabs, Boston, MA, USA) according to the manufacturer's recommendations, was used as a positive control. DNA methylation assessed by M.SssI was verified using the restriction enzyme *Bst*UI (New England BioLabs).

**mRNA extraction and cDNA synthesis.** mRNA was extracted using an RNeasy Mini kit (Qiagen) according to the manufacturer's recommendations. mRNA was eluted in 20 µl of diethyl pyrocarbonate (DEPC) water (Qiagen) and quantified with a NanoDrop ND-100 device (Thermo Fisher Scientific). cDNA was synthesized from 1 µg of mRNA from each sample using Moloney murine leukemia virus reverse transcriptase (M-MLV RT) and random hexamers (Promega, Madison, WI, USA). The cDNA synthesis reaction was prepared according to the manufacturer's recommendations by mixing 1 µl of 1 µg mRNA, 4 µl of 5× RT buffer, 1 µl of 500 nM oligo dT, 1 µl of 10 mM dNTP, 0.5 µl of RNasin, 1 µl of M-MLV RT, and 11.5 µl of distilled water in PCR tubes. The mixture was incubated in 37°C for 1 h. cDNA was diluted with 20 µl of distilled water and stored at -80°C until use.

**Real-time PCR.** mRNA expression was confirmed by quantitative real-time PCR using a 7000 HT Real-Time PCR System (Applied Biosystems) according to the manufacturer's recommendations. The primers were designed using Primer3 version 0.4.0 (<http://primer3.wi.mit.edu/>). The specific primers were: *RUNX3*, 5'-CAG AAG CTG GAG GAC CAG AC-3' (sense) and 5'-TCG GAG AAT GGG TTC AGT TC-3' (antisense); *ACTB*, 5'-AGA GCT ACG AGC TGC CTG AC-3' (sense) and 5'-AGC ACT GTG TTG GCG TAC AG-3' (antisense). The product sizes of *RUNX3* and *ACTB* were 179 bp and 184 bp, respectively. The PCR reaction was performed in a final volume of 20 µl using an optical 96-well tray. The reaction mixture consisted of 5 µl of 2× Maxima® SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific), 250 nM of each primer, and 100 ng of cDNA template. The real-time PCR program was initiated at 50°C for 2 min and 95°C for 10 min, followed by 35 cycles of 95°C for 15 s and 60°C for 1 min. The thermal melt profile was examined to assess the homogeneity of the PCR application. Each DNA sample was analyzed in duplicate, and the mean quantity was used for further analysis. The relative levels of amplified mRNA in each sample were quantified by measuring the threshold cycle ( $C_T$ ) values of the target genes and *ACTB*. The mean quantity of each gene was divided by the mean quantity of *ACTB* and was used for the normalization of input DNA. cDNA of a known concentration was prepared at 1, 1/4, 1/16, and 1/64 *via* serial dilutions and used as the standard curve for quantification.

**Statistical analysis.** MTT assay results were quantified using SoftMax® Pro software (Molecular Devices, LLC). The methylated intensity ratio of QMSP was determined as the percentage of methylated reference (PMR), and the PMR value was defined as  $[(\text{GENE})_{\text{sample}}/(\text{ACTB})_{\text{sample}}]/[(\text{GENE})_{\text{M.SssI}}/(\text{ACTB})_{\text{M.SssI}}] \times 100$ . The significance of the differences in PMR values was defined by the chi-squared test, Fisher's exact test, and ANOVA using Sigma Stat (SPSS Inc., Chicago, IL, USA). In all statistical tests, *p*-Values of <0.05 were considered statistically significant. Data from real-time PCR analysis were analyzed using Sigma Stat (SPSS Inc.). *p*-Values of <0.05 were considered statistically significant.

## Results

**Vincristine promotes *RUNX3* demethylation in the DLD-1 colon cancer cell line.** The methylation status of *RUNX3* was examined in DLD-1 colorectal adenocarcinoma cells and normal CCD18Co colon cells using QMSP. *RUNX3* was hypermethylated in DLD-1 cells, but not in CCD18Co cells. Hypermethylated *RUNX3* in DLD-1 cells was significantly demethylated after treatment with 5-Aza-dC and vincristine. No significant change was observed in CCD18Co cells (Figure 1A).

To determine the effects of vincristine on cell viability of DLD-1 and CCD18Co cells, cell viability was examined using the MTT assay after treatment with 1-1,000 nM of vincristine. Cell viability was not affected by treatment with 100 nM vincristine, but treatment with 300 nM or more of vincristine significantly reduced cell viability of DLD-1 and CCD18Co cells (Figure 1B). These results show that vincristine treatment affects the viability of colon cancer cells.

***RUNX3* methylation and expression are inversely correlated in DLD-1 cells.** To examine the correlation between *RUNX3* methylation and expression in the presence and absence of vincristine, the methylation status and mRNA levels of *RUNX3* were assessed by MSP and real-time PCR analysis, respectively, in DLD-1 and CCD18Co cells after vincristine treatment. *RUNX3* was significantly demethylated in DLD-1 cells after treatment with 100 nM vincristine for two days, but no change in methylation status was detected in CCD18Co cells (Figure 2A). After treatment with 100 nM vincristine and 30 µM 5-Aza-dC for two days, the expression of *RUNX3* mRNA was restored in DLD-1 cells, as shown by real-time PCR, but the level of *RUNX3* mRNA did not change significantly in CCD18Co cells (Figure 2B).

In addition, we investigated the level of *RUNX3* mRNA in DLD-1 cells after treatment with various concentrations of vincristine for two days. After treatment with vincristine, the expression of *RUNX3* mRNA was restored in a concentration-dependent manner; the optimal dose of vincristine was 100 nM, but the levels of *RUNX3* mRNA did not change significantly in CCD18Co cells (Figure 2C). These results suggest that vincristine promotes the demethylation of

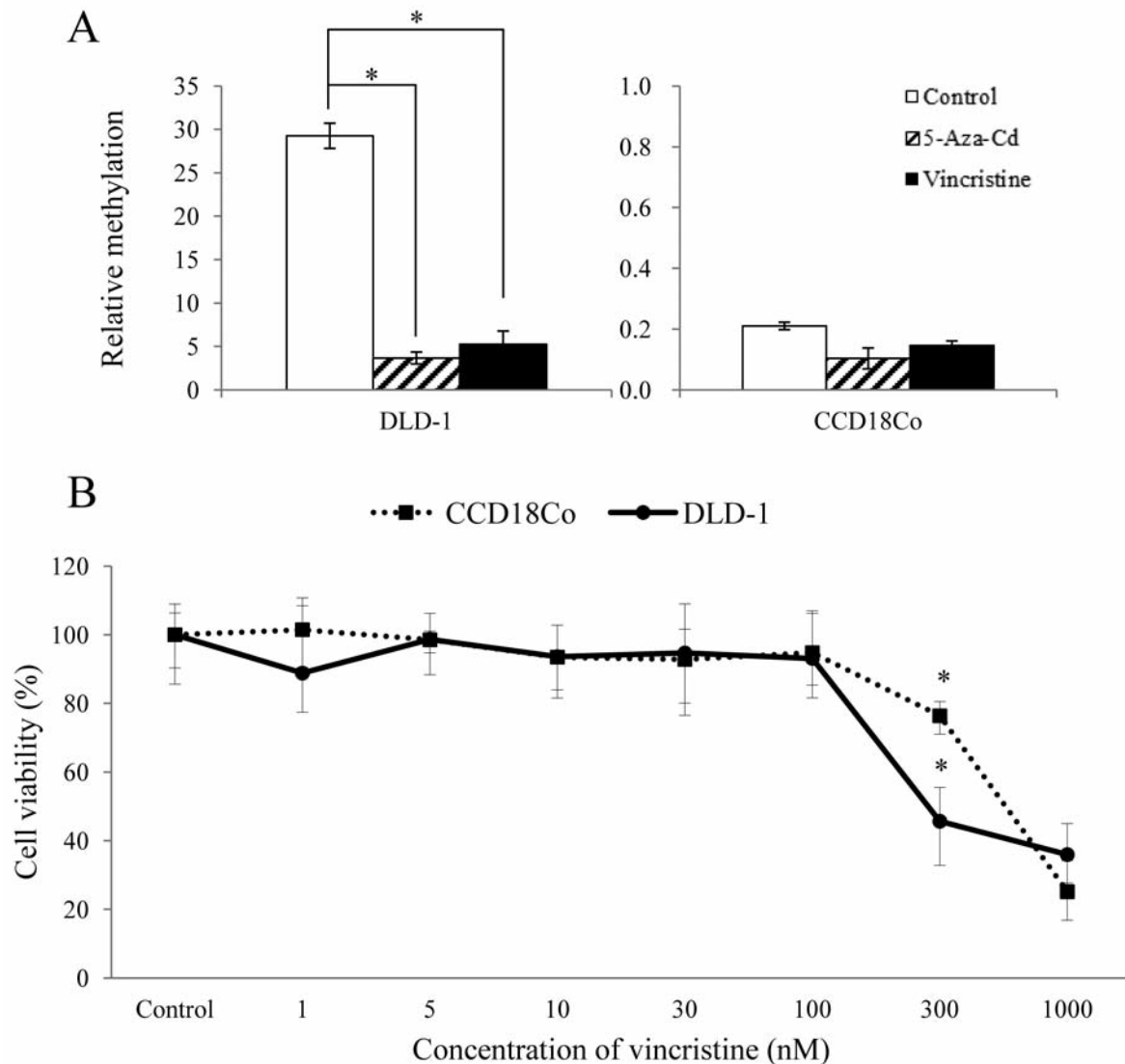


Figure 1. The methylation status of runt-related transcription factor-3 (*RUNX3*) after treatment with 5-Aza-2'-deoxycytidine (5-Aza-dC) and vincristine. A: The methylation status of *RUNX3* in DLD-1 and CCD18Co cells was assessed using quantitative methylation PCR (QMSP). *RUNX3* was hypermethylated in DLD-1 cells, and was significantly demethylated by treatment with 5-Aza-dC and vincristine. On the other hand, *RUNX3* was also demethylated in CCD18Co cells by treatment with 5-Aza-dC and vincristine, but the change was not significant. B: Cell viability of DLD-1 and CCD18Co cells after treatment with vincristine for two days was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay. The cell viability in DLD-1 and CCD18Co cells did not change significantly after treatment with concentrations of vincristine less than 100 nM for two days, but the cell viability was significantly reduced after treatment with concentrations of vincristine greater than 300 nM. \*p-Values of <0.05 were considered statistically significant. 5-Aza-dC, treated with 30  $\mu$ M 5-aza-2'-deoxycytidine; vincristine, treated with 100 nM vincristine.

*RUNX3* and that the methylation-mediated silencing of *RUNX3* is reversed by vincristine in colon cancer cells.

*RUNX3* is frequently hypermethylated in CRC tissues. To validate the hypermethylation of *RUNX3* in CRC tissues, we performed QMSP analysis in 105 CRC tissues and 11 normal colon tissues. Hypermethylation of *RUNX3* was detected in 70 out of 105 (67%) CRC tissues compared to two out of 11

(18.2%) normal tissues ( $p=0.003$ ) (Figure 3A). We then applied statistical analysis to examine the relationship between DNA methylation status in tumor specimens and CRC risk factors. The PMR value of *RUNX3* was significantly higher in colon cancer than in rectal cancer ( $p=0.017$ ) (Table I). This indicates that hypermethylation of *RUNX3* is frequently present in CRC and more closely associated with colon cancer than with rectal cancer.

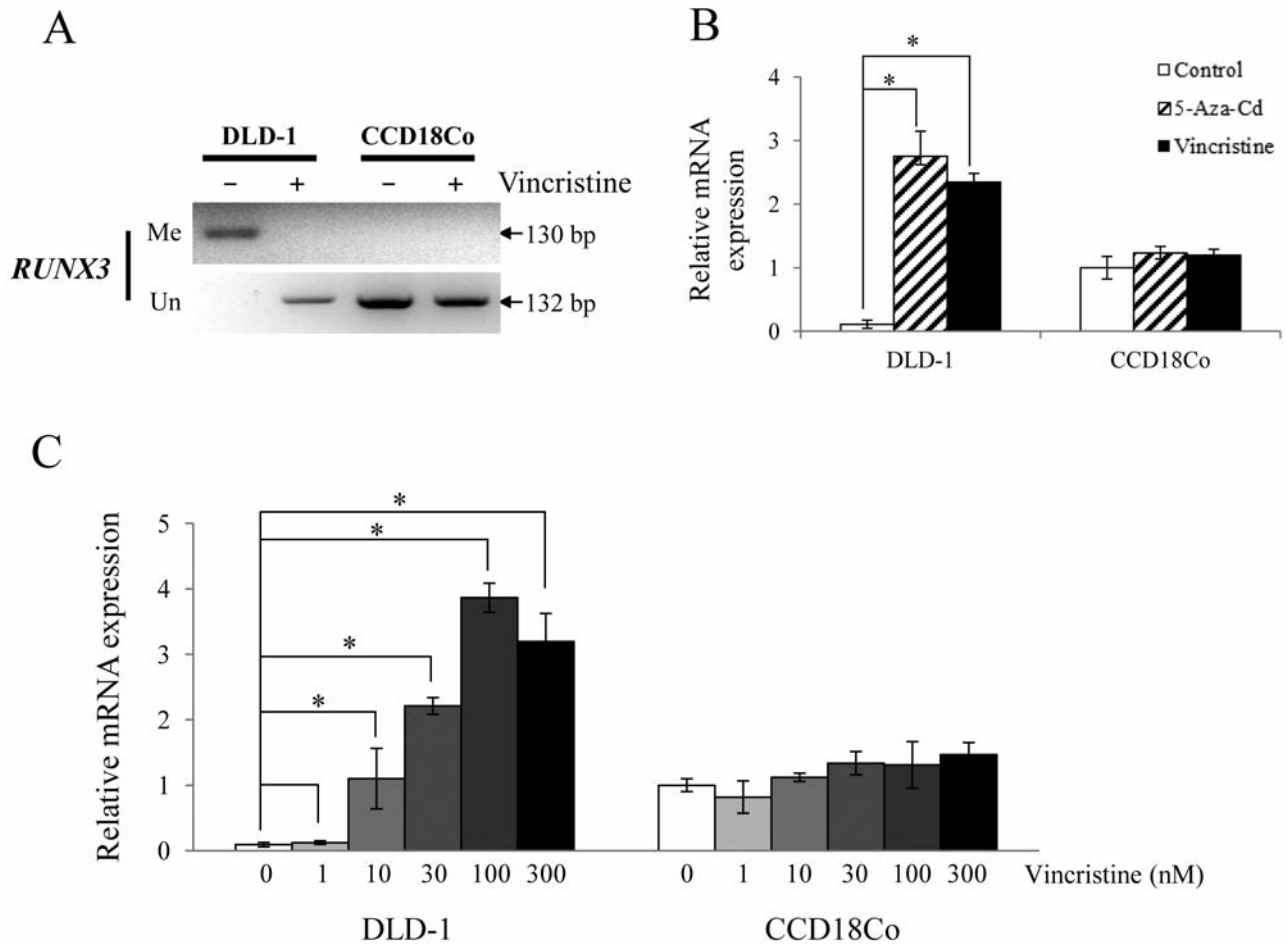


Figure 2. Changes in runt-related transcription factor-3 (*RUNX3*) methylation in DLD-1 and CCD18Co cells after vincristine treatment. The methylation status (A) and mRNA expression (B) of *RUNX3* were determined by methylation-specific polymerase chain reaction (MSP) and real-time reverse transcription polymerase chain reaction (real-time PCR) analysis, respectively, after treatment of DLD-1 and CCD18Co cells with vincristine. The methylation of *RUNX3* decreased, and the expression of *RUNX3* increased in DLD-1 cells after treatment with 100 nM vincristine for two days, but levels did not change in CCD18Co cells. (C) The expression of *RUNX3* mRNA in DLD-1 cells was assessed after treatment with vincristine for two days. After treatment with vincristine, the expression of *RUNX3* mRNA was significantly restored in DLD-1 cells in a concentration-dependent manner. 5-Aza-dC treatment was used as a positive control for demethylation. Expression of beta actin (*ACTB*) was used as a control for mRNA integrity. \**p*-Values of <0.05 were considered statistically significant. Un, Unmethylated gene; Me, methylated 5'CpG region; +, treated with 100 nM vincristine; -, not treated with vincristine; 5-Aza-dC, treated with 30  $\mu$ M 5-aza-2'-deoxycytidine; vincristine, treated with 100 nM vincristine.

Expression of *RUNX3* mRNA is significantly down-regulated in CRC. To examine the relationship between the methylation and expression of *RUNX3* and the clinical implications, the levels of *RUNX3* mRNA was measured in 105 CRC tumor tissues and 11 normal tissues using real-time PCR. The expression of *RUNX3* mRNA was reduced in 68 out of 105 (64.8%) tumor tissues compared to two out of 11 (18.2%) normal tissues ( $p=0.017$ ; Figure 4A). The expression of *RUNX3* mRNA was significantly lower. In addition, the down-regulation of *RUNX3* was greater in colon cancer than in rectal cancer (Table II, Figure 4B). The results demonstrate that methylation-mediated down-regulation of

*RUNX3* is significantly correlated with colon cancer, rather than with rectal cancer.

## Discussion

The aberrant DNA methylation of a promoter region is an important epigenetic mechanism for gene silencing in cancer. DNA hypermethylation leads to the down-regulation and silencing of tumor-suppressor genes during the pathogenesis of various types of human cancer (2-5, 25, 26). DNA methylation is reversible; gene expression can be restored by demethylation agents such as 5-Aza-dC (8). In addition,

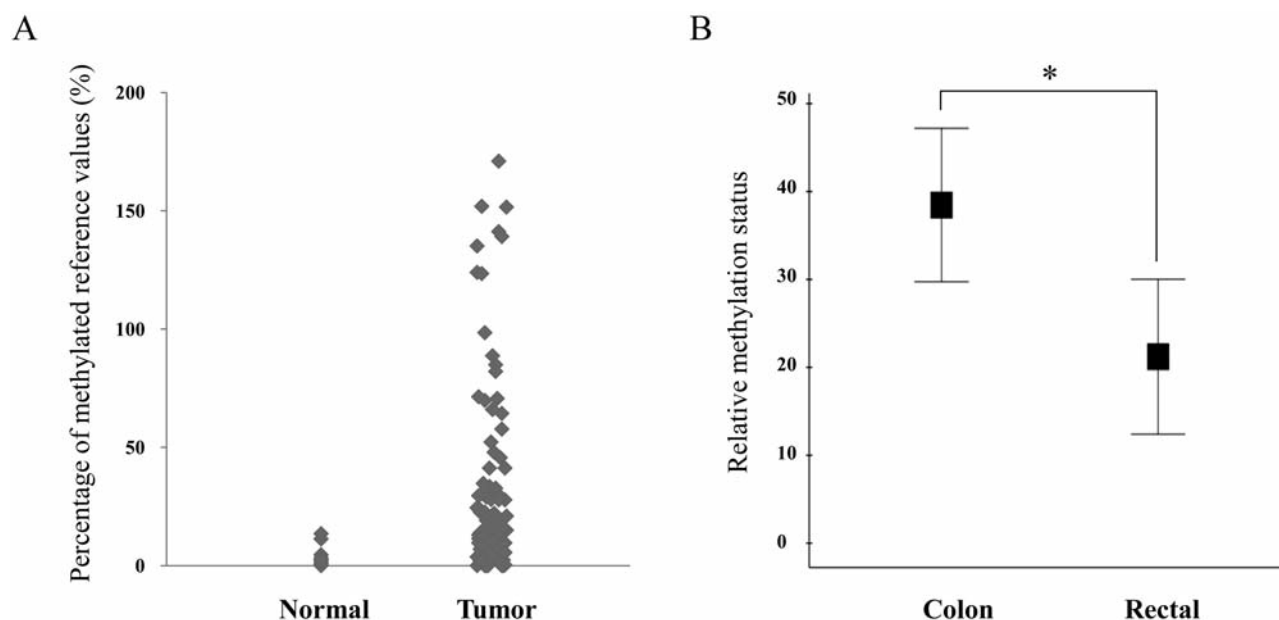


Figure 3. The methylation status of runt-related transcription factor-3 (*RUNX3*) in normal colon tissues and colorectal cancer (CRC) tissues. The methylation status of *RUNX3* was investigated in 11 normal colon tissues and 105 CRC tissues by quantitative methylation-specific polymerase chain reaction (QMSP). A: Hypermethylation of *RUNX3* was detected in 70 out of 105 (67%) CRC tissues ( $p=0.003$ , compared with normal tissues). B: The relative percentage of methylated reference (PMR) value of *RUNX3* was significantly higher in colon cancer than in rectal cancer ( $p=0.017$ ). \* $p$ -Values of  $<0.05$  were considered statistically significant. Normal, Normal colon tissue; Tumor, colorectal cancer; Colon, colon cancer; Rectal, rectal cancer.

Table I. Patients' characteristics and methylation status of runt-related transcription factor-3 (*RUNX3*).

Characteristic	No. of cases	PMR value of <i>RUNX3</i>	
		Median (range)	$p$ -Value
Age, years			0.715
≤65	44	34.50 (±5.60)	
>65	61	31.98 (±4.22)	
Gender			0.713
Female	39	34.66 (±5.80)	
Male	66	32.07 (±4.17)	
Differentiation			0.303
Moderate	73	30.74 (±4.03)	
Well	32	38.57 (±6.42)	
Location			0.017†
Colon	72	38.46 (±4.36)	
Rectum	33	21.20 (±4.41)	
Stage			0.171
I, II	46	38.29 (±5.13)	
III, IV	59	28.94 (±4.45)	
Size, mm			0.554
≤20	53	35.06 (±4.76)	
>20	51	31.00 (±4.92)	
Invasion			0.221
No	85	35.04 (±3.80)	
Yes	20	24.49 (±7.24)	

Differences between PMR values were evaluated by analysis of variance (ANOVA). † $p$ -Values of  $<0.05$  were considered statistically significant. PMR, Percentage of methylated reference.

Table II. Runt-related transcription factor-3 (*RUNX3*) mRNA expression.

Tissue type	No. of cases	Expression of <i>RUNX3</i> mRNA	
		Median (range)	$p$ -Value
Normal	11	2.7 (±0.79)	
Colorectal cancer	105	1.1 (±0.16)	0.005†
Colon cancer	72	1.0 (±0.16)	0.012†
Rectal cancer	33	1.3 (±0.36)	0.070

Differences between percentage of methylated reference (PMR) values were assessed by analysis of variance (ANOVA). † $p$ -Values of  $<0.05$  were considered statistically significant.

several anticancer drugs are associated with changes in DNA methylation in human tumor cells (9).

In the present study, we hypothesized that conventional anticancer drugs might affect DNA methylation in colorectal adenocarcinoma cells. In previous studies, the DNA methylation status of some genes in colorectal adenocarcinoma cells did change after vincristine treatment, but there was no change in the DNA methylation status of genes after treatment with the mitotic inhibitor paclitaxel (data not shown). Recently, patients with primary colonic lymphoma were successfully treated with COP chemotherapy, which included vincristine (16). In addition, DLD-1 colorectal

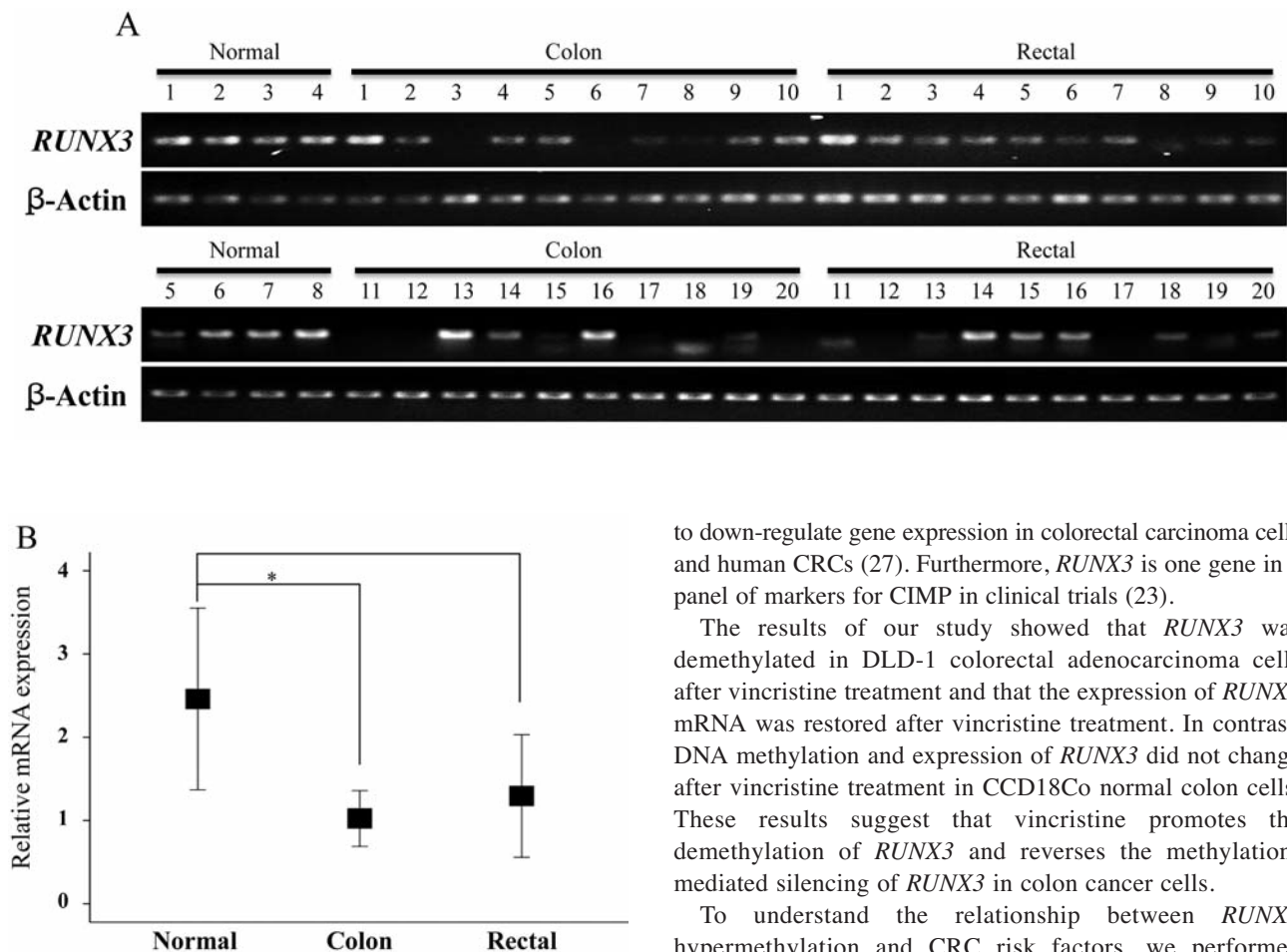


Figure 4. Expression of runt-related transcription factor-3 (*RUNX3*) mRNA in normal colon tissues and colorectal (CRC) tissues. The expression of *RUNX3* mRNA in 11 normal colon tissues and 105 CRC tissues was assessed by real-time reverse transcription polymerase chain reaction (real-time PCR). A: The expression of *RUNX3* mRNA was lower in colorectal cancer tissues than in normal colon tissues. B: *RUNX3* mRNA expression was significantly lower in colon cancer tissues than in rectal cancer tissues.  $\beta$ -Actin was used as a control for mRNA integrity. \*p-Values of <0.05 were considered statistically significant. Normal, Normal colon tissue; Tumor, colorectal cancer; Colon, colon cancer; Rectal, rectal cancer.

adenocarcinoma cells, used in the present study, originated from patients with lymph node metastasis. Therefore, an epigenetic-based therapeutic approach using vincristine in DLD-1 cells was investigated. Among the several genes demethylated by vincristine in DLD-1 cells, *RUNX3* was chosen as the target gene because both vincristine and *RUNX3* promote cell-cycle arrest and inhibit proliferation. *RUNX3* induces cell-cycle arrest through TGF- $\beta$  signaling and inhibits proliferation by regulating Wnt signaling pathways in human colorectal adenocarcinomas (20, 22). In addition, hypermethylation of the *RUNX3* promoter has been reported

to down-regulate gene expression in colorectal carcinoma cells and human CRCs (27). Furthermore, *RUNX3* is one gene in a panel of markers for CIMP in clinical trials (23).

The results of our study showed that *RUNX3* was demethylated in DLD-1 colorectal adenocarcinoma cells after vincristine treatment and that the expression of *RUNX3* mRNA was restored after vincristine treatment. In contrast, DNA methylation and expression of *RUNX3* did not change after vincristine treatment in CCD18Co normal colon cells. These results suggest that vincristine promotes the demethylation of *RUNX3* and reverses the methylation-mediated silencing of *RUNX3* in colon cancer cells.

To understand the relationship between *RUNX3* hypermethylation and CRC risk factors, we performed QMSP and real-time PCR in primary CRCs. Interestingly, the results showed that hypermethylation of *RUNX3* was frequently present in CRC and more closely associated with colon cancer than with rectal cancer. Additionally, hypermethylation of *RUNX3* in colon cancer significantly correlated with down-regulation of *RUNX3* mRNA, but the correlation was not significant in rectal cancer. The results suggest that hypermethylation of *RUNX3* may play an important role in colorectal carcinogenesis. Furthermore, methylation-mediated inactivation of *RUNX3* may be location-dependent in CRC.

In conclusion, our *in vitro* studies of hypermethylated *RUNX3* and vincristine suggest an epigenetic-based therapeutic approach for treating colorectal cancer. Our results may provide critical information for the development of sensitive drug therapies for CRC.

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