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Vorinostat, SAHA, Represses Telomerase Activity via Epigenetic Regulation of Telomerase Reverse Transcriptase in Non-Small Cell Lung Cancer Cells

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ABSTRACT

Vorinostat (suberoylanilide hydroxamic acid), a class of histone deacetylase inhibitors, represents an emerging class of anticancer agents currently progressing in clinical trials. It causes cell growth inhibition, differentiation, and apoptosis of many tumor types in vitro and in vivo. Recently, it was reported that hTERT is one of the targets for cancer therapy in cancer cells. Telomerase repeat amplification protocol assay was used to analyze the expression of hTERT after vorinostat treatment in the A549 lung cancer cells. Vorinostat inhibited telomerase activity by reducing the expression of human telomerase reverse transcriptase (hTERT) in A549 human lung cancer cells. The epigenetic regulation mechanism is responsible for the repression of hTERT by vorinostat, analyzed through the methylation-specific PCR and bisulfite sequencing of the hTERT promoter. Vorinostat induced the demethylation of site-specific CpGs on the promoter region of hTERT, which was caused by the down-regulation of DNA methyltransferases. DNA methyltransferases (DNMT1 and DNMT3b) were also decreased in vorinostat-treated A549 cancer cells. Furthermore, chromatin immunoprecipitation analysis of the hTERT promoter revealed that vorinostat decreased the level of inactive chromatin markers dimethyl-H3K9, and the declined binding of DNMT1 and DNMT3b were associated. The novel insights showed that vorinostat down-regulated telomerase via epigenetic alteration in lung cancer to vorinostat-mediated cancer-specific therapies. J. Cell. Biochem. 112: 3044–3053, 2011.

KEY WORDS: VORINOSTAT; LUNG CANCER; TELOMERASE ACTIVITY; HTERT; EPIGENETIC REGULATION

The turnover of histone acetylation is controlled by the opposing enzymatic activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs) [Marks et al., 2001]. Epigenetic changes can be reversed by small molecules, like HDAC inhibitors (HDACIs), which have been shown to induce cell-cycle arrest, terminal differentiation, and apoptosis in numerous tumor-derived cell lines, as well as inhibit tumor growth [Johnstone et al., 2002]. Several HDACi are in various stages of development, including clinical trials as monotherapy and in combination with other anti-cancer drugs and radiation [Marks and Xu, 2009]. A number of structurally divergent classes of HDAC inhibitors have been identified, including (1) short chain fatty acids, (for example, butyrates) (2) hydroxamic acids, [for example, trichostatin A (TSA) and vorinostat (also known as SAHA, suberoylanilide hydroxamic acid)] (3) cyclic tetrapeptides, and (4) benzamides [Bouchain and Delorme, 2003; Curtin and Glaser, 2003]. In recent reports, HDAC

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inhibitors were Sp1-mediated for transcriptional regulation to up-regulate histone H3 lysine 4 (H3K4) methylation and then to functionally regulate histone modifications [Huang et al., 2010]. HDAC inhibitors were associated with the inhibition of DNA methyltransferase (DNMT) which enhances apoptosis [Zhu et al., 2001]. One of HDAC inhibitors, TSA, was associated with a significant decrease in global methylation by increasing histone acetylation, which occurs even when DNA replication is blocked by hydroxyurea, and thus supporting the replication-independent mechanism of demethylation [Ou et al., 2007]. Another of HDAC inhibitors, scriptaid, resulted in the modifications of core histone tails that are important in euchromatin structure by increasing acetyl-H3-K9 and dimethyl-H3-K4 and decreasing dimethyl-H3-K9 on p16 promoter [Lee et al., 2008]. Therefore, the correlation between vorinostat and the regulation of DNA methylation on the promoter has not yet been elucidated.

Telomerase is reactivated in a cancer cell-specific manner but not in normal somatic cells, and contributes to the immortality of cancer cells by maintaining the lengths of the ends of chromosomes [Kyo et al., 2008]. The human telomerase reverse transcriptase (hTERT) gene, which encodes the catalytic subunit of telomerase holoenzyme, is a promising candidate for cancer therapeutics and an important biomarker for the diagnosis of malignancy [Komata et al., 2002; Wu et al., 2003; Kyo et al., 2008]. The hTERT promoter is regulated by dynamic processes through histone acetylation and promoter methylation. Because the hypermethylation of the hTERT promoter was found exclusively in hTERT-expressing telomerase-positive samples and was absent in telomerase-negative samples, they confirmed that the hTERT promoter methylation truly occurred in tumor cells and methylation is involved in the regulation of hTERT gene expression [Bechter et al., 2002; Guilleret et al., 2002]. The activation of hTERT is related to DNA methylation of the promoter region, in contrast to most known tumor suppressor genes which were down-regulation by DNA promoter methylation [Guilleret and Benhattar, 2003]. However, it remains incompletely understood how the repression or activation of the hTERT gene in normal cells. In previously study, TSA, an inhibitor of HDAC, can down-regulation of DNMT1 and cause demethylation of a CTCF-binding site on the hTERT promoter. And TSA represses the expression of hTERT via recruitment of CTCF to the promoter [Choi et al., 2010].

The hTERT promoter region is embraced in a CpG island (positions −1,100 to +1,500), and this region is mostly hypermethylated through specific DNMTs in cancer cells [Renaud et al., 2007]. In addition, it was demonstrated that the DNA methylation patterns of the hTERT promoter (−150 to +150 around the transcription start) are consistent with the usual dynamics of gene expression in that the absence of methylation in this region and the association with active chromatin marks allow for the continued expression of hTERT [Zinn et al., 2007]. In addition, the aberrant methylation pattern in the hTERT 5′-regulatory region prevents the binding of the methylation-sensitive CTCF repressor to the first exon of hTERT, but partial hypomethylation of the core promoter is necessary for hTERT expression [Renaud et al., 2007]. This hTERT gene is the opposite of the general model of gene regulation, in which the presence of methylated cytosines in a promoter typically inhibits gene transcription.

Vorinostat was found to have antiproliferative properties by causing the varied cancer cells to undergo cell cycle arrest, apoptosis, and differentiation [Munster et al., 2001; Arnold et al., 2007]. Likewise, the combination of vorinostat with other chemotherapeutic agents possessed synergistic cytotoxicity properties against human cancer cells [Fedier et al., 2007; Witta et al., 2009]. We evaluated the changes in hTERT expression and explored DNA methylation of the hTERT promoter as one of the possible mechanisms of regulation of hTERT expression by vorinostat. Our results showed that vorinostat repressed telomerase activity through decreasing expression of hTERT mRNA in A549 lung cancer lines in a time- and dose-dependent manner. Meanwhile, vorinostat induced demethylation of CpGs on the hTERT promoter via the down-regulation of DNA methyltransferase 1 (DNMT1) and DNA methyltransferase 3b (DNMT3b) to inhibit the hTERT transcription. Vorinostat modified chromatin structure by the down-regulation of chromatin markers dimethyl-H3K9. Taken together, our data provide evidence for a reversible crosstalk between histone acetylation and DNA demethylation. These findings suggest that demethylation of CpGs can epigenetically regulate the expression of tumor-related genes by vorinostat and may provide significant implications on how to improve cancer-specific therapeutic strategies.

MATERIALS AND METHODS

CELL LINES AND CELL CULTURE

A549 human lung adenocarcinoma cells, WI-38 human normal lung fibroblasts, and BEAS-2B human bronchial epithelium cells were obtained from the American Type Culture Collection. A549 and WI-38 were maintained at 37°C in a 5% CO2-humidified atmosphere on Dulbecco's modified Eagle's medium (DMEM) and Minimum Essential Eagle's Medium (MEM) containing 10% fetal bovine serum (FBS) and 100 ng/ml each of penicillin and streptomycin. BEAS-2B was maintained in an LHC-9 medium (Life Technologies, Inc.).

REAGENTS

Penicillin, streptomycin, Minimum Essential Eagle's Medium (MEM), and Dulbecco's modified Eagle's medium were obtained from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was supplied by Gibco (Carlsbad, CA).

TOTAL RNA EXTRACTION, RT-PCR ANALYSIS, AND QUANTITATIVE RT-PCR

Total RNA was isolated from A549 cells in 6-cm culture dishes treated with vorinostat in the presence of RareRNA (Genepure Technology, Taiwan) for the indicated time, according to the protocol of the manufacturer. RNA concentration was spectrophotometrically determined at 260 nm. First-strand cDNA synthesis was performed with 2 μg of total RNA using random hexamers as primers in a final volume of 20 μl (100 ng/μl random hexamers, 1 mM dNTPs, 1 U/μl RNasin®, and 5 U/μl Moloney murine leukemia virus reverse transcriptase). The reaction was carried out at 42°C for 90 min. cDNAs encoding β-actin, DNMT1, DNMT3b, and hTERT were amplified from 1 μl of the cDNA reaction mixture using

specific gene primers. Oligonucleotide primers for β-actin, DNMT1, DNMT3b, and hTERT were as follows: β-actin:5′-TCATCAC-CATTGGCAATGAG-3′ (sense), 5′-CACTGTGTTGGCGT ACAGGT-3′ (antisense); hTERT: 5′-AGTTCCTGCACTGGCTGATGAG-T-3′ (sense), 5′-CTCGGCCCTCTTTTCTCTGCG-3′ (antisense); DNMT1:5′-CCCCT -GAGCCCTACCGAAT-3′ (sense), 5′-CTCGCTGGAGTGGACTTGTG-3′ (antisense); DNMT3b: 5′-TCG ACCTCACAGACGACACA-3′ (sense), 5′-CAC -GACGCACCTTCGACTTAT-3′ (antisense). DNA amplification was achieved by annealing at 60°C for hTERT, and at 55°C for β-actin. hTERT and β-actin sequences were amplified for 32 and 28 cycles, respectively. The PCR protocol for DNMT-1 and DNMT-3b entailed 5 min at 95°C; 32 cycles of 45 s at 95°C, 45 s at 55°C, and 45 s at 72°C; with 10 min final extension at 72°C. The PCR products were analyzed on 1.5% agarose 0.5× TBE gel containing ethidium bromide and their sizes were compared to a molecular weight marker. Real-time PCR was performed in triple replicates of each sample using TaqMan primer probes (assays on demand; Applied Biosystems, Foster City, CA) with a total reaction volume of 20 μl. It contained 10 μl 2× TaqMan Universal PCR Master Mix, 10 or 100 ng cDNA template (diluted in 9 μl DNase-free H2O), and 1 μl 20× Assays-on-Demand Gene Expression Assay Mix. After 2 min at 50°C and 10 min at 95°C, 40 cycles of 15 s at 95°C, and 1 min at 60°C were run and data acquisition followed each cycle. Reactions were performed with the Mthe ABI Prism 7700 Sequence Detection System (Applied Biosystems) and CT values of each sample were determined. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as positive control and normalizer. TaqMan assays had the following identification numbers and reporter sequences: hTERT, Hs00162669_m1; DNMT3b, Hs00171876_m1; DNMT1, Hs00945899_m1; and GAPDH, Hs99999905_m1

ASSAY FOR TELOMERASE ACTIVITY

Pelleted cells were lysed with 100 μl of 1× CHAPS lysis buffer [10 mM Tris–HCl (pH 7.5), 1 mM EGTA, 0.5% CHAPS, 10% (v/v) glycerol, 5 mM β-2-mercaptoethanol, and 0.1 mM phenylmethyl-sulfonyl fluoride)], incubated on ice for 30 min and centrifuged (13,000 g, 4°C, 30 min). Supernatant extracts were quantified for protein with a BSA Protein Assay Kit (Pierce, IL). Assay for telomerase activity was measured with the telomerase repeat amplification protocol (TRAP) assay. TRAP assay was performed as previously described [Yan et al., 1999] with only minor modifications, with a set of primers (TS,5′-AATCCGTCGAGCAGAGTT-3′; ACX,5′-GCGCGGCTTACCCTTACCCTTACCCTAACC-3′; NT,5′-ATC-GCTTCTCGGCCTTTT-3′), and an internal standard, TSNT (5′-AAT-CCGTCGAGCAGAGTTAAAAGGCCGAGAAGC GAT-3′). Reaction mixtures were incubated (25°C, 30 min) for telomerase-mediated extension and the samples were heated to 85°C (10 min). Taq polymerase was added and each sample was amplified by 29 cycles of polymerase chain reaction (PCR) amplification (94°C for 30 s, 60°C for 30 s, and 72°C for 90 s) in a DNA thermal cycler (GeneAmp PCR System 2400, PerkinElmer Co., Norwalk, CT). TRAP products were resolved by 12.5% (w/v) non-denaturing polyacrylamide gel electrophoresis (PAGE) and visualized by staining with ethidium bromide. The activity of each sample was normalized to that of 50 ng of total cellular protein. The signal intensity in each lane was measured by the area integration of the first six ladders from the bottom of the gel with MultiImageTM (Alpha Innotech Corporation). Relative telomerase activities were quantified by comparing signal intensities among lanes and with the positive control (extract of untreated cells) as 100%.

BISULFITE SEQUENCING

Genomic DNA was isolated from cells using GenoMarker (Genepure Technology) following the manufacturer's instructions. Bisulfite modification was done as described previously [Herman et al., 1996]. Briefly, 4 μg of genomic DNA was denatured by NaOH (final concentration, 0.2 mol/L) for 10 min at 37°C. Hydroquinone (10 mmol/L, 30 μl) and 520 μl of 3 mol/L sodium hydroxide (pH 5.0) were added, and samples were incubated at 50°C for 16 h. Modified DNA was purified using Wizard DNA Clean-Up System following the manufacturer's instructions (Promega) and eluted into 100 μl water. DNA was treated with NaOH (final concentration, 0.3 mol/L) for 15 min at room temperature, ethanol precipitated, and resuspended in 20 μl water. The modified DNAs were used immediately or stored at −20°C. The modified DNAs were then amplified by PCR using a 5′-AGTGGATTCGCGGGTATAGAC-3′ and 5′-GCCGCACGAACGT AACCAAC-3′ primer set. Amplification for PCR was performed using Pro Taq polymerase (Protech Technology, Taiwan) and the following PCR conditions: 40 cycles of 95°C for 30 s, 55°C for 45 s and 72°C for 60 s. Following PCR amplification, purified PCR products were then cloned using a T&A cloning kit according to the manufacturer's instructions (Yeastern Biotech Corp., Taipei, Taiwan). Plasmid DNA was isolated using a plasmid miniprep kit according to the manufacturer's instructions (Protech Technology). Plasmid DNAs were sequenced using the M13 universal reverse primer.

METHYLATION-SPECIFIC PCR AND QUANTITATIVE PCR

Primers specific for unmethylated and methylated alleles of hTERT were used to amplify bisulfite-modified DNA. Methylation-specific PCR (MSP) primers were designed as described previously about the hTERT promoter [Zinn et al., 2007]. PCR was done using 2 μl of bisulfite-treated DNA and Pro Taq polymerase (Protech Technology). PCR products were analyzed by 3% agarose. For methylation-specific quantitative PCR the IQ^2 FAST EVA Green QPCR Kit (biogenesis Technology, Taiwan) and primers specific for fully methylated hTERT promoter sequences were taken [Zinn et al., 2007]. For each 25 μl PCR, 2 μl eluate containing the bisulfite-converted DNA, 12.5 μl 2× IQ^2 FAST EVA Green QPCR Master Mix, 0.75 μl each forward and reverse primer (concentrations 10 μM resulting in final concentrations of 0.3 μM), and 9 μl DNase-free water were used. To correct cycles of threshold (CT) values for input of DNA, a primer pair corresponding to a specific β-actin sequence was chosen [Eads et al., 2000]. Real-time PCR conditions were 95°C for 3 min followed by 45 cycles of 94°C for 15 s, 61°C for 30 s, 72°C for 30 s with data acquisition after each cycle. At the end, properties of real-time PCR conditions and amplification products were checked by melting curve analysis: 95°C for 1 min, 60°C for 1 min, followed by 80 cycles with increasing incubation temperature for 10 s, starting at 60°C, and ending at 95°C (increment 0.5°C) with data acquisition after each cycle. PCRs were done in triple replicates of each sample by the laser detector of the ABI Prism 7000

Sequence Detection System and the CT values of each sample were determined.

CHROMATIN IMMUNOPRECIPITATION

A549 were cultured in complete medium until 60% confluent (1×10^6 cells/100 mm dish) and vorinostat were added for 48 h. The ChIP assay protocol was carried out following the manufacturer's protocol (chromatin immunoprecipitation assay kit (ChIP), catalog no. 17–295; Upstate Biotechnology Inc., Lake Placid, NY). Briefly, 100 μg chromatin per antibody was resuspended in SDS lysis buffer and sonicated (Branson Sonifier, Danbury, CT) with twenty 10-s pulses to shear DNA to 200–800 bp. Antibodies for dimethyl-H3K9, Sp1, and total histone H3 were purchased from Upstate and used to capture protein–DNA complexes. Antibodies for DNMT3b and DNMT1 were purchased from Cell Signaling Technology (Danvers, MA) and (Genetex, San Antonio, TX). ChIP PCR analysis was done by using 5 μl of ChIP DNA and primers spanning the region of hTERT promoter. Purified DNA was analyzed by PCR with specific primers for the amplification of either the first exon of the hTERT gene to generate a 144 bp fragment (Chip-F 5′-AGCCCCTCCCCTTCCTTTCC-3′ and Chip-R 5′-AGCGCACGGCTCGG CAGC-3′) [Zinn et al., 2007]. The location of chip primer contains the region of the first exon the hTERT minimal promoter and from −68 to +76. PCR products were analyzed on 2% agarose gel [Zinn et al., 2007].

STATISTICAL ANALYSIS

Results are reported as means ± SD. Statistical analysis was carried out using Bonferroni/Dunn test. A value of $P < 0.05$ was considered statistically significant.

Results

VORINOSTAT REPRESSED TELOMERASE ACTIVITY AND HTERT MRNA EXPRESSION IN A549 LUNG CANCER CELLS

The cancer cells also express elevated levels of telomerase, which allows these cells to survive, proliferate, and bypass cellular senescence. To evaluate the role of vorinostat in the regulation of telomerase activity in A549 lung cancer cells, cells were cultured in dose- and time-dependent manner on vorinostat. Compared to untreated cells, the telomerase activity of A549 cells was significantly repressed after treatment with vorinostat in a dose-dependent (1.25, 2.5, 5, and 10 μM for 48 h; Fig. 1A,B) and time-dependent (5 μM for 0–5 days) manner (Fig. 1C). To explore the repression pattern of telomerase activity by vorinostat treatment in A549 cells, we performed semi-quantitative RT-PCR amplification of hTERT mRNA. RT-PCR and Q-PCR results showed that hTERT mRNA expression in A549 cells was significantly decreased in a dose- and time-dependent manner upon vorinostat stimulation (Fig. 2A–C).

ALTERATION OF METHYLATION STATUS ON THE HTERT PROMOTER IN A549 CELLS BY VORINOSTAT

Previous studies have shown that hTERT is epigenetically regulated and the chromatin structure via the DNA methylation or modulation of nucleosome histones are important for the regulation of the hTERT promoter [Kyo et al., 2008]. It is well established that vorinostat is a kind of HDAC inhibitor, which is one of the contributing factors for histone acetylation [Huang et al., 2007]. Whether vorinostat induces DNA demethylation via a reversible crosstalk with histone acetylation is unclear, especially in terms of which mechanisms are involved. To confirm the mechanism by which DNA methylation in the promoter region of hTERT affects its expression, we next investigated whether vorinostat could influence

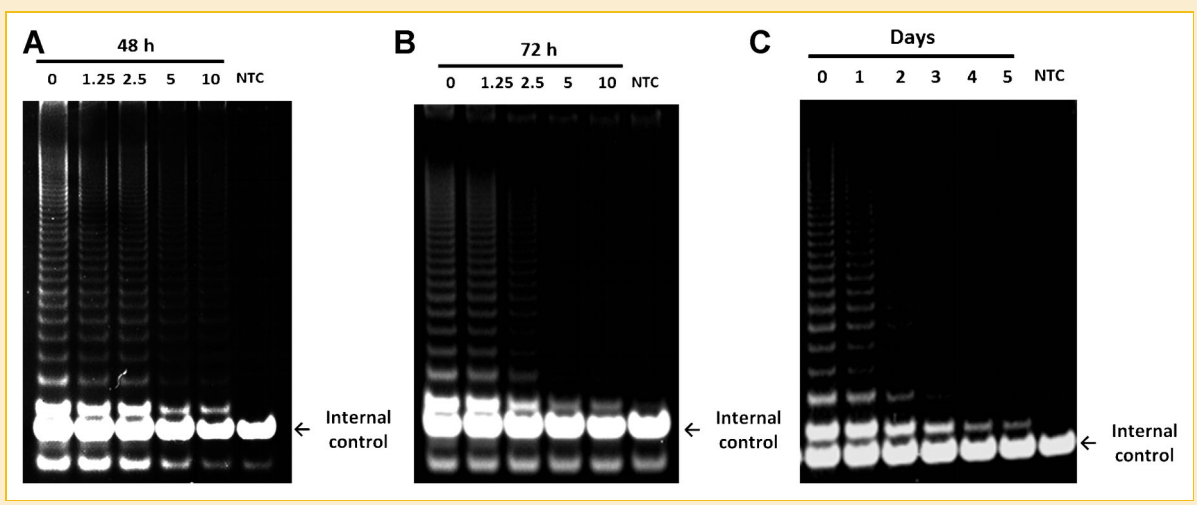

Fig. 1. Evaluation of the effect of vorinostat on telomerase activity in A549 cells in dose- and time-dependent manner. A549 cells were treated with varying concentrations (0, 1.25, 2.5, 5, and 10 μM) of vorinostat for (A) 48 h, (B) 72 h, and (C) the indicated times (0, 1, 2, 3, 4, and 5 days) with 5 μM vorinostat. Telomerase activity was detected on TRAP assays and the 36-base pair internal standard was used as a control. The data are representative of three independent experiments. NC (negative control, right lane): no telomerase extract was added.

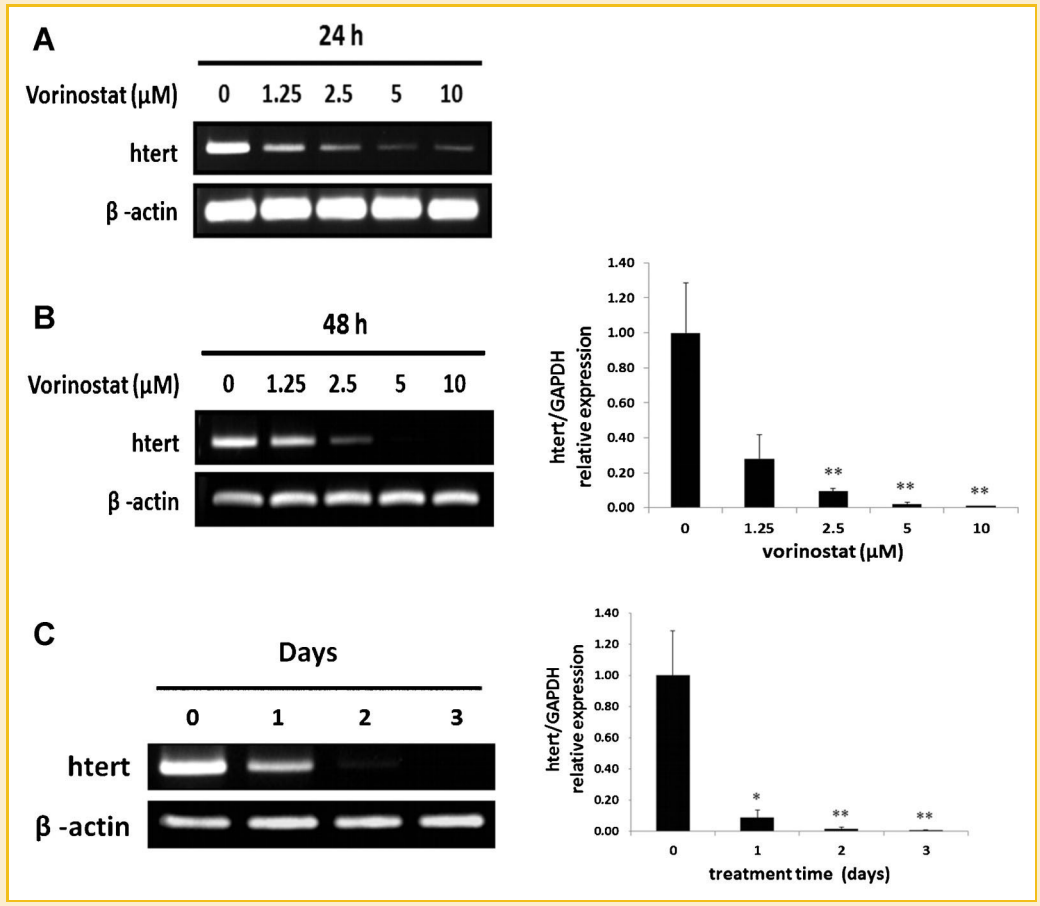

Fig. 2. Inhibition of hTERT mRNA expression in A549 cells by vorinostat in dose- and time-dependent manner. hTERT mRNA expression was measured by semiquantitative RT-PCR and Q-PCR analysis from A549 cells treated with varying concentrations (0, 1.25, 2.5, 5, and 10 μM) of vorinostat in (A) 24 h, (B) 48 h, and (C) the indicated times (0, 1, and 2 days) with 5 μM vorinostat. β-actin mRNA from the same cDNA were amplified as control. The data are presented as mean ± SD of triplicate experiments. $^{*}P < 0.05$. $^{**}P < 0.005$.

the methylation status of the hTERT promoter. In earlier findings, hTERT promoter had demonstrated that methylation status truly occurred in tumor cells and methylation of hTERT promoter sequence has been involved in the regulation of the hTERT gene expression [Guilleret et al., 2002]. For an initial assessment of the methylation status of the hTERT promoter region, we used the MSP assay to analyze A549 cells after vorinostat treatment. In our study, we treated A549 cells with vorinostat for 48 h to understand the methylation status of the hTERT promoter region. In Figure 3A, A549cells were not changed in the region 600 bp upstream of the transcription start site using MSP up primer set, vorinostat-treated or not. On the other hand, the region around the transcription start site (MSP down primer set) was changed from having a methylated status to an unmethylated status in a dose-dependent manner (Fig. 3A). In the Q-PCR, in order to demonstrate this correlation, we also used the demethylating agent 5aza-2′-deoxycytidine (5azadC) to confirm our experiments (Fig. 3A). The results are also show in the Q-MSP assay on the Down of hTERT promoter (Fig. 3B). The results had a similar pattern in a time-dependent manner (Fig. 3C).

To study the methylation patterns in more detail, we used bisulfite sequencing analysis to confirm the hTERT promoter region (from −203 to +106) in vornostat-treated cells. A total of 41 CpG sites containing many transcription factor-binding sites were analyzed for site-specific methylation status (Fig. 4A). In control (non-vorinostat-treated) A549 cells, the overall pattern of CpG methylation is in a very intense hypermethylated state around the promoter region (1st–10th CpGs) and the transcription start site (17th–26th CpGs) of hTERT. Conversely, the CpGs at the translation start site (28th–34th CpGs) tended to be hypomethylated (Fig. 4B). However, putative CTCF-binding sites were partially methylated in untreated A549 cells. In our data, the transcription start site in the hTERT regulatory region underwent dramatic demethylation with different doses of vorinostat treatment. In particular, the CpG island in the region contained the binding sites of several factors such as SP1 and DNMT. The vorinostat treatment of A549 cells also had a dramatic demethylation effect near the translation start site (35th–40th CpGs) of the hTERT regulatory region.

DECREASING METHYLATION LEVEL ON HTERT PROMOTER THROUGH VORINOSTAT-MEDIATED DNMT1 AND DNMT3B DOWN-REGULATION

DNA 5′-cytosine-methyltransferases (DNMTs), which are enzymes that methylate the cytosine residue of CpGs, are involved in many

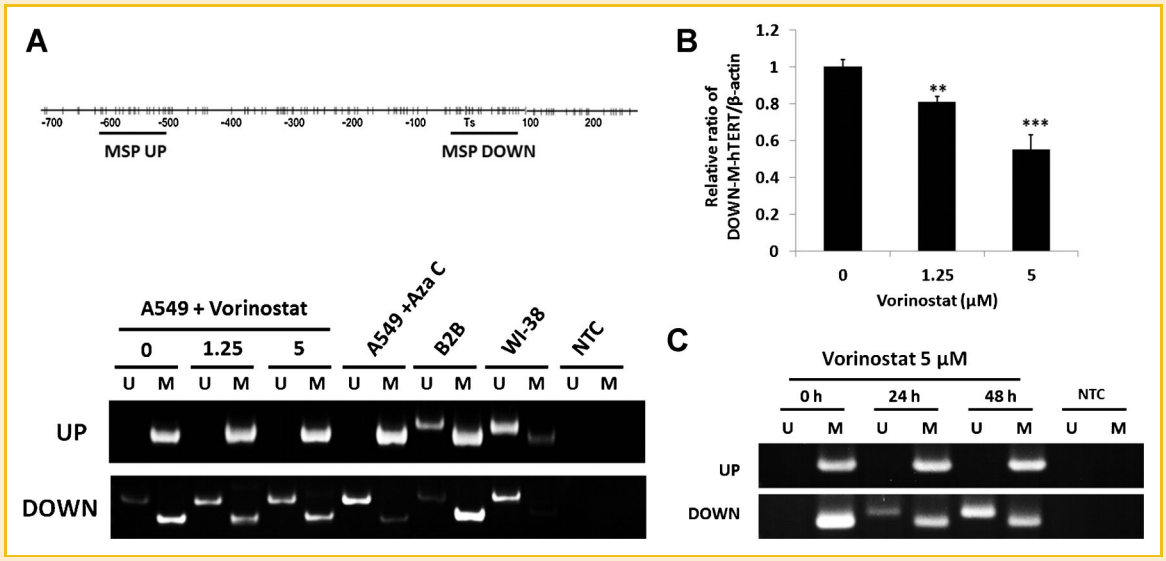

Fig. 3. Vorinostat induced methylation status exchange on the hTERT promoter regions in A549 lung cancer cells. A: MSP results for vorinostat-treated A549 cells in hTERT promoter. MSP analysis of hTERT promoters A549 cells treated with indicated dose of vorinostat for 48 h. Normal fibroblast (WI-38) and human bronchial epithelium (Beas-2B) are controls. Primers and annealing temperature are listed in Materials and Methods section. PCR products were resolved in 3% agarose gel and stained with ethidium bromide. Results using the MSP up primer set; bottom, results using the MSP down primer set. U, unmethylated DNA; M, methylated DNA. B: MSP results for vorinostat-treated A549 cells in hTERT promoter with 5 μM vorinostat for the (0, 1, and 2 days). The data are presented as mean ± SD of triplicate experiments. $^{**}P < 0.005$. $^{***}P < 0.001$.

kinds of cancer. Several DNMTs have been identified, including DNMT3a, DNMT3b, and DNMT1 [Ting et al., 2006]. Of these, DNMT1, belongs to a maintenance methyltransferase and attaches a methyl group on hemimethylated DNA during replication. DNMT3a and DNMT3b exhibit de novo activity and they participate in the biosynthesis of methyl cytosine in CpG. To further understand the epigenetic modulations that occurred in the hTERT promoter region, we assessed epigenetic-related enzymatic expression of DNMTs (DNMT1 and DNMT3b) in A549 cells with vorinostat treatment. In our study, we discovered that vorinostat can considerably inhibit DNMT1 and DNMT3b expression in a dose- and time-dependent manner. As indicated in Figure 5A, vorinostat in dose inhibited DNMT1 and DNMT3b expression in A549 cells from 100% to 19% and 11% (vorinostat 10 μM). In a time-dependent manner (Fig. 5B), 5 μM vorinostat at third day inhibited DNMT1 and DNMT3b expression from 100% to 25% and 22%, respectively. These results suggested that down-regulation of DNMT1 and DNMT3b in A549 cells by vorinostat associate with the promotion of demethylation of CpGs on the promoter of hTERT, which may be one of the major mechanisms of vorinostat-mediated repression of hTERT in cancer cells.

VORINOSTAT-INDUCED CHROMATIN MODIFICATION OF THE HTERT PROMOTER VIA DOWN-REGULATED BINDING OF DNMT AND UP-REGULATED BINDING OF SP1

Since methylation changes were found at varied compounds in the hTERT regulatory region, we sought to determine changes in histone modification of the hTERT regulatory region by vorinostat-treatment in A549 cells. In Figure 6, we found that vorinostat-induced chromatin alterations such that the facilitation of the enrichment of transcriptional inactive chromatin markers such as dimethyl-H3K9 was decreased. We also observed that the binding of DNMT1 and DNMT3b were decreased by vorinostat. Our results suggest that vorinostat-reduced inactive chromatin markers were caused by the decreased binding of the DNMT1 and DNMT3b on hTERT promoter.

DISCUSSION

Telomerase reactivation occurs preferentially in varied cancers with certain cell cycle regulatory defects and that telomerase activity levels are related to such specific defects [Kageyama et al., 1997; Landberg et al., 1997]. For the high-risk stage I non-small cell lung cancer (NSCLC) patients, telomerase activity is an important prognostic factor that should be considered in future prospective trials of adjuvant therapy [Marchetti et al., 1999]. Telomerase activity can be detected in about 85% of different malignant tumors, but is absent in most normal cells [Abdul-Ghani et al., 2000]. Hypermethylation of the hTERT promoter was found exclusively in hTERT-expressing telomerase-positive samples and was absent in telomerase-negative samples [Guilleret et al., 2002]. In addition, the regulation of hTERT expression through chromatin remodeling including acetylation and methylation-modulated chromatin structure can be accessed through a number of transcription factors, including c-MYC and MAD1 [Meeran et al., 2010]. RBP2, which is a histone demethylase and recruited by Mad1, is responsible for the inhibition of chromatin inactive markers, thereby contributing to a stable repression of hTERT expression [Ge et al., 2010]. Numerous studies have also reported that DNA methylation plays important roles in hTERT transcriptional regulation [Li et al., 2009].

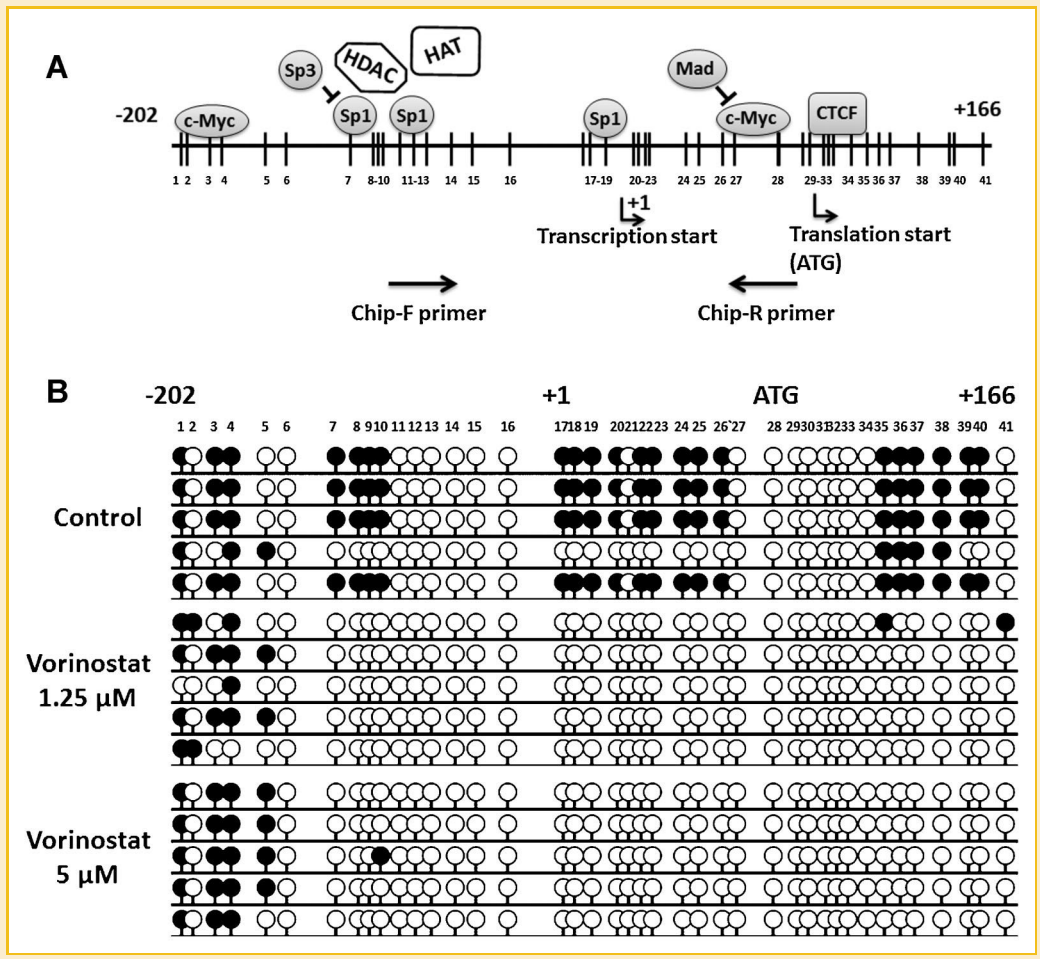

Fig. 4. DNA methylation on hTERT promoter upon vorinostat treatment in A549 cells. A: A total of 41 CpG sites on the promoter and 5′ proximal exon regions of hTERT in A549 cells were analyzed by bisulfate modification sequencing. Each strait on the scale represents a CpG site in the analyzed region. The transcriptional site means as +1. The CpG islands were predicated by the software, CpG Island Searcher. B: Methylation status of the hTERT promoter and 5′ proximal exon (−202 to +166 nucleotide) of lung cancer A549 cells treated with vorinostat (0, 1.25, 5 μM) for 48 h. After PCR amplification of bisulfite-modified DNA and cloning into T-A vector, several clones for each treatment were analyzed by DNA sequencing. Each circle represents one CpG site. Methylation at each CpG site is indicated as follows: Black circle, methylated CpG; white circle, unmethylated CpG.

Inhibition of histone deacetylation at the hTERT promoter did not prevent hTERT repression or nucleosomal deposition after HDAC inhibitor treatment [Wang et al., 2010]. Several other compounds that have HDAC inhibition activity, such as sulforaphane, can mediate epigenetic down-regulation of telomerase in breast cancer [Meeran et al., 2011]. Genistein [Li et al., 2009], a natural isoflavone found in soybean products, has been reported to down-regulate telomerase activity through three major DNMT1, 3a, and 3b and that this prevents cancer and contributes to the apoptosis of cancer cells [Li et al., 2009]. Several reagents including differentiation-inducing and antineoplastic agents are known to inhibit telomerase activity. Demethylating reagents, including 5-azacytidine (5-aza-CR), have recently been used as potential antineoplastic drugs for some types of cancers [Kitagawa et al., 2000] and these can also reduce telomerase activity through epigenetic regulation. (−)-epigallocatechin-3-gallate (EGCG) and (−)-epigallocatechin (EGC), which belong to tea polyphenols, have inhibitive effects on the repression of hTERT transcription that might be linked to the inhibition of carcinoma cell growth [Lin et al., 2006].

Trichostatin A (TSA), a HDAC inhibitor, derepresses the telomerase reverse transcriptase (hTERT) gene by physical interaction of Sp1 with HDAC1 [Hou et al., 2002]. In the previous study, TSA repressed hTERT expression by recruiting CTCF into the demethylated core promoter region of hTERT by inducing DNMT1 down-regulation in HCT116 cells [Choi et al., 2010]. In our present study, the CTCF-binding site is partially methylated in untreated A549 cells. Vorinostat repressed hTERT expression may recruit Sp1/Sp3 and interfere with DNMT binding. The dual activity of Sp1 as an activator or as a repressor may be dependent on cell type and cell species. Although the Sp1 transcription factor has been identified as a potential positive activator of the hTERT transcription [Kyo et al., 2000], Sp1 and Sp3 recruit HDAC to repress the transcription of human telomerase reverse transcriptase (hTERT) in human normal

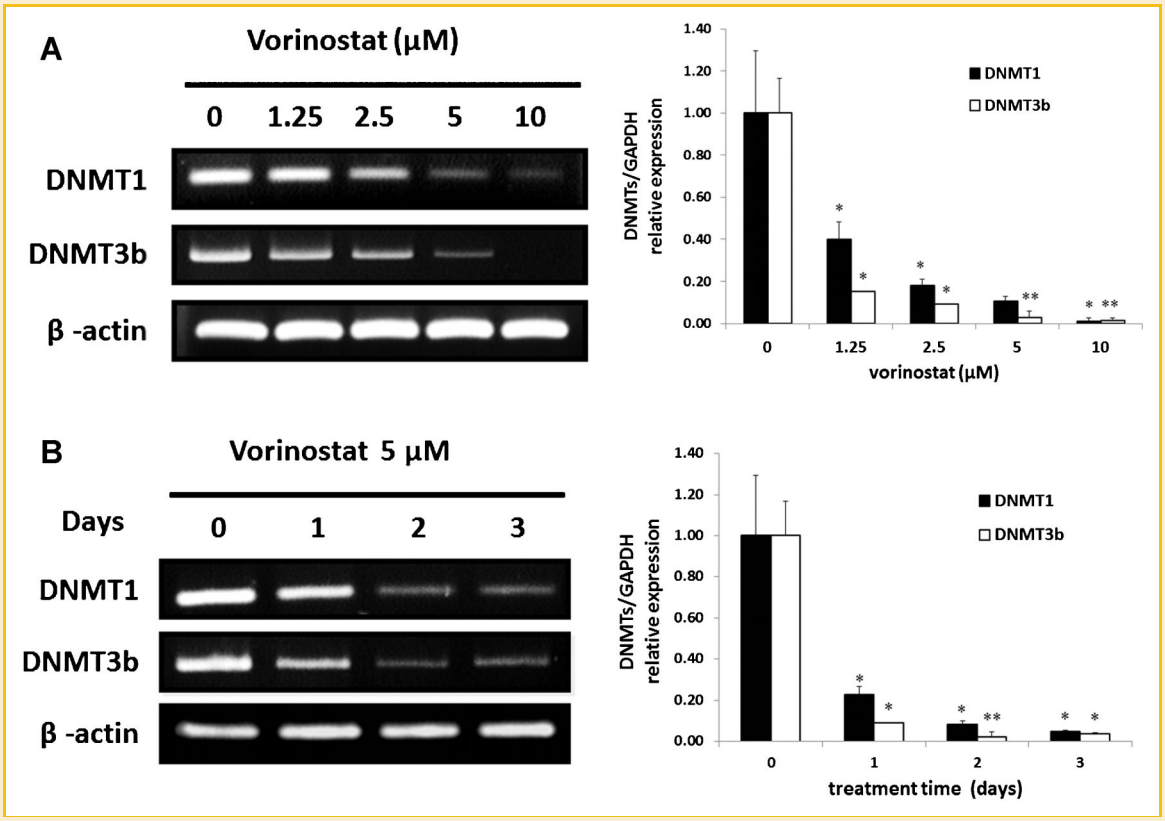

Fig. 5. Inhibition of DNMT1 and DNMT3b mRNA expression in A549 cells by vorinostat in dose- and time-dependent manner. DNMT1 and DNMT3b mRNA expressions were measured by semiquantitative RT-PCR analysis from A549 cells treated with (A) varying doses (0, 1.25, 2.5, 5, and 10 μM) for 48 h and (B) days (0, 1, 2, and 3) of vorinostat. β-actin mRNA from the same cDNA were amplified as a control. The data are presented as mean ± SD of triplicate experiments. $^{*}P < 0.05$. $^{**}P < 0.005$.

somatic cells [Won et al., 2002; Hsin et al., 2010]. Nevertheless, regarding the alteration of the methylation status of these specific CpGs by vorinostat, the methylation status may control or recruit the binding of other unknown regulatory proteins to CpGs.

Recently, several chemotherapeutic agents are currently being used to treat tumors to regulate cell growth and promote apoptosis. One of them is HDACIs, including Trichostatin A (TSA) and vorinostat (SAHA; Zolinza^{TM}), which regulate tumor suppressor genes that promote cell cycle arrest (e.g., p21) and/or establish competency for apoptosis [Choi, 2005; Komatsu et al., 2006]. Vorinostat is an oral HDACI and is in phase trials in varied tumor cell. The earliest vorinostat clinical trials are those in phases II and III for refractory cutaneous T-cell lymphoma (CTCL) [Duvic et al., 2007]. In recent years, vorinostat was assessed as a therapy with chemotherapeutic or targeted agents in metastatic breast cancer [Luu et al., 2008]. In addition, there is a phase I/II trial of vorinostat combined with 5-fluorouracil for patients with metastatic colorectal cancer who previously failed 5-FU-based chemotherapy [Wilson et al., 2010]. In non-small cell lung cancer cells, vorinostat enhances the antitumor effect in combination with carboplatin and paclitaxel [Owonikoko et al., 2010]. Therefore, it is unclear which molecular mechanisms are involved in vorinostat that reduces cancer cell viability. In our study, we associated vorinostat with having HDAC inhibition activity and demethylated the hTERT control region in the process of anti-carcinogenesis.

Another important discovery of this study is that vorinostat reduced the binding activities of DNMTs (DNMT1 and DNMT3b) in human lung cancer cells. DNMTs catalyze the methylation of genomic DNA. Levels of DNMT1 protein increased significantly during carcinogen exposure and were associated with the detection of promoter hypermethylation of genes [Damiani et al., 2008]. TSA can induce DNMT1 down-regulation to repress hTERT expression via recruiting CTCF into demethylated core promoter region of hTERT in HCT116 cells [Choi et al., 2010]. DNMT1 or DNMT3b knockout reduced dimethylated lysine-9 (diMe-H3K9) levels on special cancer/germline antigen genes [James et al., 2006]. Convincingly, we found that voriostat can decrease dimethylated lysine-9 (diMe-H3K9) levels and down-regulating DNMTs.

In the present study, we demonstrated that vorinostat induced the down-regulation of telomerase in lung cancer cells via epigenetic mechanisms such as demethylation at the first exon of hTERT to repress hTERT expression. It is important to point out that hTERT gene control is unique and the proposed mode of action is not the only way that HDACI inhibits acetylation in cancer cells. Vorinostat also can play a role in demethylation.

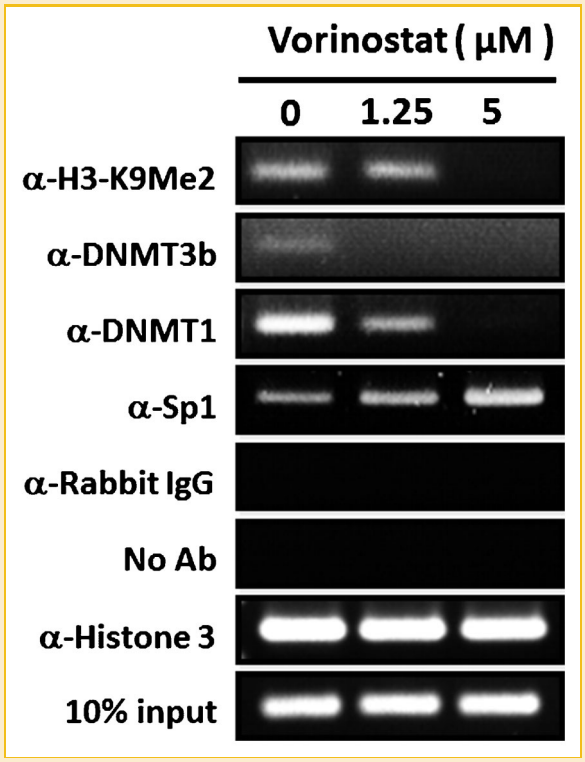

Fig. 6. Effect of vorinostat on dimethyl H3K9, DNMT3b, and DNMT1 binding activities in A549 cells by chromatin immunoprecipitation assay. A549 cells were incubated with the indicated concentrations of vorinostat for 48 h, and then cross-linked chromatin was prepared and immunoprecipitated with or without antibody (Ab) before amplification of the hTERT gene region containing the methylation related site. Immunoprecipitates were analyzed by PCR for the presence of the hTERT gene promoter sequence using the primer pair described in Materials and Methods section. The DNA purified from the sonicated chromatin was directly analyzed by PCR using the same primer set, which was used as an input control (input) and rabbit IgG as negative controls. The figure shows the representative results from three independent experiments.

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