

DNA demethylation is superior to histone acetylation for reactivating cancer-associated genes in ovarian cancer cells

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Abstract. To investigate the contributions of histone H3 lysine 9 acetylation and DNA methylation to p16, hMLH1 and MGMT silencing in ovarian cancer cells, we treated three ovarian cancer cell lines with Trichostatin A (TSA) and 5-aza-2'-deoxycytidine and examined the status of mRNA expression, DNA methylation and histone H3 lysine 9 acetylation at the promoter of p16, hMLH1 and MGMT. The results showed that the hypermethylated silenced tumor-related genes in the ovarian cancer cells were characterized by hypoacetylated histone H3 lysine 9. Treatment with TSA resulted in the increase of histone H3 lysine 9 acetylation at the hypermethylated promoter, but with little effects on gene expression. TSA did not contribute to DNA demethylation. 5-Aza-2'-deoxycytidine treatment caused DNA demethylation, increased histone H3 lysine 9 acetylation at the hypermethylated promoter and resulted in reactivation of p16, hMLH1 and MGMT. Combined treatments synergistically increased histone H3 lysine 9 acetylation accompanied by the re-expression of the hypermethylated genes. To conclude, in ovarian cancer cells, DNA methylation and histone deacetylation act synergistically for the silencing of cancer-associated genes. DNA demethylation is superior to histone acetylation for reactivating cancer-associated genes.

Introduction

Ovarian cancer is the most lethal malignancy of the female reproductive tract, and its survival rate remains much worse than the 61.5% overall cancer survival rate for women (1). Hence, novel approaches to understanding the etiology of ovarian cancer are warranted. Inactivation of genes crucial for

control of normal cell growth is a hallmark of cancer cells (2). Silencing of cancer-associated genes by allelic loss and somatic mutation has been reported in ovarian cancer, but these factors do not seem to be the main mechanism of silencing (3,4).

Epigenetic silencing is the major alternative to accomplish cancer-associated gene inactivation (5). These epigenetic mechanisms mainly include crosstalk between DNA methylation and histone modification. Methylation of CpG islands has been associated with the silencing of a growing number of cancer-associated genes in numerous human cancers and has been proposed as the most common mechanism for gene regulation in cancer (6,7). Methylation of a variety of cancer-associated genes has been reported in ovarian cancers (8-10).

Apart from DNA methylation, histone acetylation plays an important role in transcriptional regulation of a number of cancer-associated genes (11,12). Different results have been reported in regards to the function of histone acetylation on gene expression. In *Neurospora crassa* and *Brassica napus*, the histone deacetylase inhibitor alone was found to reactivate methylated genes (13,14). In addition, in human cancer, histone deacetylase inhibitors can reactivate several genes, including the estrogen receptor genes, FMR1, ARHI and MGMT (15-17). By contrast, several groups have shown that the DNA demethylating agent, but not the histone deacetylase inhibitor, reactivates the expression of hypermethylated tumor-suppressor genes in human colorectal cancer (18,19).

The aim of this study was to investigate the effects of histone acetylation and DNA methylation on cancer-associated gene silencing and explore the relationship of histone H3 lysine 9 (H3-K9) acetylation and DNA methylation in ovarian cancer. Various genes are frequently hypermethylated and silenced in certain cancer types. They include the tumor-suppressor gene, p16, a mismatch repair gene, human mutL homolog 1 (hMLH1), and a DNA repair gene, O6-alkylguanine-DNA alkyltransferase (MGMT). The expression of these genes was examined following treatment with Trichostatin A (TSA), a histone deacetylase inhibitor and 5-aza-2'-deoxycytidine (5-aza-CdR), a DNA demethylating agent, in ovarian cancer cells. We found that histone deacetylation and DNA methylation act synergistically for the silencing of tumor-related genes. Furthermore, DNA demethylation is superior to histone acetylation for reactivating tumor-related genes.

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Table I. Primers and PCR conditions used in MSP.

Gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Product size (bp)	Annealing temp. °C (No. of PCR cycles)
MGMT	M: GGTCGTTTGTACGTTTCGC	GACCGATACAAACCGAACG	116	60 (3), 58 (4), 56 (5), 54 (23)
	U: GTAGGTTGTTTGTATGTTTGT	AACCAATACAAACCAACA	122	
P16	M: TTATTAGAGGGTGGGGCGGATCGC	GACCCCGAACCGCGACCGTAA	150	55 (35)
	U: TTATTAGAGGGTGGGGTGGATTGT	CAACCCCAAACCACAACCATAA	151	55 (35)
hMLH1	M: GATAGCGATTTTAAACGC	TCTATAAATTACTAAATCTCTTCG	74	52 (35)
	U: AGAGTGGATAGTGATTTTAAATGT	ACTCTATAAATTACTAAATCTCTTCA	115	52 (35)

M, methylated sequence; U, unmethylated sequence.

Table II. Primers and PCR conditions used in RT-PCR.

Gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Product size (bp)	Annealing temp. °C	PCR cycle (no.)
MGMT	CGAAATAAAGCTCCTGGGCA	GAACTCTTCGATAGCCTCGGG	151	55	30
P16	CAACGCACCGAATAGTTACGG	GCGCAGTTGGGCTCCG	106	55	38
hMLH1	AGTGGCTGGACAGAGGAAGA	GATCAGGCAGGTTAGCAAGC	463	55	38
GAPDH	TCCCATCACCATCTTCCAG	ATGAGTCCTCCACGATACC	309	55	25

Materials and methods

Cell culture conditions. The human ovarian cancer cell line CaOV3 (20), CoC1 and its DDP-resistant subline, CoC1/DDP (21), was provided by the Oncology Institute of China Medical University. All of the cells were cultured in RPMI-1640 medium (Gibco-BRL, USA) supplemented with 10% fetal bovine serum (Gibco) and incubated in a humidified incubator containing 10% CO₂ at 37°C.

Treatment of cells with 5-aza-CdR and TSA. Cells were cultured for 24 h prior to the following treatments. 5-aza-CdR (1 μM; Sigma, USA) was applied for 72 h. Medium containing 5-aza-CdR was replaced every 24 h. TSA (300 nM; Sigma) was applied for only 24 h. 5-aza-CdR (1 μM) was applied for 48 h followed by TSA (300 nM) for an additional 24 h. Control cells were incubated without 5-aza-CdR or TSA, with replacement of fresh medium on the same schedule as that used for the drug-treated cells.

Methylation-specific polymerase chain reaction (MSP). Genomic DNA from the CaOV3, CoC1 and CoC1/DDP cells, treated as described above, was extracted with phenolchloroform:isoamyl alcohol and collected by ethanol precipitation. A 5-μg sample of genomic DNA was treated with sodium bisulfite (Sigma) as previously described (22). Briefly, DNA was denatured by incubation with 0.3 mol/l NaOH at 37°C for 30 min, followed by incubation with 10 mmol/l hydroquinone (Sigma) and 3 mol/l sodium bisulfite (Sigma) at 55°C for 16-20 h. Modified DNA was purified using a Wizard DNA Clean-Up System according to the manufacturer's protocol (Promega Corp., Madison, WI, USA).

The primers used for MSP are located in the promoter region of the genes. The CpG map of the promoter and the location of primers used in this study were based on a previous study (23). The primers used for MSP and additional PCR conditions are shown in Table I. Methyltransferase (Sss-I)-treated and untreated peripheral blood cell DNA from healthy adults were used as positive and negative controls, respectively. PCR products were separated by electrophoresis on 2% agarose gels. The gels were photographed using a ChemImager 5500 automatic formatter (Alpha Innotech, San Leandro, USA). The experiment was repeated three times.

RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA from the CaOV3, CoC1 and CoC1/DDP cells, treated as described above, was extracted with TRIzol (Invitrogen, Carlsbad, USA) according to the manufacturer's protocol. cDNA was synthesized from 2 μg total RNA using a Reverse Transcription System (Promega Corp.). The primers used for PCR and PCR conditions are shown in Table II. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control. PCR products were resolved on 2% agarose gels and quantitated using the FluorChem 2.0 system. The expression level was determined by quantifying the intensities of the PCR product versus GAPDH. The experiment was repeated three times.

Real-time reverse transcriptase-polymerase chain reaction. Quantitative real-time PCR was carried out under optimized conditions using the following intron-spanning primers and Taqman probe, as shown in Table III. The PCR product was cloned into a pMD18-T vector and confirmed by sequencing. Purified recombinant plasmid DNA was quantified using a UV

Table III. Primers and Taqman probes used in real time RT-PCR.

Gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Taqman probe
MGMT	CTC TTC ACC ATC CCG TTT TC	AGG GCT GCT AAT TGC TGG TA	GAA GGT TGT GAA ATT CGG AGA AGT GAT TTC
P16	GGCTCCTCATTCTCTTCCT	TCAGGTAGCGCTTCGATTCT	CAGAAGGGGTTTGTAAATCACAGACCTCCT
hMLH1	CAGAGGAAGATGGTCCCAA	CAATCAGGTTCCCTTCTCA	TGAGTTTCTGAAGAAGAAGGCTGAGATGC

Table IV. Primers and Taqman probes used in real time ChIP-PCR.

Gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Taqman probe
MGMT	CCCCATCTCCAAATAAGGTCA	CCTAGACACTGCCAGAGCCTG	CTGGTGGACACAATTCAACTCCTAATAACG
P16	GGCTCCTCATTCTCTTCCT	TCAGGTAGCGCTTCGATTCT	CAGAAGGGGTTTGTAAATCACAGACCTCCT
hMLH1	CAGAGGAAGATGGTCCCAA	CAATCAGGTTCCCTTCTCA	TGAGTTTCTGAAGAAGAAGGCTGAGATGC

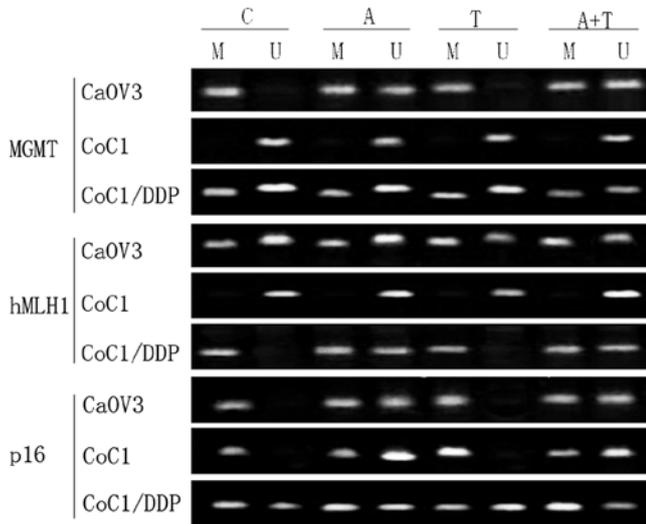


Figure 1. MSP analysis of DNA methylation at the MGMT promoter region before and after treatment of CaOV3, CoC1 and CoC1/DDP cells with 5-aza-CdR (A), TSA (T), or the combination (A+T). Lane M indicates the presence of methylated alleles. Lane U indicates the presence of unmethylated alleles. At least two independent experiments were performed with similar results. C, control.

spectrophotometer and then serially diluted to a final concentration range of 2×10^4 to 2×10^8 copies of genome equivalents/ml. Aliquots ($5 \mu\text{l}$) of each dilution (100 - 10^6 genome equivalents/reaction) were used for real-time PCR to create the standard curve used to quantify the experimental samples. Each sample was amplified in triplicate to obtain average copy numbers. Reactions without cDNA templates were used as a negative control.

Chromatin immunoprecipitation assay (ChIP). ChIP assays were performed as previously described with some modifications (24). Briefly, $\sim 1.75 \times 10^7$ cells, treated as described above, were fixed with 1% formaldehyde at 37°C for 20 min, resuspended in lysis buffer (1% sodium dodecyl sulfate, 10 mmol/l EDTA, 50 mmol/l Tris-HCl, pH 8.1) and sonicated to generate ~ 500 -bp DNA fragments. The main soluble chromatin frac-

tion was immunoprecipitated using an antibody against Lys-9 acetylated histone H3 (Upstate Biotechnology, Lake Placid, NY). The remaining soluble fraction was incubated with normal rabbit IgG (negative control) and used as a DNA input control. The cross-linking between DNA and proteins was reversed by heating the samples at 65°C for 5 h, followed by proteinase K digestion. DNA was then extracted with phenol/chloroform. ChIP experiments were repeated three times.

Real-time PCR analysis of immunoprecipitated DNA. Real-time PCR was performed using immunoprecipitated DNA, a negative control and a DNA input control. The primer probes for the CpG islands within the promoter region and Taqman probe are shown in Table IV. PCR was performed under optimized conditions. The segment of cDNA was cloned into the pMD18-T vectors and confirmed by sequencing. PCR reactions were carried out as described above.

Statistical analysis. The ratio results were expressed as mean \pm standard deviation (SD). Significance between controls and treated samples was calculated by the Student's t-test. Significance between the controls in the different cell lines was calculated using one-way ANOVA and q-tests. Statistical calculations were performed using SPSS version 10.0 (SPSS Inc., Chicago, IL, USA). p-values < 0.05 were considered statistically significant.

Results

Differences in histone H3-K9 acetylation status under different promoter DNA methylation conditions. We investigated the status of DNA methylation and histone H3-K9 acetylation at the promoter region by using MSP and ChIP, respectively. The three cell lines had a characteristic DNA methylation status at the promoter region. As seen in Fig. 1, MGMT was hypermethylated (both alleles are methylated) in the CaOV3 cells, partially methylated (only one allele is methylated) in the CoC1/DDP cells, but was unmethylated (neither allele is methylated) in the CoC1 cells. p16 was hypermethylated in the CaOV3 and CoC1 cells, but was unmethylated in the CoC1/DDP cells. hMLH1 was hypermethylated in CoC1/DDP cells,

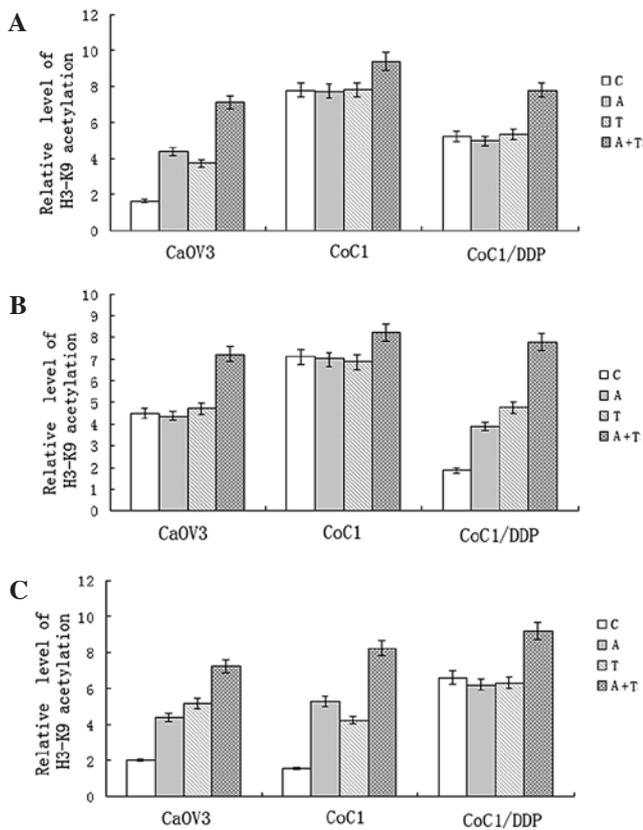


Figure 2. ChIP analysis of H3-K9 acetylation before and after treatment of the CaOV3, CoC1 and CoC1/DDP cells with 5-aza-CdR (A), TSA (T), or the combination (A+T). Real-time PCR experiments were repeated three times. The averaged ratios of precipitated DNA over input DNA shown on the y-axis represent the relative values of H3-K9 acetylation. Average H3-K9 acetylation levels are shown along with their standard error bars. (A) Summary of quantitative real-time PCR analysis of ChIP assays, using an antibody that recognizes acetyl-H3-K9 at the p16 promoter region. (B) Summary of quantitative real-time PCR analysis of ChIP assays, using an antibody that recognizes acetyl-H3-K9 at the hMLH1 promoter region. (C) Summary of quantitative real-time PCR analysis of ChIP assays, using an antibody that recognizes acetyl-H3-K9 at the MGMT promoter region. C, control

partially methylated in CaOV3 cells, but was unmethylated in the CoC1 cells.

Comparing Fig. 2 to Fig. 1, we found that acetylation of H3-K9 at the promoter region of the p16, hMLH1 and MGMT genes was inversely correlated with DNA methylation status. As shown in Fig. 2A, acetylation of H3-K9 at the promoter region of the MGMT gene was apparently higher in CoC1 (unmethylated) than in CoC1/DDP (partially methylated) cells and in CaOV3 cells (hypermethylated). Similar results were noted for the hMLH1 gene (Fig. 2B), which showed a low degree of H3-K9 acetylation in every part of the promoter region in the CoC1/DDP cells (hypermethylated). The highest degree of H3-K9 acetylation was detected in the CoC1 cells (unmethylated). An intermediate degree of H3-K9 acetylation was detected in the CaOV3 cells (partially methylated). In the CoC1/DDP cells, acetylation of H3-K9 at the promoter region of the p16 gene was apparently higher than that in the CoC1 and CaOV3 cells (Fig. 2C).

Gene silencing is associated with DNA methylation and H3-K9 acetylation at the promoter region. We examined

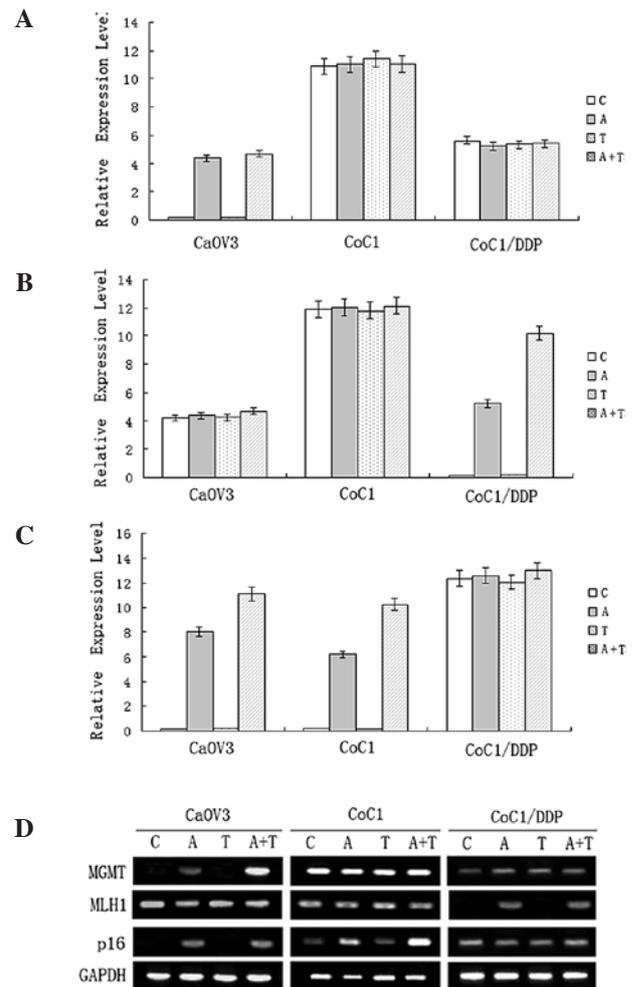


Figure 3. Real-time RT-PCR analysis of mRNA expression before and after treatment of CaOV3, CoC1 and CoC1/DDP cells with 5-aza-CdR (A), TSA (T), or the combination (A+T). Real-time RT-PCR experiments were repeated three times, with expression levels normalized to each sample's respective GAPDH expression. Error bars were calculated as standard error of the mean from three independent experiment. (A) Summary of quantitative real-time PCR analysis of p16. (B) Summary of quantitative real-time PCR analysis of hMLH1. (C) Summary of quantitative real-time PCR analysis of MGMT. (D) Representative images of the PCR assay. C, untreated control.

mRNA expression of p16, hMLH1 and MGMT in three human ovarian cancer cell lines using RT-PCR. We found that DNA hypermethylation and H3-K9 hypoacetylation at the promoter region had a transcriptional silencing function in the p16, hMLH1 and MGMT genes. The promoter region in the gene-expressing cells was usually DNA hypomethylated and H3-K9 acetylated, whereas that of the gene-silenced cells was DNA hypermethylated and H3-K9 hypoacetylated. As shown in Fig. 3A and D, the MGMT gene was silenced in the CaOV3 cells, but expressed in the CoC1 and CoC1/DDP cells. hMLH1 was expressed in the CaOV3 and CoC1 cells, but silenced in the CoC1/DDP cells (Fig. 3B and D). p16 was silenced in the CaOV3, CoC1, but expressed in the CoC1/DDP cells (Fig. 3C and D).

5-aza-CdR reactivates expression of the p16, MGMT and hMLH1 genes with DNA demethylation and an increase in H3-K9 acetylation at the DNA hypermethylated promoter. As

shown in Figs. 2 and 3 treatment with 5-aza-CdR resulted in restoration of MGMT expression in hypermethylated CaOV3 cells, accompanied with DNA demethylation and an increase in H3-K9 acetylation. In CoC1 and CoC1/DDP cells (CpG island was partially methylated or unmethylated), treatment with 5-aza-CdR minimally affected the expression of MGMT or the status of DNA methylation or the status of H3-K9 acetylation. 5-aza-CdR treatment resulted in demethylation, an increase in H3-K9 acetylation and re-expression of p16 in the CaOV3 and CoC1 cells, and similar results were noted for hMLH1 in the CoC1/DDP cells.

TSA increases H3-K9 acetylation at the DNA hypermethylated promoter without gene reactivation. We used ChIP assay to measure the effect of the treatment with TSA on the acetylation status of H3-K9 in ovarian cancer cell lines. Fig. 2 presents the results of ChIP assays in three ovarian cancer cell lines after treatment with TSA. Treatment with TSA alone had no effect on histone H3-K9 acetylation in cells with unmethylated DNA, but moderately increased histone H3-K9 acetylation in cells with hypermethylated DNA or partially methylated DNA. However, the combination of 5-aza-CdR and TSA increased histone H3-K9 acetylation effectively regardless of the DNA methylation status. Although TSA alone increased H3-K9 acetylation, it not only did not contribute to DNA demethylation, but also did not reactivate the expression of MGMT in the CaOV3 cells. The combined treatment resulted in demethylation of MGMT and restoration of MGMT expression in CaOV3 cells. In CoC1 and CoC1/DDP cells (CpG island was partially methylated or unmethylated), treatment with TSA or a combination of the two agents minimally affected the status of H3-K9 acetylation, the expression of MGMT or the status of DNA methylation. The combined treatment resulted in an increase in H3-K9 acetylation, DNA demethylation and re-expression of p16 in CaOV3 and COC1 cells, and similar results were noted for hMLH1 in the CoC1/DDP cells. By contrast, TSA alone increased H3-K9 acetylation, but did not affect the DNA methylation status or the expression of p16 and hMLH1.

Discussion

In the present study, we showed that histone acetylation is correlated with transcriptional activity and is important for the establishment of initial silencing at the hypermethylated promoter. However, once silencing is established, inhibition of histone deacetylation does not result in activation of gene expression. DNA demethylation is superior to histone deacetylation for reactivating certain tumor-related genes at the hypermethylated promoter.

We found that the loss of expression of the p16, hMLH1 and MGMT genes was related to the epigenetic regulation of both DNA methylation and histone acetylation. Furthermore, histone H3-K9 acetylation in different regions of the promoters correlated well with the DNA methylation status of each gene. We found that, when the CpG island was hypermethylated, the lowest levels of histone H3-K9 acetylation were detected. When the CpG island was hypomethylated, the highest levels of acetylation were detected. When all CpG islands were partially methylated, the intermediate levels of acetylation were found.

From our data, we conclude that histone acetylation correlates with transcriptional activity and is important for the establishment of initial silencing at the hypermethylated promoter. The methylated CpG-binding protein MeCP2 was found to be closely related to the inhibition complex which consists of the pontine proteins Sin3A and HDAC (25). MeCP2 bonds to chromatin by a DNA methylation-dependent pathway. The methylation of the promoter region and histone deacetylation are synergistic in the inhibition of gene transcription.

To deeply understand the contributions of histone acetylation and DNA methylation to gene silencing and to explore their relationship, we treated cells with 5-aza-CdR, TSA and performed ChIP assays. 5-aza-CdR, a demethylating agent, covalently bonds to DNA methyltransferases (DNMTs), reduces the biological activity of DNMTs, and thus lowers the methylation level regulating gene expression (26,27). After the action of 5-aza-CdR, the transcription inhibitor methyl-CpG binding domain (MBD) breaks away from the demethylated DNA or the transcription repression complex that originally binds to MBD, and then forms to its activated conformation of chromatin body (28). ChIP is a powerful tool for identifying proteins, including histone proteins and non-histone proteins, associated with specific regions of the genome by using specific antibodies that recognize a specific protein or a specific modification of a protein. The present ChIP assays demonstrated that the addition of 5-aza-CdR to ovarian cancer cells resulted in the demethylation of the p16, hMLH1 and MGMT genes accompanied with the re-expression of mRNA in the silenced cells.

Specific inhibition of HDACs by their inhibitors leads to the hyperacetylation of histones (29). Trichostatin A, a Streptomyces product originally discovered as a fungistatic antibiotic (30), was one of the first HDAC inhibitors identified. In the present study, inhibition of HDAC activities caused induction of histone deacetylation at the hypermethylated promoter. However, TSA hardly altered the mRNA expression level of p16, hMLH1 or MGMT. Our data support the observations that histone acetylation correlates with transcriptional activity and is important for the establishment of initial silencing at the hypermethylated promoter (31-33). However, once silencing is established, inhibition of histone deacetylation does not result in activation of gene expression (24). In the present study, at least we can conclude that DNA demethylation is superior to histone deacetylation in reactivating various tumor-related genes at the hypermethylated promoter, which is consistent with the previous observations (34).

Inhibition of DNA methylation by 5-aza-CdR caused induction of histone deacetylation at the hypermethylated promoter and restored its mRNA expression in silenced ovarian cancer cells. The results strengthen the idea that there is some interdependence between reversal of histone acetylation and reactivation of a gene silenced by aberrant DNA hypermethylation. Furthermore, that a combination of TSA and 5-aza-CdR was able to activate the expression of p16, hMLH1 and MGMT with an increase in the histone acetylation level, and showed a synergetic effect, suggests that the TSA-mediated positive regulation demands the initial promoter demethylation by 5-aza-CdR.

In our previous study, we demonstrated that TSA alone was able to reactivate the expression of the MGMT gene, without

altering its DNA methylation status (35). At the same time, we noted that the reactivation of MGMT was associated with the initial status of DNA methylation at the promoter region. We found that when the promoter region was hypermethylated before drug treatment, TSA alone was able to reactivate the silenced gene. When the promoter region was partially methylated or unmethylated, TSA alone was not able to reactivate the silenced gene. The results that MGMT, as well as p16 and hMLH1, can be regulated by a DNA methylation-dependent pathway suggests that DNA methylation and histone deacetylation may be tightly coupled in transcriptional regulation.

In conclusion, in ovarian cancer cells, DNA methylation and histone deacetylation act synergistically for the silencing of cancer-associated genes. DNA methylation played the leading role in the gene transcriptional regulation. The combination of 5-aza-CdR and TSA resulted in markedly increased histone H3-K9 acetylation in all cells and was most effective at restoration of gene expression. Our findings may provide a foundation to explore the molecular mechanisms of ovarian cancer and to utilize these epigenetic modification targets for pharmacologic intervention in ovarian cancer patients.

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