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# Promoter histone H3 lysine 9 di-methylation is associated with DNA methylation and aberrant expression of p16 in gastric cancer cells

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**Abstract.** In the course of gastric cancer development, gene silencing by DNA hypermethylation is an important mechanism. While DNA methylation often co-exists with histone modifications to regulate gene expression, the function of histone modifications in gene silencing in gastric cancer has not been evaluated in detail. p16, a well-known tumor suppressor gene, is frequently silenced in DNA hypermethylation manner in gastric cancer. Accordingly, we chose p16 to clarify whether there is a correlation among histone H3 lysine 9 (H3-K9) di-methylation, H3-K9 acetylation, DNA methylation and p16 expression in human gastric cancer. Three gastric cancer cells, MKN-45, SGC-7901 and BGC-823, were treated with 5-aza-2'-deoxycytidine (5-Aza-dC) and/or trichostatin A (TSA). We investigated p16 promoter DNA methylation status, p16 mRNA levels, regional and global levels of di-methyl-H3-K9 and acetyl-H3-K9 in four groups: i) 5-Aza-dC, ii) TSA, iii) the combination of 5-Aza-dC and TSA and iv) control group with no treatments. p16 silencing is characterized by DNA hypermethylation, H3-K9 hypoacetylation and H3-K9 hypermethylation at the promoter region. Treatment with TSA, increased H3-K9 acetylation at the hypermethylated promoter, but did not affect H3-K9 di-methylation or p16 expression. By contrast, treatment with 5-Aza-dC, reduced H3-K9 di-methylation, increased H3-K9 acetylation at the hypermethylated promoter and reactivated the expression of p16. Combined treatment restored the expression of p16 synergistically. In addition, 5-Aza-dC and the combined treatment did not result in global alteration of H3-K9 di-methylation. These results suggest that H3-K9 di-methylation, H3-K9 acetylation and DNA methylation work in combination to silence p16 in gastric cancer. The decreased H3-K9 di-methylation correlates with DNA demethylation and reactivation of p16. H3-K9 di-methylation as well as DNA methylation related to p16 silencing is limited to the promoter region. In addition to its effect on DNA methylation, 5-Aza-dC can act at histone modification levels to reactivate p16 expression in region-specific and DNA methylation-dependent manner.

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*Key words:* DNA methylation, gastric cancer, histone H3 lysine 9 methylation, histone H3 lysine 9 acetylation, p16, gene expression

## Introduction

DNA hypermethylation at the promoter region is associated with the aberrant transcriptional silencing of tumor suppressor genes in cancer and is involved in many classic tumor suppressor genes (1). p16 (also known as INK4A), an inhibitor of the cyclin D-dependent protein kinase 4/6 and a cell cycle regulator involved in the inhibition of $G_1$ phase progression, is one of the most common tumor suppressor genes (2). Loss of function of p16 leads to disruption of cell cycle regulation and accelerates cell growth. In gastric cancer, the frequency of p16 inactivated by homozygous deletions ranged from 0 to 9%, the frequency of p16 inactivated by mutation ranged from 0 to 2% and the frequency of p16 inactivated by DNA methylation ranged from 32 to 42% (3-7), which suggests that DNA methylation is a major mechanism for p16 inactivation in gastric cancer.

Histone modification is closely associated with DNA methylation status and is also important for gene regulation (8). Acetylation of histone H3 lysine 9 (H3-K9) is associated with active gene transcription and di-methylation of H3-K9 is associated with gene repression. Interaction between DNA methylation and various histone modifications is now being investigated. In *Neurospora* and *Arabidopsis*, genetic evidence indicates that H3-K9 methylation is a prerequisite for DNA methylation to occur (9,10). Loss of Suv39H1/2 in knockout mouse cells also altered the DNA methylation pattern of their pericentric heterochromatin (11). On the other hand, examples of ablation of DNA methylation affecting H3 methylation and other histone modifications have also been found in *Arabidopsis* and human cells (12,13). It appears that DNA and histone methylation likely have a mutually reinforcing relationship and both are required for stable and long-term epigenetic silencing.

Although DNA hypermethylation at the promoter of p16 is involved in gastric tumorigenesis, to the best of our knowledge, the functions of histone modifications in gastric cancer have not yet been evaluated in detail. In order to elaborate a possible function of epigenetic modifications in

p16 expression, we analyzed its mRNA expression, DNA methylation, regional and global di-methyl-H3-K9 and acetyl-H3-K9 following the treatments with 5-Aza-dC and TSA in three human gastric cancer cell lines, MKN-45, SGC-7901 and BGC-823.

## Materials and methods

*Cell lines and culture conditions*. Three cell lines derived from human gastric cancer, MKN-45 (14), SGC-7901 (15) and BGC-823 (16), were cultured in RPMI-1640 (Gibco BRL, USA) supplemented with 10% fetal bovine serum (Gibco) and incubated in humidified incubator containing 10% $CO_2$ at 37˚C.

*Treatment of cells with 5-Aza-dC and TSA*. MKN-45, SGC-7901 and BGC-823 cells were cultured for 24 h prior to treatment. A total of 4 experimental groups were set up: i) 5-Aza-dC group: cells were supplemented with 5 $\mu$mol/l 5-Aza-dC (Sigma Chemical Co, St. Louis, MO) for 72 h, which was refreshed with the same concentration every 24 h; ii) TSA group: 300 nmol/l TSA (Sigma) was supplemented for 24 h; iii) 5-Aza-dC/TSA group: 5 $\mu$mol/l 5-Aza-dC was supplemented for 48 h (which was refreshed with the same concentration every 24 h) and subsequently 300 nmol/l TSA was supplemented for another 24 h; iv) control group: cells of the same batch were treated without any agent. The dose, time and sequence of 5-Aza-dC and/or TSA were based on similar preliminary studies (17-19).

*DNA modification and methylation-specific PCR (MSP)*. Genomic DNA from MKN-45, SGC-7901 and BGC-823 cells untreated or treated with 5-Aza-dC, TSA, or both was extracted with phenol-chloroform-isoamyl alcohol and was collected by ethanol precipitation. DNA concentration and purity were determined with UV spectrometer. Each genomic DNA sample (5 $\mu$g) was treated with sodium bisulfite (Sigma), as described previously (20,21). Briefly, DNA was denatured by treatment with 0.3 M NaOH and incubated at 37˚C for 30 min, followed by incubation with 10 mM hydroquinone (Sigma) and 3 M sodium bisulfite (Sigma) at 55˚C for 16 to 20 h. Modified DNA was purified using a Wizard DNA Clean-Up System (Promega Corporation, Madison, WI), recovered by ethanol precipitation and dissolved in distilled water. PCR amplification was performed using 2.0 $\mu$l bisulfite-modified DNA in a 20 $\mu$l reaction. The primers used for MSP are located in the promoter region and the CpG map was based on a previous study (18). The methyltransferase (Sss I)-treated and untreated peripheral blood cell DNA from the healthy adults were used as the positive and negative controls, respectively, while the double-distilled water as the blank control. PCR products were separated on 2% agarose gel electrophoresis and images were captured with Alpha Image 2000. Each experiment was perfomed in triplicate.

*Chromatin immunoprecipitation assay (ChIP)*. The ChIP assays were performed as described previously with some modifications (22,23). Briefly, cells untreated or treated with 5-Aza-dC, TSA, or both were fixed by addition of formaldehyde directly to the culture medium to a final concentration of 4 g/l for 20 min at 37˚C. After washing, the cell pellets were resuspended in 350 $\mu$l of lysis buffer and sonicated to generate DNA fragments in 500 bp. The lysate was divided into three fractions. The first and second (100 $\mu$l each) were diluted in 900 $\mu$l of lysis buffer and the third one (100 $\mu$l) was used for input control. The first lysate was incubated with 5 $\mu$l of anti-Lys-9 acetylated histone H3 antibody (Upstate Biotechnology, Lake Placid, NY), or 5 $\mu$l of anti-Lys-9 di-methylated histone H3 antibody (Upstate) at 4˚C overnight. The second lysate was incubated with 5 $\mu$l of normal rabbit IgG at 4˚C overnight as a negative control. Immune complexes were collected with protein A/G plus-Agarose (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at 4˚C with agitation. The cross-links were then reversed by heating the sample at 65˚C for 5 h. After elution, the samples were digested with proteinase K. DNA was extracted by phenol extraction, ethanol precipitated and resuspended in Tris-EDTA buffer.

*PCR analysis of immunoprecipitated DNA*. To ensure that PCR amplification was in the exponential range, every reaction was initially set up at different dilutions of DNA for different amplification cycle numbers and the PCR conditions were decided accordingly. Amplification was carried out with 2 $\mu$l of an immunoprecipitated DNA, a negative control or input DNA in a 20 $\mu$l reaction. The primers used are located in the promoter region and were based on a previous study (18): 5'-CATTCGCTAAGTGCTCGGAGT-3' (sense) and 5'-CTC CTCTTTCTTCCTCCGGTG-3' (antisense). PCR products were resolved on 2% agarose gels (Promega) and quantified with the Bio-Rad Gel Doc 1000 system and Molecular Analyst software. The level of H3-K9 acetylation and H3-K9 di-methylation in each immunoprecipitation was determined by quantifying the intensities of the PCR product in immunoprecipitated DNA vs. input DNA. The ChIP experiment was repeated at least two times and three independent PCR analyses of each were done.

*Real-time PCR analysis of immunoprecipitated DNA*. Real-time PCR was also performed using immunoprecipitated DNA, a negative control or a DNA input control. The primers and probe for CpG islands within the promoter region was as follows: 5'-CATTCGCTAAGTGCTCGGAGT-3', 5'-CTCC TCTTTCTTCCTCCGGTG-3' and 5' Fam-AGCGAGTGCT CGGAGGAGGTGCTATTA-3' Tamra. PCR was performed under optimized conditions. The segment of p16 cDNA was cloned into the pMD18-T vectors and confirmed by sequencing. The purified recombinant plasmid DNA was quantified by UV spectrophotometer and then serially diluted to a final concentration ranging from $2x10^4$ to $2x10^8$ copies of genome equivalents/ml. Aliquots (5 $\mu$l) of each dilution (100 to $10^6$ genome equivalents/reaction) were used for real-time PCR to create the standard curve and used as quantification standards for experimental samples. Each sample was amplified in triplicate to obtain average copy numbers. Reactions without cDNA templates were used as a negative control.

*RNA extraction and real-time RT-PCR*. Total RNA from MKN-45, SGC-7901 and BGC-823 cells untreated or treated with 5-Aza-dC, TSA, or both was extracted with TRIzol (Invitrogen, Carlsbad, USA) according to the manufacturer's

Then RNA was treated with DNAase I to exclude genomic DNA contamination and its concentration and purity were determined with UV spectrometer. Total RNA (2 $\mu$g) was used as the template to synthesize cDNA with Reverse Transcription System (Promega). Then quantitative real-time PCR was carried out using the following primers combined with a Taq Man probe: sense 5'-GGCTCCTCATT CCTCTTCCT-3', antisense 5'-TCAGGTAGCGCTTCGAT TCT-3', Taq Man probe: 5' Fam-CAGAAGGGGTTTGTAA TCACAGACCTCCT-3' Tamra. Real-time PCRs were carried out following the above protocol.

*Histone extraction and Western blot analysis*. Histone from MKN-45 cells untreated or treated with 5-Aza-dC, TSA, or both was extracted as described previously with some modifications (24,25). Briefly, MKN-45 cell pellets were resuspended in lysis buffer (HEPES pH 7.9, 1.5 mmol/l $MgCl_2$, 10 mmol/l KCl, 0.5 mmol/l DTT and 1.5 mmol/l phenylmethylsulfonyl fluoride) and hydrochloric acid (HCl) were added to a concentration of 0.2 mol/l. After incubation on ice for 30 min and centrifugation at 11,000 x g for 10 min at 4˚C, the supernatant was mixed with trichloroacetic acid (TCA) in 4:1 ratio making a 20% TCA solution, incubated for 1 h at 4˚C and centrifuged at 12,000 x g for 10 min. The pellets were washed with 0.5 ml of acetone/0.02 N HCl, centrifuged at 12,000 x g for 5 min and dried under the hood for 30 min. The dried pellets were resuspended in water and sonicated. After centrifugation at 12,000 x g for 10 min, the supernatant was collected and stored at -80˚C.

Equal amounts (30 $\mu$g) of proteins were subjected to 15% SDS-PAGE and transferred onto PVDF membrane. After blocking with 5% non-fat dried milk for 1 h at room temperature, membranes were incubated overnight at 4˚C with primary antibody with the dilution of 1:2000 for anti-Lys-9 di-methylated histone H3 (Upstate). Actin was used as a loading control. These dilutions were optimized for different antibodies respectively. After washing with TBST solution three times, membranes were incubated with goat anti-rabbit IgG HRP conjugated secondary antibody (Santa Cruz Biotechnology) with 1:1000 dilution for 1 h at room temperature. The protein-antibody complex was detected using an enhanced chemiluminescence kit for 5 min and photographed. Protein band intensity was quantified by scanning densitometry. Data were expressed as a ratio of protein content relative to actin protein in arbitrary units. The experiments were repeated three times.

*Statistical analysis*. The ratio results are expressed as mean ± standard deviation (SD). Significance between controls and treated samples was calculated by Student's t-test. Significance between controls in different cell lines was calculated by one-way ANOVA and q-test. Statistical calculations were performed using SPSS version 10.0 (SPSS Inc., Chicago, IL, USA). $P<0.01$ was considered statistically significant.

## Results

*5-Aza-dC and TSA acted differently in demethylation of p16 in gastric cancer cells*. We used MSP to evaluate the DNA methylation status at the promoter region in MKN-45, SGC-7901 and BGC-823 cells. These three cell lines have different DNA methylation status at promoter region. As can be seen in Fig. 1, p16 is DNA hypermethylated (both alleles are methylated) in MKN-45 cells, partially DNA methylated (only one allele is methylated) in SGC-7901 cells and DNA unmethylated (neither allele is methylated) in BGC-823 cells. We evaluated the effect of 5-Aza-dC and/or TSA treatment on DNA methylation status. 5-Aza-dC and the combination of 5-Aza-dC and TSA treatment resulted in DNA demethylation of p16 in MKN-45 cells. By contrast, TSA alone did not affect the DNA methylation status of p16. In SGC-7901 and BGC-823 cells, treatments with 5-Aza-dC, TSA or both had no effects on DNA methylation.

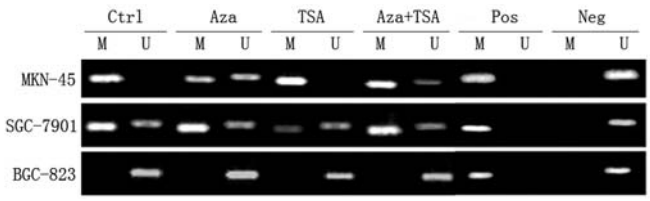


Figure 1. MSP analysis of DNA methylation at p16 promoter region before and after treatment of MKN-45, SGC-7901 and BGC-823 cells with 5-Aza-dC, TSA, or the combination. At least two independent experiments were performed with similar results. Lane M indicates the presence of methylated alleles. Lane U indicates the presence of unmethylated alleles.

*5-Aza-dC and TSA acted differently in decreasing H3-K9 di-methylation at the promoter*. We used ChIP assay to measure the status of H3-K9 di-methylation at p16 promoter in gastric cancer cell lines. Examples of these results are shown in Fig. 2A and the data are summarized in Fig. 2B. MKN-45, which had the highest degree of DNA methylation, had the highest H3-K9 di-methylation at promoter region. BGC-823, which had DNA unmethylation at this locus, had the lowest H3-K9 di-methylation. SGC-7901, which had partial DNA methylation, showed intermediate degrees of H3-K9 di-methylation. This detection indicated that DNA methylation might be related to the level of H3-K9 di-methylation.

We determined whether the level of H3-K9 di-methylation could be altered by the treatment with 5-Aza-dC and/or TSA. In MKN-45 cells (DNA hypermethylated), 5-Aza-dC decreased the level of H3-K9 di-methylation significantly ($P<0.01$). 5-Aza-dC reduced H3-K9 di-methylation significantly in SGC-7901 which promoter showed partial DNA methylation ($P<0.01$), but had no significant effect on H3-K9 di-methylation in BGC-823 cells which promoter showed DNA unmethylation. TSA alone had no significant effect on the level of H3-K9 di-methylation regardless of DNA methylation status. The combination of 5-Aza-dC and TSA had significant effects on H3-K9 di-methylation similar to those of 5-Aza-dC. Decreased H3-K9 di-methylation was consistent with an increase in p16 expression in silenced MKN-45 cells.

*5-Aza-dC and TSA acted similarly in increasing H3-K9 acetylation at the promoter*. We also used ChIP assay to measure the effect of treatment with 5-Aza-dC and/or TSA on the acetylation status of H3-K9 in gastric cancer cell lines.

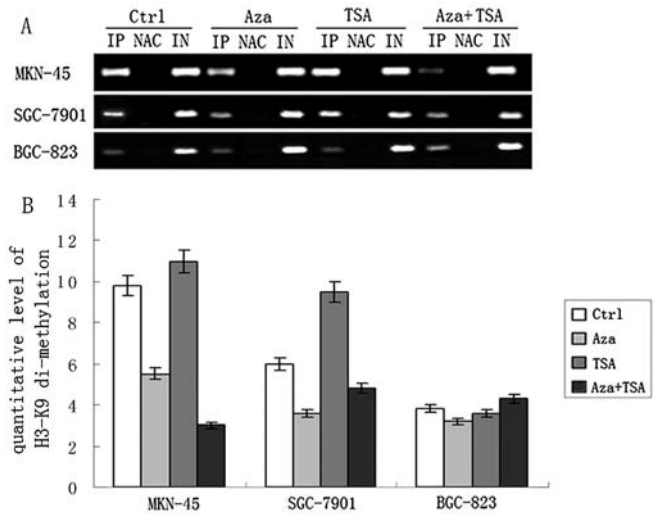


Figure 2. ChIP analysis of H3-K9 di-methylation before and after treatment of MKN-45, SGC-7901 and BGC-823 cells with 5-Aza-dC, TSA, or the combination. Three independent ChIPs were performed, using an antibody that recognizes di-methyl-H3-K9 at p16 promoter region. (A) Representative graphs of PCR assay. (B) Summary of quantitative real-time PCR analysis of ChIP assays. 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 di-methylation. Average H3-K9 di-methylation levels are shown along with their standard error bars. (Ctrl, no treatment; IP, immunoprecipitated DNA; NAC, no-antibody control; IN, input DNA from whole-cell lysate).

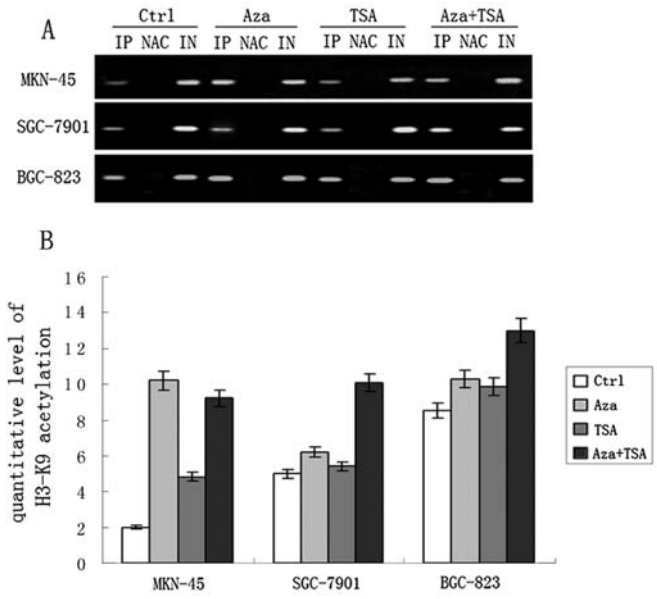


Figure 3. ChIP analysis of H3-K9 acetylation before and after treatment of MKN-45, SGC-7901 and BGC-823 cells with 5-Aza-dC, TSA, or the combination. Three independent ChIPs were performed, using an antibody that recognizes acetyl-H3-K9 at p16 promoter region. (A) Representative graphs of PCR assay. (B) Summary of quantitative real-time PCR analysis of ChIP assays. 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 (Ctrl, no treatment; IP, immunoprecipitated DNA; NAC, no-antibody control; IN, input DNA from whole-cell lysate).

Examples of these results are shown in Fig. 3A and the data are summarized in Fig. 3B. Before treatment, the promoter region of the p16 gene showed a higher degree of H3-K9 acetylation in SGC-7901 cells (partial DNA methylated) and BGC-823 cells (DNA unmethylated) compared to MKN-45 cells (DNA hypermethylated) ($P<0.01$). Treatment with TSA alone had no significant effect on H3-K9 acetylation in SGC-7901 and BGC-823 cells, but significantly increased H3-K9 acetylation in MKN-45 cells ($P<0.01$). 5-Aza-dC increased H3-K9 acetylation in MKN-45 cells but had no effect on H3-K9 acetylation in SGC-7901 and BGC-823 cells. However, the combination of 5-Aza-dC and TSA increased H3-K9 acetylation effectively regardless of DNA methylation status ($P<0.01$).

*H3-K9 di-methylation as well as DNA methylation at the promoter was associated with p16 expression in gastric cancer cells*. To determine whether histone modifications were associated with p16 expression in human gastric cancer cells, we determined p16 expression by using RT-PCR (Fig. 4A) and real-time PCR (Fig. 4B). p16 was expressed in SGC-7901 cells and BGC-823 cells, but was silenced in MKN-45 cells. To further determine the relationship between p16 expression and histone modifications, we determined the effects of treatment with 5-Aza-dC and TSA on p16 expression. In MKN-45 cells which exhibited high level of H3-K9 di-methylation as well as DNA hypermethylation at the promoter, we found that treatment with 5-Aza-dC resulted in restoration of p16 expression. TSA alone had little effect. When a combination of 5-Aza-dC and TSA treatment was used, expression of p16 was detected. By contrast, SGC-7901 and BGC-823 cells exhibited lower level of H3-K9 di-methylation at the promoter, treatment with 5-Aza-dC, TSA or a combination of the two agents minimally affected the expression of p16.

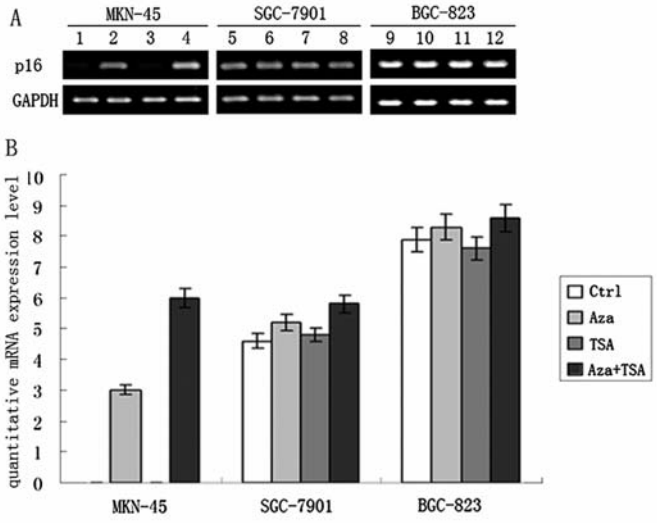


Figure 4. p16 mRNA expression before and after treatment of MKN-45, SGC-7901 and BGC-823 cells with 5-Aza-dC, TSA, or the combination. (A) Examples of RT-PCR results. Cells were treated with 5-Aza-dC (lanes 2, 6 and 10), TSA (lanes 3, 7 and 11), a combination of 5-Aza-dC and TSA (lanes 4, 8 and 12) and no drug as a control (lanes 1, 5 and 9). (B) 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. Ctrl indicates the control (without any treatments).

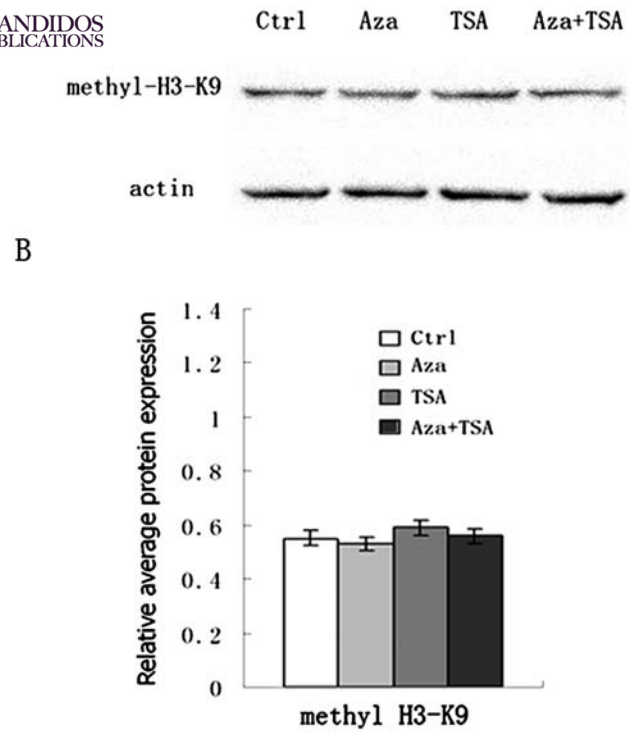


Figure 5. (A) Representative Western blots are shown detecting the global levels of H3-K9 di-methylation and H3-K9 acetylation before and after treatment of MKN-45 cells with 5-Aza-dC, TSA, or the combination. Three independent experiments were performed. (B) Relative average protein expression, as determined by integrated densitometry, is shown below the graph, normalized to the actin loading control.

*The treatments had no effect on the overall levels of di-methyl H3-K9*. The results of ChIP assays indicated that H3-K9 di-methylation associated with p16 expression was likely limited to the promoter region in MKN-45 cells. To investigate whether alteration of histone deacetylases with TSA and DNA methylation with 5-Aza-dC resulted in a global change in cellular H3-K9 di-methylation, Western blot analysis was performed using protein isolated from untreated MKN-45 cells, in addition to those treated with 5-Aza-dC or/and TSA. Immunoblots probed with di-methyl H3-K9 antibody showed that treatments with 5-Aza-dC, TSA or the combination did not result in a statistically significant change of the global levels of di-methyl-H3-K9, compared to the untreated cells (Fig. 5).

## Discussion

In the present study, we report for the first time that H3-K9 di-methylation plays a crucial role in p16 expression in gastric cancer cells. The decreased di-methylation of H3-K9 correlates with DNA demethylation and reactivation of p16. H3-K9 di-methylation consistant with DNA methylation in p16 silencing is limited in the promoter region.

The silencing of many tumor suppressor genes has been related to the epigenetic regulation of both DNA methylation and histone modifications in a variety of malignancies (26-29). These include the genes of $p21^{WAF/CIP1}$, p27, $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$, which encode cyclin-dependent kinase inhibitors. Reactivation of the expression of the INK family members can be restored by 5-Aza-dC but not TSA. 5-Aza-dC not only demethylates these promoters but also resets the histone modification. TSA does not facilitate promoter demethylation and inhibition of histone deacetylation can generally not result in activation of gene expression (30,31). TSA may synergize 5-Aza-dC-mediated gene activation, depending on the particular gene or cell type analyzed (18,22). In this case, the TSA-mediated positive regulation via histone acetylation relies on initial promoter demethylation by 5-Aza-dC. Several groups have induced re-expression of DNA hypermethylated and silenced cancer genes through 5-Aza-dC-induced DNA demethylation (32-36). In the present study, we found p16 can be regulated by DNA-methylation-dependent mechanisms and the activity is related to the methylation status of the CpG islands at the p16 promoter. When CpG islands were partially methylated or unmethylated, 5-Aza-dC had no effect on p16 expression. If CpG islands were hypermethylated, a DNA methyltransferase inhibitor alone was sufficient to activate p16. However, inhibition of histone deacetylase alone was not sufficient to restore p16. The combination of demethylation and histone deacetylase inhibition achieved high reactivation of p16 expression. Thus, our findings suggest that DNA methylation is dominant over histone deacetylation in maintaining a silent state at hypermethylated promoter.

To exploit the reversible nature of epigenetics is a better approach to further understand the relationship of DNA methylation and histone modifications in aberrant gene silencing. Several groups have induced re-expression of DNA hypermethylated, silenced cancer genes through 5-Aza-dC-induced DNA demethylation and noted the reappearance of the active marks, acetylated H3-K9 and the decrease of the silencing mark, di-methylated H3-K9 (32-36). In our study, we found that TSA and 5-Aza-dC acted differently with regards to histone modification. We demonstrate that 5-Aza-dC, but not TSA, can reactivate expression of the silenced gene and reverse histone H3-K9 di-methylation at the gene promoter. The decreased di-methylation of H3-K9 was consistent with DNA demethylation and the up-regulation of p16. While TSA increased histone acetylation at hypermethylated CpG islands, it had little effect on gene expression. Thus, we speculated that H3-K9 di-methylation might be coupled to DNA methylation in gene silencing in gastric cancer cells. Furthermore, the results suggested that reactivation of the silenced gene correlates much better with decreased H3-K9 di-methylation than with increased H3-K9 acetylation.

In colorectal cancer, ChIP assays indicate that H3-K9 di-methylation directly correlates and H3-K9 acetylation inversely correlates with DNA methylation of p16, MLH1 and MGMT (18). We explored these findings further in gastric cancer. Before the drug treatment, we demonstrated that H3-K9 di-methylation in the promoter correlated and H3-K9 acetylation inversely correlated, very well with DNA methylation of p16 in MKN-45, SGC-7901 and BGC-823 cells. After the drug treatment, an increase in the level of H3-K9 acetylation was accompanied with a decrease in the level of H3-K9 di-methylation in CpG islands. Accordingly, our findings revealed that there is a trend toward an inverse correlation between H3-K9 acetylation and H3-K9 di-methylation.

Because the primers used for ChIP-PCR were located in the promoter region, the modified histones tested by ChIP were regional. In order to make out fully whether the change of H3-K9 di-methylation associated with p16 expression was global or regional, we performed Western blot analysis to test the overall levels of H3-K9 di-methylation. Because there were no changes in the overall levels of di-methylated H3-K9, the chromatin alteration associated with p16 silencing was region-specific rather than global. Data from this study suggest that certain regions of a gene are more vulnerable than others to heterochromatinization and may serve as foci for the seeding of aberrant DNA methylation and H3-K9 di-methylation. It has been postulated that transcription through a gene region may facilitate *de novo* cytosine methylation (37,38). The data in this study indicate that CpG islands are more heterochromatic when they are located in the promoter region.

In conclusion, in gastric cancer cells, H3-K9 di-methylation as well as DNA methylation related to silencing of p16 is limited to the promoter region. In addition to its effect on DNA methylation, 5-Aza-dC can act at histone modification levels to reactivate p16 expression in region-specific and DNA methylation-dependent manner. Our findings may provide a foundation to explore the molecular mechanisms of gastric cancer and to make these epigenetic modifications targets for pharmacologic intervention in gastric cancer patients.

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