Basic Research Paper

Effects of 5-Aza-2'-deoxycytidine and trichostatin A on DNA methylation and expression of hMLH1 in ovarian cancer cell line COC1/DDP

Chun-Feng Meng, Dong-Qiu Dai, and Ke-Jun Guo

1Department of Surgical Oncology; Department of General Surgery; and 2Department of Gynecology; The First Affiliated Hospital; China Medical University; Shenyang, Liaoning P.R. China

Key words: ovarian neoplasm, drug resistance, COC1 cell, COC1/DDP cell, cisplatin, DNA methylation, hMLH1, 5-Aza-2'-deoxycytidine, trichostatin A

Background and Objective: Cisplatin (DDP) can cause DNA damage in cells. DNA mismatch repair proteins serve to detect DDP-caused DNA damage by generating an injury signal which eventually contributes to the triggering of tumor cell apoptosis. As a member of the mismatch repair system, the absence of hMLH1 expression contributes to the resistance of tumor cells to DDP. This study explores the role of hMLH1 expression and DNA methylation in DDP-resistance of human ovarian cancer, and evaluates the reversal effects of 5-Aza-2’-deoxycytidine (5-Aza-dC) and Trichostatin A (TSA) on DDP-resistance. Methods: We cultured the human ovarian cancer cell line COC1 and its DDP-resistant subline, COC1/DDP. We treated the two cell lines with 5-Aza-dC and TSA. DNA methylation at the hMLH1 gene promoter was detected by methylation-specific polymerase chain reaction (MSP). The expression of hMLH1 was detected by reverse transcription-polymerase chain reaction (RT-PCR) and western blot. The inhibition rate of cell proliferation was detected by MTT assay. Results: In COC1 cells, both hMLH1 mRNA and protein were detected, while no DNA methylation of the hMLH1 gene was detected. 5-Aza-dC. TSA used alone or in combination had no effects on DNA methylation, hMLH1 mRNA or protein expression (p > 0.05), or cell proliferation. In COC1/DDP cells, DNA hypermethylation of the hMLH1 gene was detected, while no hMLH1 mRNA or protein was detected; 5-Aza-dC resulted in DNA demethylation and restoration of hMLH1 expression; TSA had no effect on DNA demethylation or restoration of hMLH1 expression; 5-Aza-dC plus TSA also resulted in DNA demethylation, restored hMLH1 expression more than 5-Aza-dC (p < 0.05) and restricted the proliferation of COC1/DDP cells. Conclusions: Hypermethylation of DNA promoter is related to the silencing of hMLH1 in ovarian cancer COC1/DDP cells. The use of 5-Aza-dC alone or in combination with TSA results in DNA demethylation of hMLH1 gene, restoration of hMLH1 expression, and reversal of DDP-resistance of COC1/DDP cells.

The therapeutic paradigm for ovarian cancer is the comprehensive surgery combined with chemotherapy with platinum agents being one of the most effective chemotherapeutic drugs. Yet 20%–30% of tumors are primarily resistant to cisplatin (DDP),1 one of the major causes of ovarian cancer relapse and therapy failure.2 Overcoming and reversing such resistance is currently a hot topic.

Resistance can be mediated by repair of injury in tumor cells wherein the absence of hMLH1 expression, as a member of the mismatch repair system, contributes to the resistance of tumor cells to DDP.3 This study selects human ovarian cancer cell line COC1 and cisplatin-resistant cell line COC1/DDP to test hMLH1 gene and protein expression in both cell lines as well as DNA methylation at the promoter region, to explore the role of hMLH1 expression and DNA methylation in the DDP-resistant ovarian cancer cell line and to evaluate the reversal effects of demethylative agent 5-Aza-2’-deoxycytidine (5-Aza-dC) and histone deacetylase inhibitor Trichostatin A (TSA) on hMLH1 expression and DNA methylation.

Materials and Methods

Reagents. Human ovarian cell line COC1 and DDP-resistant cell line COC1/DDP were purchased from Cancer Research Institute of China Medical University. RPMI-1640 culture medium
Effects of 5-Aza-2'-deoxycytidine and trichostatin A on DNA methylation and expression of hMLH1 in ovarian cancer cell line COC1/DDP

was purchased from Gibco, while 5-Aza-dC, TSA and sodium bisulfate were purchased from Sigma. The Wizard DNA Clean-up system and RT-PCR reverse transcription kit were purchased from Promega. Trizol was purchased from Invitrogen. Taq enzyme and dNTP were purchased from Takara. The hMLH1 antibodies were purchased from Santa Cruz. Horseradish peroxidase labeled goat-anti-rabbit secondary antibodies were purchased from Beijing Dingguo Biotechnology. An ECL kit from Applygen was purchased from Technologies Inc. Pre-stained protein electrophoresis molecular weight markers and methylase were purchased from New England Biolabs. PVDF film was purchased from Millipore and primers were from Beijing Sun Biotechnology.

Cell culture. COC1 cells and COC1/DDP cells were cultured in RPMI-1640 culture media containing 10% fetal bovine serum, 100 U/L penicillin G and 100 μg/L streptomycin and incubated at 37°C, at the humidity of 95% and at the atmosphere of 5% CO₂.

Cell treatment. COC1 and COC1/DDP cells at logarithmic growth phase were harvested and seeded into 250 mL flask at the density of 5 x 10⁶/mL for 24 h. A total of 4 experimental groups were set up: (1) 5-Aza-dC group: cells were supplemented with 5 mmol/L 5-Aza-dC for 72 h, which was refreshed with the same concentration every 24 h; (2) TSA group: 300 nmol/L TSA was supplemented for 24 h; (3) 5-Aza-dC/TSA group: 5 mmol/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; (4) control group: cells of the same batch were treated without any agent.

Detection of DNA methylation in hMLH1 gene promoter region with methylation-specific PCR. Cells were harvested and the genome DNA was extracted and modified with sodium bisulfate. The DNA was purified with Wizard DNA Clean-Up System as instructed by the user manual. PCR amplification was performed on the template of modified DNA. The sequence for hMLH1 methylation primer was as follows: sense 5'-GAT AGC GAT TTT TAA CGC-3', antisense 5'-TCT ATA AAT TAC TAA ATC TCT TCG-3'. Amplification fragment was 124 bp. The reaction system was as follows: cDNA 2 mL, 10x Buffer 2.5 mL, dNTP 1 mL, Taq enzyme 0.2 mL, sterile water 14.3 mL, primer F 0.5 mL and primer R 0.5 mL at the total volume of 20 mL; PCR reaction conditions: predenaturation at 95°C for 5 min, denaturation at 95°C for 30 s, annealing at 52°C for 45 s, elongation at 72°C for 60 s for the total 35 cycles and final elongation at 72°C for 10 min. PCR products were separated on 1.5% agarose gel electrophoresis and images were captured with Alpha Image 2000 and quantified with Fluorchem V 2.0. The ratio of hMLH1 products to GAPDH products was used to analyze the expression of mRNA. Each experiment was triplicated.

Western blot of hMLH1 protein. Total cellular proteins were extracted, with culture media discarded, rinsed in PBS twice, supplemented with pre-cooled RIPA lysis buffer (1x PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 mg/mL PMSF) on ice and the concentration of cellular proteins were determined using the BCA method. Cellular protein sample was mixed with 2x SDS loading buffer of equal volume in water bath of 100°C for 5 min. An equal amount of protein (30 μg) was loaded from each cellular protein sample and separated on 10% polyacrylamide gel electrophoresis (PAGE). Proteins on the gel were electroblotted onto polyvinylidene fluoride (PVDF) membrane. The membrane was placed on 5% skim milk for blocking for 1 h at room temperature, then incubated with hMLH1 polyclonal antibody (1:1,000, 85 kDa) and actin monoclonal antibody (1:800, 43 kDa) at 4°C overnight, rinsed, incubated with secondary antibody (1:2,000) at room temperature, then incubated with hMLH1 polyclonal antibody (1:2,000) at room temperature for 1 h, rinsed and colorized with enhanced chemiluminescence (ECL). Images were captured with an electrophoresis imaging system (BIS303PC) and quantified with Fluorchem V 2.0. The ratio of hMLH1 products to GAPDH products was used to analyze the expression of mRNA. Each experiment was triplicated.

Detection of cellular proliferation inhibition rate with MTT. Cells from both experimental and control groups were seeded onto a 96-well plate at the density of 1 x 10⁶/well. The well without cells but with culture media was taken as a zeroing well. The absorbance (A) of each well was measured, with cellular proliferation inhibition rate = (1 - Aexp/Actr) x 100%. The absorbance (A) of each well was measured, with cellular proliferation inhibition rate = (1 - Aexp/Actr) x 100%.

Statistical analysis. SPSS 10.0 was used for statistical analysis. All data were expressed as mean ± SD. A student-t test was used as inter-group comparison and the level of significance was taken as (p = 0.05).

Results

DNA methylation profiles in hMLH1 promoter region. For COC1/DDP cells, hypermethylation of DNA was present in the hMLH1 promoter region (methylated band present only) while non-methylation was present in COC1 cells (non-methylated band present only). For COC1/DDP cells, DNA was demethylated
Effects of 5-Aza-2’-deoxycytidine and trichostatin A on DNA methylation and expression of hMLH1 in ovarian cancer cell line COC1/DDP

5-Aza-dC was more significantly inhibited in cellular proliferation than COC1 cells and negative control cells (p < 0.05); after being treated by TSA, the cellular proliferation inhibition rates didn’t vary among COC1/DDP cells, COC1 cells and negative control cells in a statistically significant manner (p > 0.05) (Table 2).

Discussion

The DNA mismatch repair system is a security assurance system to repair DNA base pair mismatch, consisting of a series of mismatch repair genes. The functions of the DNA mismatch repair system are to correct the erroneous uptake of nucleotide acid, enhance the fidelity of DNA replication, decrease the spontaneous mutation incidence, maintain the stability of microsatellite loci or even the whole genome and guarantee the high fidelity of DNA replication. Any mutation in the gene family would result in mismatch repair defect, genetic instability and susceptibility to tumors. Our study found that hMLH1 was a key element of mismatch repair system as the sensor of DNA injury, capable of recognizing the mismatch loci, whose absence would result in the resistance of tumor to DDP through the following mechanism: (1) by reducing the recognition of DNA injury and disabling the trigger of apoptotic signal; (2) by increasing the genomic instability and inducing the mutant resistant to chemotherapeutic agents.

The results showed that the hMLH1 gene was silenced and protein expression was absent in DDP-resistant COC1/DDP cells, while it was expressed in non-resistant COC1 cells, indicating the association between DDP-resistance and hMLH1 gene silencing in DDP-resistant ovarian cancer cells. The comparison of the single use of 5-Aza-dC and the combined use of 5-Aza-dC and TSA showed that previously absent hMLH1 was re-expressed in DDP-resistant cells, indicating the potential of 5-Aza-dC to reverse the resistance of ovarian cancer cells to DDP.

Inhibition of 5-Aza-dC and TSA on cellular proliferation. After being treated by 5-Aza-dC with or without TSA, COC1/DDP cells was more significantly inhibited in cellular proliferation than COC1 cells and negative control cells (p < 0.05); after being treated by TSA, the cellular proliferation inhibition rates didn’t vary among COC1/DDP cells, COC1 cells and negative control cells in a statistically significant manner (p > 0.05) (Table 2).

Expression profiles of hMLH1 mRNA. The hMLH1 was not expressed in COC1/DDP cells but expressed in COC1 cells. The use of 5-Aza-dC activated re-expression of silenced hMLH1 in COC1/DDP cells (Fig. 2A) but had no effect on hMLH1 expression in COC1 cells (p > 0.05) (Table 1). TSA had no effect on gene expression (p > 0.05). Compared to the single treatment with 5-Aza-dC, the combined treatment with 5-Aza-dC and TSA had more significant effect on hMLH1 mRNA expression in COC1/DDP cells (p < 0.05).

Expression profiles of hMLH1 protein. Our study found that hMLH1 protein was expressed in COC1 cells other than in COC1/DDP cells (Fig. 2B). The hMLH1 protein was expressed in COC1/DDP cells after being treated with 5-Aza-dC. TSA had no effect on protein expression (p > 0.05) (Table 1). Compared to the single treatment with 5-Aza-dC, the combined treatment with 5-Aza-dC and TSA had more significant effect on hMLH1 protein expression in COC1/DDP cells (p < 0.05).

Inhibition of 5-Aza-dC and TSA on cellular proliferation. After being treated by 5-Aza-dC with or without TSA, COC1/DDP cells was more significantly inhibited in cellular proliferation than COC1 cells and negative control cells (p < 0.05); after being treated by TSA, the cellular proliferation inhibition rates didn’t vary among COC1/DDP cells, COC1 cells and negative control cells in a statistically significant manner (p > 0.05) (Table 2).
The treatment of histone deacetylase (HDAC) inhibitor Trichostatin A didn’t change DNA methylation in the hMLH1 promoter region or hMLH1 gene expression. The results showed that chromatin remodeling played a critical role in regulation of gene transcription while modification of histone through acetylation and deacetylation had significant effects on chromatin remodeling. Histone acetylation status depended on the activity competition between histone acetyltransferase and histone deacetylase (HDAC).\(^5\) HDAC could remove the acetyl on histone H3/H4 lysine residual to expose positively charged lysine residual and the interaction between positively charged lysine residual and DNA could restrict the movement of nucleosome on DNA, making transcriptional regulatory element inaccessible for promoter. Previous results showed that demethylation of histone played a critical role in initial gene silencing of the hypermethylated promoter region while acetylation of histone could no longer activate the gene if silenced.\(^6,7\)

Our results were consistent with the previous results in which promoter hypermethylation could result in hMLH1 gene silencing.\(^8-10\) while promoter hypermethylation was also associated with the resistance of tumor cells to DDP.\(^3\) DNA methylation played an important role in gene expression regulation, cellular proliferation, differentiation and development and tumorgenesis. As a biochemical modification, DNA methylation was mainly defined as the attachment of a methyl group to 5’C in cytosine with the action of DNA methyltransferase.\(^11\) DNA methylation of CpG sites in promoter region typically resulted in inhibition of gene transcription through the mechanism possibly associated with the following factors: (1) methylation of cytosine likely to interfere with the binding of transcription factors to CpG sequence in the recognizing sites; (2) transcription inhibition likely to be associated with two protein complexes (MeCP1 and MeCP2), which were capable of binding to methylated CpG sequence specifically to interfere with the binding of transcription factors to CpG sequence.\(^12\) Our study found that 5-Aza-dC was a DNA methyltransferase inhibitor, capable of binding to DNA methyltransferase and reducing the bioactivity of DNA methyltransferase to decrease the methylation level and regulate the gene expression.\(^10,13\)

We found that 5-Aza-dC was not only used for cellular demethylation in vitro but also for treatment of leukemia, myelodysplastic syndrome (MDS) and non-small cell lung cancer with excellent efficacy achieved.\(^14-16\) In 2004, 5-Aza-dC was approved by the FDA for treatment of MDS.\(^15\) Our results showed that 5-Aza-dC was able to demethylate the hMLH1 promoter region in resistant cells and activate the gene transcription. Thus, demethylation is an effective target to reverse the resistance as methylation could silence DNA mismatch repair genes resulting in resistance to chemotherapy, which can be reversed by demethylating agents. This undoubtedly provides an effective strategy for the increasing intensifying resistance of ovarian cancer, and it should be further studied by detailed procedures in clinical trials.

### References