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Delineating the effect of demethylating agent 5-aza-2'-deoxycytidine on human Caco-2 colonic carcinoma cells

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Abstract. Aberrant epigenetic changes are known to contribute to various phases of tumor development. The gene function loss caused by aberrant methylation is analogous to genetic mutations. Unlike genetic mutations, epigenetic alterations can be reversed. 5-Aza-2'-deoxycytidine (5-aza-CdR) has been approved by the Food and Drug Administration for the treatment of certain types of cancer, such as MDS and leukemia. The aim of the present study was to determine whether 5-aza-CdR has the potential to be used in the treatment of colon cancer using a human Caco-2 colonic carcinoma cell line. The effect of 5-aza-CdR on cell proliferation, cell cycle, apoptosis and reversal of aberrant methylation of the Ras association domain family 1A (*RASSF1A*) gene was also examined. The 5-aza-CdR was prepared at different concentrations in sterile tri-distilled water at 0.4, 1.6, 6.4, 25.6 and 102.4 μmol/l and employed to treat the human Caco-2 colonic carcinoma cells. An MTT assay was used to detect the effect of 5-aza-CdR on cell proliferation. Flow cytometry was used to examine the cell cycle and apoptosis. The *RASSF1A* mRNA transcript level was examined by reverse transcription-polymerase chain reaction. The results showed that 5-aza-CdR inhibited the proliferation of Caco-2 cells in a time- and concentration-dependent manner ($p<0.01$). The 5-aza-CdR treatment affected the cell cycle and caused accumulation of cells in the G0/G1 phase and this effect was concentration-dependent ($p<0.05$). 5-aza-CdR treatment caused an increase in the number of cells undergoing apoptosis and reactivated the *RASSF1A* tumor suppressor gene that was silenced by hypermethylation in Caco-2 cells. In conclusion, 5-aza-CdR inhibited growth and promoted apoptosis in Caco-2 cells by upregulating the epigenetically silenced tumor suppressor *RASSF1A* gene.

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Introduction

The biological consequence of gene function loss caused by DNA methylation is analogous to the consequences of gene mutation (1). Unlike gene mutation, DNA hypermethylation can be reversed pharmacologically using DNA demethylating agents. An association between DNA methylation of tumor suppressor genes and the development of colorectal cancer has been previously reported (2-5). The re-activation of tumor suppressor genes that are silenced by DNA hypermethylation is commonly termed epigenetic therapy, which is a feasible and achievable strategy for cancer treatment. Previous *in vitro* experiments identified that, 5-aza-2'-deoxycytidine (5-aza-CdR) can reactivate epigenetically silenced tumor suppressor genes, thereby restoring their inherent anti-cancer effect.

The Ras association domain family 1A (*RASSF1A*) gene is located in the short arm of chromosome 3, originally found as a novel candidate tumor suppressor in lung cancer (6,7). The aim of the present study was to examine the effect of 5-aza-CdR on proliferation, cell cycle and apoptosis in Caco-2 cells *in vitro*. In addition, a semi-quantitative analysis of *RASSF1A* transcripts was carried out to determine the reactivation of the tumor suppressive function and whether 5-aza-CdR can be extended to treat colon cancer.

Materials and methods

Cell lines and culture. Human Caco-2 colon adenocarcinoma cells, purchased from Shanghai Jiahe Biotechnology Co., Ltd., Shanghai, China, were cultured in RPMI-1640 medium supplemented with 100 ml/l calf serum (Wisent, Nanjing, China), 100 kU/l streptomycin (Wisent), and 100 kU/l penicillin (Wisent) at 37˚C with 5% CO_2. Subsequently, 5-aza-CdR (Sigma, St. Louis, MO, USA) was dissolved in tri-distilled water and stored at 70˚C. The desired concentration of 5-aza-CdR was obtained by serial dilution of the stock solution.

Monoplast suspension was obtained by digesting the Caco-2 cells in the logarithmic phase using trypsin (2.5 g/l). This monoplast suspension was cultured and passaged to obtain the concentration of $2x10^6$/l. The cell suspension was then treated with 5-aza-CdR at different concentrations of 0.4, 1.6, 6.4, 25.6 and 102.4 μmol/l. At every 24 h, the medium was aspirated and replaced with fresh RPMI-1640 medium containing

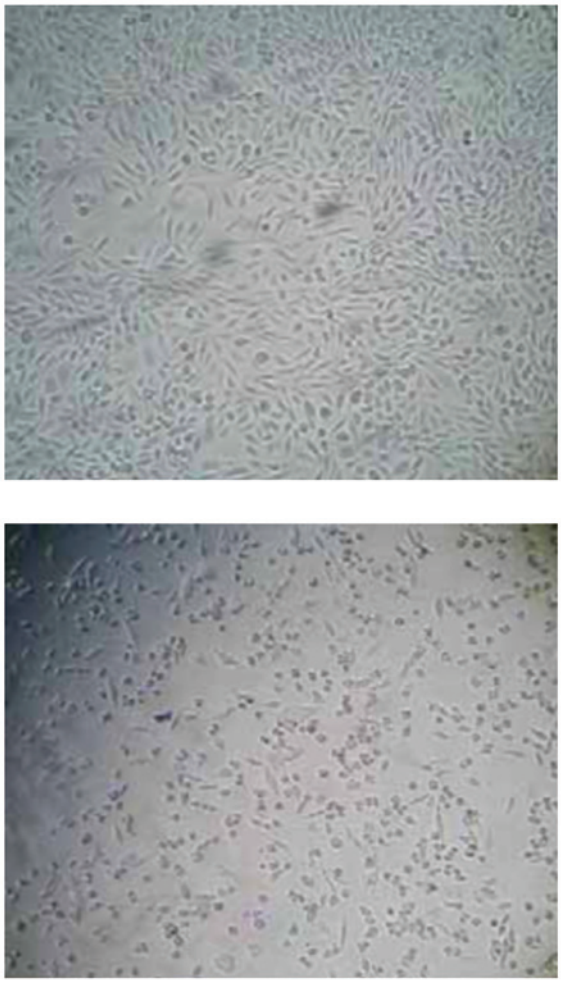

Figure 1. (A and B) Caco-2 cell morphology following treatment with 6.4 μmol/l 5-aza-2'-deoxycytidine for 3 days. A decrease in volume and density was identified.

Table I. Inhibition of cell proliferation by 5-aza-CdR in Caco-2 cells.

	Duration of exposure of cells to 5-aza-CdR				
Concentration of 5-aza-CdR, μmol/l	Day 1 (%)	Day 2 (%)	Day 3 (%)	Day 4 (%)	Day 5 (%)
0.4	97	94	92	90	89
1.6	90	84	75	66	50
6.4	80	74	68	56	40
25.8	71	43	30	11	6
102.4	48	40	9	7	5

Concentration (F=44.079, p<0.01), duration of exposure (F=12.250, p<0.01). 5-aza-CdR, 5-aza-2'-deoxycytidine.

Table II. Cell cycle and apoptosis in Caco-2 cells treated with 5-aza-CdR.

	Cell cycle			
Concentration of 5-aza-CdR, μmol/l	Sub-G1 phase	G1 phase	S phase	G2 phase
0	1.78	56.21	22.01	15.27
0.4	18.90	57.21	15.99	8.94
1.6	37.81	45.31	9.98	7.54
6.4	49.25	38.46	9.35	5.09
25.6	38.21	44.67	6.72	3.75

t=3.98, p<0.05 vs. 0 μmol/l. 5-aza-CdR, 5-aza-2'-deoxycytidine.

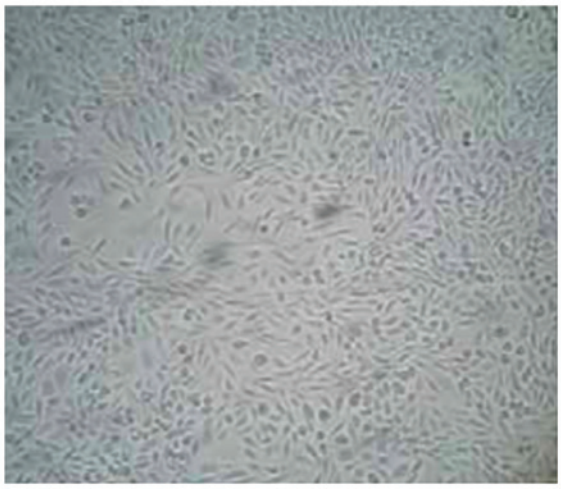

Figure 2. Caco-2 cell morphology without treatment.

the same concentration of 5-aza-CdR and this process was repeated for 3 days. The RPMI-1640 medium containing the drug was then replaced by complete culture medium and incubated for 4-days. The same procedure as described above was performed with the exception of 5-aza-CdR in cultured cells, which served as the control. During the incubation process, morphological changes in the cells treated with 5-aza-CdR were observed using phase contrast microscope (Aipuda, Shanghai,China).

Growth curve using MTT assay. Caco-2 cells were seeded in a 96-well plate at a density of $3x10^3$ to a final volume of 200 μl. Cell culture medium containing a concentration of 5 g/l of 5-aza-CdR was changed regularly. A negative control (without 5-aza-CdR) and a blank control (without cells) were included in each plate. MTT (20 μl) was added to each well and incubated for 4 h at 37˚C. Following incubation, MTT was aspirated and the cells were rinsed twice with PBS. This step was followed by the addition of 150 μl of DMSO and incubation for 15 min. The optical density (OD) was determined at 570 nm in an ELISA reader (Perlong, Beijing, China). Cell proliferation was calculated according to the formula: Cell proliferation = (OD of treated - OD of blank)/(OD of the negative control-OD of the blank) x 100%.

Cell cycle and apoptosis. The 5-aza-CdR-treated cells were collected and rinsed twice in PBS. The cells were adjusted to

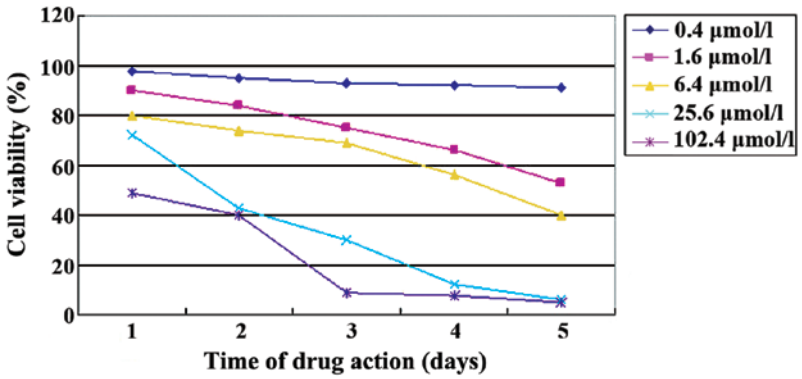

Figure 3. Cell growth curve at different concentrations of 5-aza-2'-deoxycytidine.

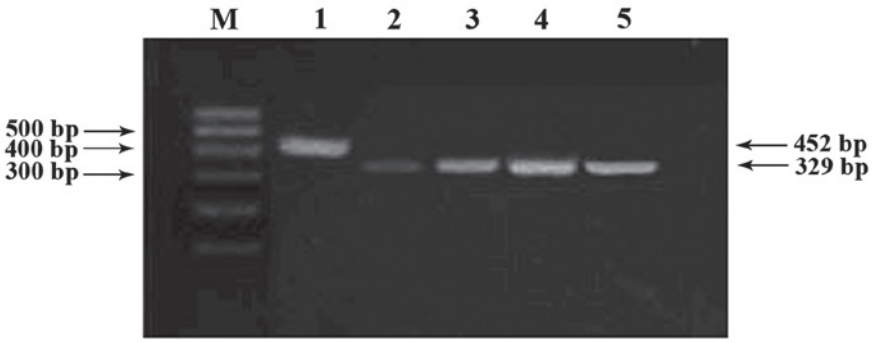

Figure 4. Ras association domain family 1A expression in 5-aza-2'-deoxycytidine (5-aza-CdR)-treated cells. Lane M, DNA molecular weight marker; lane 1, GAPDH; lane 2, 0.4 μmol/l (5-aza-CdR); lane 3, 1.6 μmol/l; lane 4, 6.4 μmol/l; and lane 5, 25.6 μmol/l.

contain a cell density of $1x10^9$/l in a flask. Subsequently, 5 ml of ice cold hexanol (700 ml/l) was added to immobilize the cells for 24 h. RNase A (Solarbio, Beijing, China) then was added (1 g/l). Propidium iodide (Leagene, Beijing, China) was added at a final concentration of 50 mg/l and incubated for 30 min at 37˚C. The cell cycle and apoptosis were determined in a flow cytometer (Potenov, Bejing, China).

Reverse transcription-polymerase chain reaction (RT-PCR). TRIzol® reagent (Leagene, Beijing, China) was used to extract total RNA from the treated and untreated cells. The extracted total RNA was then reverse transcribed. Briefly, 2 μg of total RNA was added to the pre-existing mixture of 1 μl 10X reaction buffer (Leagene) with $MgCl_2$ and 1 μl DNase I. The non-specific inhibitor diethylpyrocarbonate (DEPC)-treated water was added to increase the volume to 10 μl, followed by incubation for 30 min at 37˚C. Subsequently, 1 μl Oligo dT18 was added and mixed gently. A centrifugal separation step at 1,000 x g was performed after 5 min incubation at 70˚C. The tube was kept on ice. For reverse transcription, 5 μl 5X Moloney Murine Leukemia Virus (M-MLV) buffer, 1.25 μl deoxyribonucleotide (dNTP) mixture, 1 μl M-MLV, 0.5 μl RNasin and DEPC-treated water were added until a total volume of 25 μl was achieved. Incubation was performed again for 15 min at 72˚C. RT-PCR was performed in a total volume of 25 μl and the constituents used were: 2.5 μl 10X PCR buffer, 0.5 μl dNTP mixture, 0.625 μl MBI TaqDNA polymerase, 1 μl primer 1 (10 μmol/l), 1 μl primer 2 (10 μmol/l), 1.5 μl $MgCl_2$, 1 μl cDNA and sterile distilled water (final volume of 25 μl). *RASSF1A*-specific primers were used to achieve PCR amplification. GAPDH was selected as a reference owing to its stable expression (8). *RASSF1A* primers were selected from a previously published study (9). The primers used were: forward: 5'-GGCGTCGTGCGCAAAGGCC-3' and reverse: 5'-GGGTGGCTTCTTGCTGGAGGG-3'. The primer sequences for GAPDH were: forward 5'-ACCACA GTCCATGCCATCAC-3' and reverse 5'-TCCACCACCCTG TTGCTGTA-3'. The PCR amplification step consisted of initial denaturation at 95˚C for 5 min, 35 cycles of denaturation at 94˚C for 30 sec, annealing at 56˚C for 30 sec, extension at 72˚C for 60 sec and a final extension at 72˚C for 5 min. Thus the PCR amplicons were visualized on 2% agarose gel (Novelab, Shanghai, China).

Statistical analysis. Experimental data were processed using SPSS software (IBM, Armonk, NY, USA) and F-test and T-tests were performed.

Results

Morphology and cell proliferation. Morphological changes in Caco-2 cells prior to and following 5-aza-CdR treatment were observed under an inverted microscope (Dygx, Shanghai, China). The treated cells decreased in volume and density, and died (Fig. 1A and B). No such abnormalities were observed in the untreated cells (Fig. 2). The MTT assay showed that 5-aza-CdR inhibited Caco-2 cell proliferation. The number of cells in which proliferation was inhibited by 5-aza-CdR

was elevated with an increasing concentration of 5-aza-CdR ($F=44.079$, $p<0.01$) and exposure time ($F=12.250$, $p<0.01$, Table I, Fig. 3) was observed.

Cell cycle and apoptosis. 5-aza-CdR treatment induced cell cycle arrest and caused accumulation of cells in the G0/G1 phase. Accumulation of the G0/G1 phase cells was enhanced with an increasing dose of 5-aza-CdR (Table II). Flow cytometry showed that the percentage of apoptotic cells in the absence of 5-aza-CdR was 1.78% while the same increased to 49.25% when they were treated with 6.4 μmol/l of 5-aza-CdR, and this difference was statistically significant ($t=3.98$, $p<0.05$ vs. 0 μmol/l; Table II). When 5-aza-CdR concentration reached 102.4 μmol/l, cell necrosis instead of cell apoptosis occurred.

RT-PCR analysis of the RASSF1A gene. Caco-2 cells originally lacking *RASSF1A* gene expression were treated with 5-aza-CdR. Re-expressed *RASSF1A* mRNA was dependent on the concentration of 5-aza-CdR as observed in the 2% agarose gel after RT-PCR analysis (Fig. 4).

Discussion

5-aza-CdR has been identified to be effective in treating recurrent, intractable, acute and chronic myelogenous leukaemia (10,11). However, its effectiveness against solid tumors remains unclear. Hypomethylation and hypermethylation have been observed in various types of cancer (12,13). Hypomethylation can contribute to genomic instability, activation of oncogenes, or loss of imprinting. Gene-specific promoter hypermethylation in tumor suppressor genes cause silencing of tumor suppressors, which can contribute to many of the hallmarks of cancer such as evading apoptosis, insensitivity to antigrowth signals, sustained angiogenesis, limitless replicative potential and tissue invasion and metastasis. Previous findings have shown that aberrant gene methylation in cancer renders them resistant to chemotherapeutics via inhibition of apoptosis. Since methylation involves changes in gene regulation but not the DNA sequence, the change is reversible. The silenced tumor suppressor genes can be re-expressed when hypermethylation in their promoter region is removed that may ultimately repress tumor growth (14-19).

In the current study, a concentration- and time-dependent inhibition of proliferation of Caco-2 cells was identified following treatment with different concentrations of 5-aza-CdR. The percentage of Caco-2 cells in the G0/G1 phase was enhanced with an increasing the dose of 5-aza-CdR, thereby increasing the cells that undergo apoptosis. The morphological changes including decreased cell volume and density and cell death were observed at low doses of 5-aza-CdR, whereas cell disruption and necrosis were observed at higher doses of 5-aza-CdR. The changes were similar to the cytotoxic effects attributed to chemotherapeutic drugs where apoptosis occurs at lower doses and necrosis at higher doses. Additionally, the *RASSF1A* gene, which is silenced by hypermethylation in Caco-2 cells, was reactivated by the 5-aza-CdR treatment. The mRNA expression of *RASSF1A* gene was identified even after five successive generations. The reason for this might be the demethylating effect of 5-aza-CdR. The re-expression therefore contributed to the tumor suppressive function in Caco-2 cells. It is also possible that the cytotoxicity of 5-aza-CdR leads to an anticancer effect. However, 5-aza-CdR does not lead to an anticancer effect by exerting cytotoxity on cancer cells. Studies (20-22) have been carried out in which Ara-C, an equally cytotoxic drug as 5-aza-CdR, was used to determine whether the anticancer effect attributed by 5-aza-CdR was due to cytotoxicity in bladder cancer cells. Ara-C did not possess a demethylating capability. The two drugs inhibited cell proliferation although the inhibitory effect of Ara-C was not transmitted, demonstrating that inhibitory effect of 5-Aza-CdR is not derived from cytotoxicity.

It has been reported that Ras-GTPase is a member of the superfamily of molecular switches regulating proliferation and apoptosis (23-27). It performs different functions dependent on the signal molecule. Ras-GTPase interacts with a series of different downstream effector molecules to promote cell growth and differentiation, inducing cell dormancy, terminal differentiation and apoptosis in order to suppress cell growth.

RASSF1A gene methylation was found to be present in various types of cancer. Previous studies (28,29) using methylation-specific PCR examined colorectal cancer and identified that *RASSF1A* CpG-island in the neoplastic foci region methylated more frequently than the periphery of the neoplastic foci. Kuroki *et al* (30,31) and other investigators (32-34) using methylation-specific PCR analyzed esophageal carcinoma, gastric carcinoma and bladder cancer and observed that, *RASSF1A* was hypermethylated and the degree of methylation correlated closely with the clinical stages of patients. *RASSF1A* gene expression was silenced by hypermethylation of the CPG island in the promoter region of a wide range of tumors. Müller *et al* (35) analyzed the aberrant DNA methylation of *RASSF1A* in breast cancer and found that patients with aberrant *RASSF1A* methylation had a poorer prognosis.

In conclusion, the current findings suggest that *RASSF1A* can result in an antitumor effect when Caco-2 cells are treated with 5-aza-CdR. The demethylating agent 5-aza-CdR embraces good prospects in antitumor therapy, given the universality of regional hypermethylation in tumor cells.

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