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Original article

# 5-Aza-2′-deoxycytidine reactivates expression of RUNX3 by deletion of DNA methyltransferases leading to caspase independent apoptosis in colorectal cancer Lovo cells

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**Abstract**

The DNA methylation inhibitor 5-Aza-2′-deoxycytidine (5-Aza-CdR) has therapeutic value for the treatment of cancer. However, the mechanism by which 5-Aza-CdR induces antineoplastic activity is an important unresolved question. In this study, we found that 5-Aza-CdR at limited concentrations induced inhibition of colorectal cancer Lovo cell proliferation as well as increased apoptosis caused by DNA damage, which was independent of the caspase pathway. Regarding the mechanisms, for the first time, we examined that cytotoxicity against Lovo cells was regulated via down-regulation of DNA methyltransferase 3a, DNMT3b and then reactivated the expression of RUNX3. We therefore conclude that RUNX3 is a relevant target for methyltransferases dependent effects of 5-Aza-CdR on colorectal cancer Lovo cells.


*Keywords:* 5-Aza-2′-deoxycytidine; Apoptosis; Caspase 3; DNA methyltransferases; RUNX3

## 1. Introduction

Because the causal relationship between hypermethylation of the promotor of tumor suppressor genes and the development of cancer has been clearly demonstrated [1,2], specific demethylating agents are of interest for use in molecular targeting [3]. 5-Aza-cytidine and 5-Aza-2′-deoxycytidine (5-Aza-CdR) belong to a class of cytosine analogues which are developed as inhibitors of DNA methylation and have been shown to have significant cytotoxic and antineoplastic activities in many experimental tumors [3,4]. 5-Aza-CdR, however, is reported to be noncarcinogenic and incorporates into DNA but not RNA or protein [5]. Additionally, considerable evidence shows that 5-Aza-CdR has been found empirically to have more potent therapeutic effects than 5-Aza-cytidine in cell culture and animal models of human cancer. Recently, several clinical trials of 5-Aza-CdR have been reported, including a phase II study of 5-Aza-CdR in patients with metastatic prostate cancer and a phase III study of 5-Aza-CdR (decitabine) in patients with myelodysplasia [6]. Clinical trials evaluating 5-Aza-CdR as a cancer chemotherapeutic have shown promise for the treatment of leukemia but less utility against solid tumors.

The biological activity of 5-Aza-CdR is associated with its incorporation into DNA where it binds DNA methyltransferase (DNMT) in an irreversible, covalent manner, thus sequestering the enzyme and preventing maintenance of the methylation state [7]. Consequently, silenced genes induced by hypermethylation are re-expressed by depleting the cells of DNMT activity. Based on the chemical mechanism of 5-Aza-CdR activity, a number of nonmutually exclusive mechanisms of its tumor cytotoxicity have been proposed. Among these, two major mechanisms are (a) demethylation of cellular DNA, with reactivation of silenced genes; and (b) induction of DNA damage due to the formation of irreversible, covalent enzyme-DNA adducts [8]. The relative contribution of gene reactivation and enzyme-DNA adduct formation to the efficacy and toxicity of 5-Aza-CdR in vivo is an important unresolved question.

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As one of the major causes of cancer death, colorectal cancer remains threatening around the world and most patients in advanced stages need chemotherapy. In our investigation, we showed that 5-Aza-CdR was cytotoxic against colorectal cancer Lovo cells and overcame the growth and survival advantages. Mechanistic studies demonstrated that 5-Aza-CdR induced DNA damage and caused apoptosis of colorectal cancer Lovo cells, which was independent of the caspase pathway. These cytotoxic effects of 5-Aza-CdR were mediated via deletion in DNA methyltransferases 3a and 3b levels predominantly, leading to the restoration of RUNX3 expression through demethylation in Lovo cells.

## 2. Materials and methods

### 2.1. Cells and treatments

Human colorectal cancer cell line Lovo was obtained from China Center for Type Culture Collection (CCTCC). Cells were grown in DMEM containing 10% fetal bovine serum at 37 °C in a humidified atmosphere with 5% $CO_2$. For treatment with 5-Aza-CdR (Sigma), the cells were exposed to a single pulse of 0.01–100 μM of the drug. 5-Aza-CdR was dissolved in phosphate-buffered saline and fresh medium containing 5-Aza-CdR was added every 24 h.

### 2.2. MTT assay

Cell proliferation was measured using MTT assay. Cells were plated in triplicate at $1 \times 10^3$ cells per well in 96-well plates, cultured as described above, and treated in the presence of increasing concentrations of 5-Aza-CdR (or vehicle control) for 72 h and different times (24, 48, 72 and 96 h) respectively. Twenty microliters of 5 mg/ml of MTT (Amresco) was then added into each well and the cells cultured at 37 °C for an additional 4–6 h. The resulting formazan crystals were solubilized by the addition of 150 μl of DMSO to each well. The optical density level under 570 nm was measured and the percentage of cell viability was calculated using the following formula: percentage of cell viability = (absorbance of experimental well − absorbance of blank)/(absorbance of untreated control well − absorbance of blank) × 100%.

### 2.3. Colony forming assay

To measure the cell colony formation ability, indicated concentrations of 5-Aza-CdR were added to the medium. Briefly, about 30–70 cells per well were exposed to 5-Aza-CdR or without for 72 h and then the cells were allowed to grow in fresh medium for the next 10–15 days. The cells were fixed in 70% ethanol and stained with 10% (v/v) Giemsa (Merck). Colonies that consisted of more than 50 cells were counted. The inhibitory rate (IR) was calculated as follows: IR (%) = (number of colonies formed in the control group − number of colonies formed in the test group)/number of colonies formed in the control group.

### 2.4. Apoptosis assay

#### 2.4.1. Annexin V staining

Cells ($5–7 \times 10^5$) were seeded into 6-well plates and treated with 0.5, 1, 5 μM of 5-Aza-CdR and/or z-VAD-fmk (Beyotime Institute of Biotechnology) for 48 h, immediately trypsinized, washed in PBS, and then the manufacturer's instructions of the Annexin V Staining Kit (MultiSciences Biotech Co. Ltd) were followed.

#### 2.4.2. DNA ladder assay

Briefly, for the DNA ladder assay, $5 \times 10^6$ cells were collected by centrifugation and washed with phosphate-buffered saline twice. Then the cell pellets were resuspended in lysis buffer (100 mM NaCl, 10 mM Tris–HCl, 25 mM ethylene diamine tetraacetate [EDTA], and 0.5% SDS) containing 0.1 mg/ml proteinase K and then incubated overnight at 55 °C. DNA was cleared from the lysates by centrifugation at 12,000 and extracted with an equal volume of phenol/chloroform/isoamylol. After that, the DNA pellet was washed with 75% ethanol and resuspended in TE buffer containing 1 μg/ml RNase A at 37 °C for 1 h. DNA fragments were separated by 2.0% agarose gel electrophoresis, stained with ethidium bromide, and photographed under UV light.

#### 2.4.3. Morphological detection of apoptosis

Morphological evaluation of apoptotic cell death was performed using the Hoechst 33258 kit according to the manufacturer's instructions (Beyotime Institute of Biotechnology). Briefly, $5 \times 10^4$ Lovo cells were seeded onto coverslips in 6-well culture plates. After treatment with 5 μM of 5-Aza-CdR for 72 h, the cells were fixed at 4 °C overnight. Next day, the cells were stained with 500 μl of Hoechst 33258 for 5 min and then subjected to fluorescence microscopy.

### 2.5. Comet assay for detecting DNA strand breaks

The comet assay, also called single-cell gel electrophoresis, was performed as described previously [9]. In brief, slides were cleaned with acid wash and scrapped with 40 μl of 0.6% agarose. Twenty microliters of cell suspension and 80 μl of 1.1% low-melting agarose were mixed and added to the first gel layer. Immediately, the coverslip was laid and then kept at 4 °C for 15 min to allow solidification. After gently removing the coverslip, the slides were immersed in freshly prepared cold lysing solution (2.5 M NaCl, 100 mM $Na_2EDTA$, 10 mM Tris, pH 10.0) with 1% Triton X-100 and 10% DMSO for at least 1 h at 4 °C. After electrophoresis in fresh solution (1 mM $Na_2EDTA$, 300 mM NaOH, pH 13.0) for 30 min, the slides were then placed in Tris buffer (0.4 M Tris, pH 7.5) for 15 min twice. The slides were then stained with 40 μl of 0.1 mg/ml of propidium iodide (PI) and 100 randomly selected cells were counted per slide. The images were captured and scored for each sample using an image analysis software system (IMI ver. 1.0). The standard of assessing DNA single strand breaks was based on the percentage of cells with tail and tail length

(a distance from the DNA head to the end of the DNA tail) by visual estimation.

### 2.6. Measurement of caspase 3 activity

For the cell lysate, caspase 3 activity was measured using a colorimetric kit according to the manufacturer's instructions (Beyotime Institute of Biotechnology). This kit detected the hydrolysis of acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) by caspase 3, resulting in the release of p-nitroanilide (pNA). The absorbance was determined at 405 nm and the activity of caspase 3 was determined by calculating the ratio of OD405 nm of drug treated cells to untreated cells.

### 2.7. RNA preparation and RT-PCR

Total RNA was isolated using the Trizol reagent (Invitrogen) and 1 μg of RNA was used as a template for the synthesis of cDNA using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's instructions. Polymerase chain reaction (PCR) analysis was performed in a final volume of 25 μl using PCR Master Mix (Fermentas). Primer sequences and annealing temperatures are indicated in Table 1. PCR products were separated on 1.5% agarose gels, stained with ethidium bromide and photographed.

### 2.8. Western blotting

Cells were cultured with the indicated concentrations of 5-Aza-CdR for the specified times, harvested, washed, and lysed using RIPA buffer containing protease inhibitors. After normalization for total protein content (50 μg/lane), the samples were subjected to 15% SDS–PAGE and then transferred to nitrocellulose membrane (Bio-Rad). After blocking with 5% nonfat dry milk and 0.1% Tween-20 in Tris buffered saline, the membranes were incubated with mouse anti-$p21^{Waf1/Cip1}$, p53, MMP 2 (Santa Cruz), goat anti-RUNX3 (R and D), MMP 9, rabbit anti-caspase 3, β-tubulin (Santa Cruz). After extensive rinsing with TBST buffer, blots were incubated with HRP-conjugated anti-rabbit, anti-mouse or anti-goat secondary antibodies (Pierce), developed with the use of an enhanced chemiluminescence system (Millipore) and captured on a light-sensitive imaging film (Kodak, Tokyo, Japan).

### 2.9. Statistical analysis

All determinations were repeated in triplicate. Data are presented as means ± SD. Levels of significance for comparisons between samples were determined using the Student's *t*-test distribution. Statistical analyses between more than three samples were performed by ANOVA with Tukey's honest significant difference post hoc test applied to the significant main effects or interactions (SPSS 11.5 for Windows).

## 3. Results

### 3.1. Dose and duration dependent inhibition of cell proliferation by 5-Aza-CdR

In this study, human colorectal cancer Lovo cells were treated with 5-Aza-CdR at different concentrations for 72 h and the cell viability was determined by MTT assay. A dose dependent inhibition of cell proliferation was observed (Fig. 1A). Subsequently, Lovo cells were treated with 1 μM of 5-Aza-CdR for the indicated times to evaluate the duration effects of 5-Aza-CdR on cell growth. This dose was selected as it induced 50% of the cell growth inhibition in Lovo cells and was reported to be available in many investigations. In the experiment, we observed not only dose dependent growth suppression, but also time dependent manner in Lovo cells (Fig. 1B).

Another antiproliferative study for assessing the long term effects of 5-Aza-CdR on cell growth is an extremely sensitive colony formation assay. Based on the data examined above, we selected three doses of 0.01, 0.5, and 1 μM of 5-Aza-CdR for this assay. Compared with untreated cells, cells treated with 5-Aza-CdR at 0.01, 0.5, 1 μM were examined 36.33%, 63%, and 90.33% of inhibition rate respectively, as monitored by colonial formation assay (Fig. 1C). Consistent with the results of the MTT reduction assay, the colony formation assay further showed a dose dependent inhibitory effect of 5-Aza-CdR within the treatment period. Interestingly, we found that 5-Aza-CdR had much more effects on the long term in Lovo cells than short term, as the inhibition rate was 90.33% at 1 μM of 5-Aza-CdR in the colony formation assay whereas cell viability reached 53.25% in the MTT assay.

Table 1
Primers and conditions used for RT-PCR

| Primers | Sequences | Temperatures (°C) | Product length (bp) |
|---|---|---|---|
| GAPDH | F: 5′-ACGGATTTGGTCGTATTGGG-3′<br>R: 5′-TCCTGGAAGATGGTGATGGG-3′ | 56 | 211 |
| RUNX3 | F: 5′-GAAAAGGCGTAAGGGAACTC-3′<br>R: ACCTGGGACCAGCTATAACC-3′ | 56 | 395 |
| DNMT1 | F: 5′-CGCTGTATCTAGCAAGGGTCA-3′<br>R: 5′-TCGAATCTCGCGTAGTCTTG-3′ | 56 | 313 |
| DNMT3a | F: 5′-ACCACAGAGGCGGAAATACC-3′<br>R: 5′-GTCTCCCTGCTGCTAACTGG-3′ | 56 | 543 |
| DNMT3b | F: 5′-CAGGAGACCTACCCTCCACA-3′<br>R: 5′-TTACGTCGTGGCTCCAGTTA-3′ | 56 | 436 |

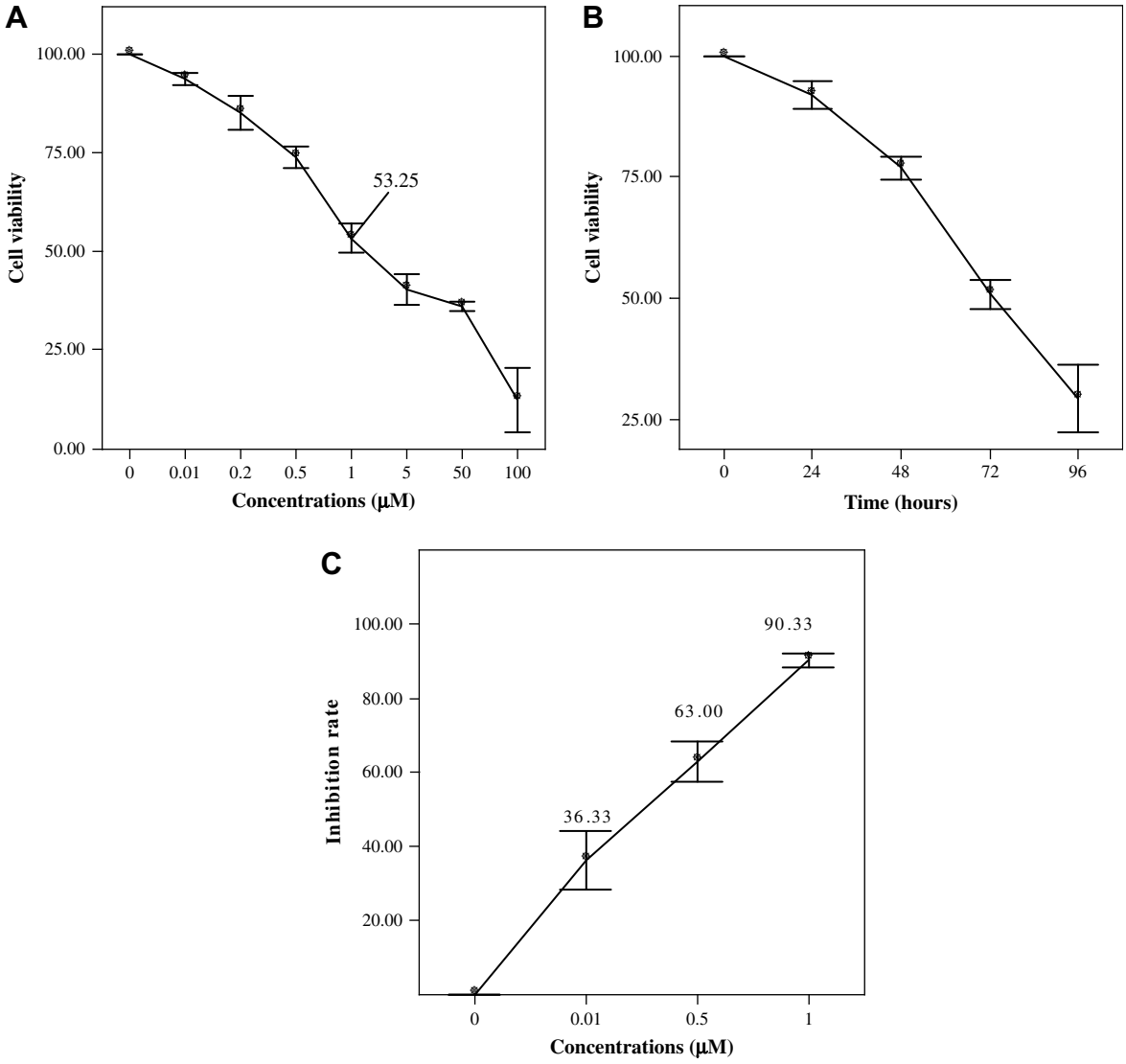


Fig. 1. 5-Aza-CdR induced cell proliferation inhibition in colorectal cancer Lovo cells. Cell viability was measured with MTT when Lovo cells were treated with 5-Aza-CdR at 0–100 μM for 72 h (A) and at 1 μM for different intervals (B). (C) 5-Aza-CdR treatment results in decreased clonogenic survival. Lovo cells were treated with 0, 0.01, 0.5 and 1 μM of 5-Aza-CdR for 72 h, followed by replacement of the medium and continued growth for 10–15 days. Results are presented as the average of quadruplicate measurements, and the bar is the standard deviation.

### *3.2. Effects of 5-Aza-CdR on cell apoptosis*

To confirm the results obtained from the MTT assays and to elucidate the mechanisms of action of the effect of 5-Aza-CdR, we analyzed the induction of apoptosis using an annexin V flow cytometry assay. In this investigation, Lovo cells were treated with 0.5, 1, and 5 μM of 5-Aza-CdR and detected after 48 h culture. Our data revealed that 5-Aza-CdR treatment increased the proportion of cells positive for annexin V from 4.6% in the pretreated cells to 16.6% in the cells treated with 0.5 μM of 5-Aza-CdR, which peaked at 5 μM (52.4%), in a dose dependent fashion. The result strongly suggested that apoptosis rather than necrosis was the mechanism of 5-Aza-CdR induced growth inhibition in Lovo cells (Fig. 2A).

As we know, apoptotic cells can be characterized by a number of morphologic, molecular, and biochemical features, including shrinkage of cells, blebbing of cells and nuclear membranes, compaction and condensation of chromatin toward the nuclear periphery, and fragmentation of DNA into oligonucleosomes. Detection of more than one of these parameters is essential for the clear characterization of apoptotic cells. Therefore, we performed DNA ladder and Hoechst 33258 staining to further confirm apoptosis of Lovo cells triggered at different concentrations of 5-Aza-CdR. As presented in Fig. 2B, the cells displayed a characteristic "DNA ladders" pattern of apoptosis in the course of treatment with 1 and 5 μM of 5-Aza-CdR compared with untreated cells. Hoechst 33258 staining, a classical way of identifying the morphology of apoptotic cells, displayed that pretreated Lovo cells appeared normal nuclear size and blue fluorescence, whereas cells incubated continuously with 5 μM of 5-Aza-CdR for 72 h started to change their shape (they shrunk and round up).

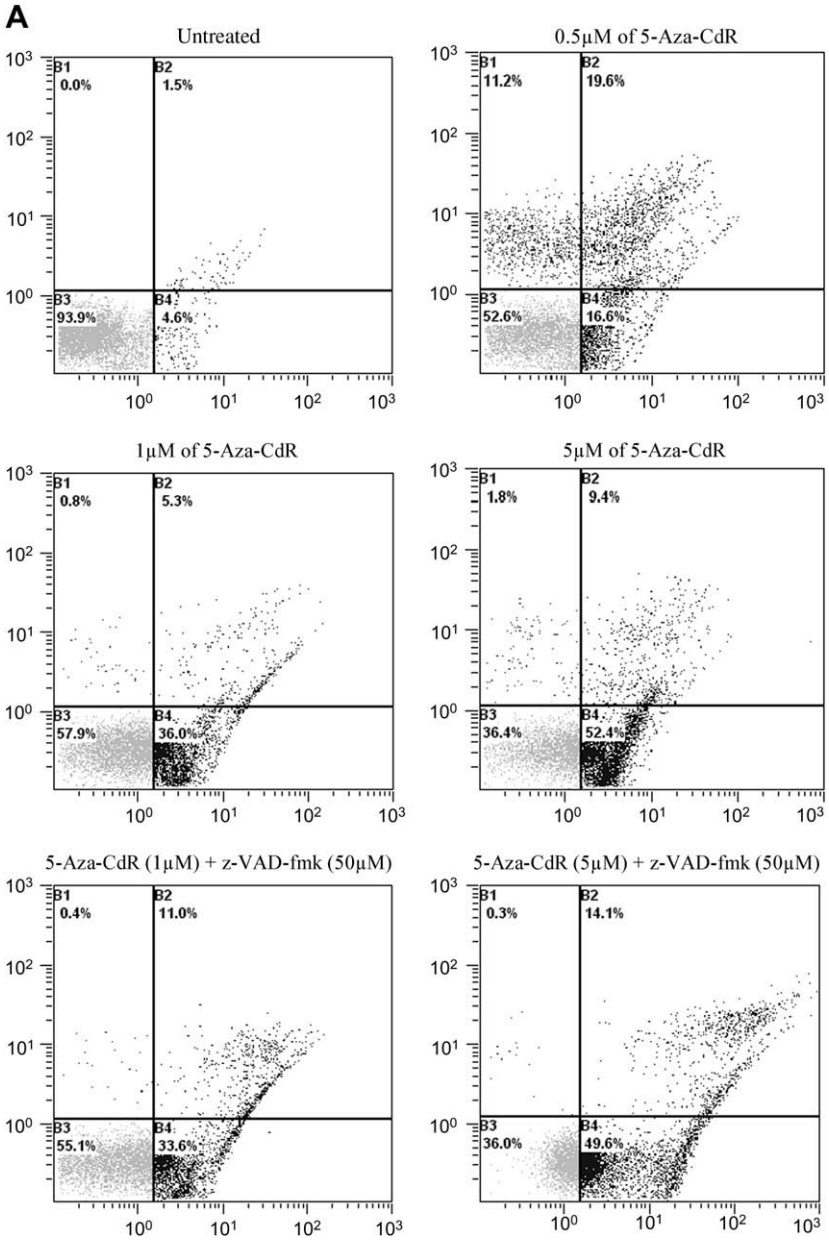


Fig. 2. The apoptotic effects of 5-Aza-CdR in Lovo cells. (A) The percentage of apoptotic cells was scored after cell exposure to 5-Aza-CdR and/or z-VAD-fmk at the indicated concentration for 48 h by the annexin V flow cytometric assay method, which can detect cells in an earlier stage of the apoptotic pathway and distinguish among apoptotic and necrotic cells. (B) Lovo cells treated with 5-Aza-CdR at higher concentration (1 and 5 μM) were observed nucleosomal DNA fragmentation significantly. Lane1: marker; lane 2: untreated. Lane 2: 0.01 μM; lane 3: 0.2 μM; lane 4: 0.5 μM; lane 5: 1 μM; lane 6: 5 μM (5-Aza-CdR). (C) After following with 5 μM of 5-Aza-CdR treatment for 72 h, Lovo cells showed the typical apoptotic nuclear morphology. The arrow indicates the condensed nucleus.

Specifically, the highly condensed chromatin of the nucleus as well as fragments of chromatin were presented after 5-Aza-CdR exposure (Fig. 2C).

### *3.3. Treatment with 5-Aza-CdR caused DNA damage*

Since 5-Aza-CdR has been reported to incorporate into DNA and not RNA [5], we studied the effect of 5-Aza-CdR on DNA damage using the comet assay. Lovo cells were treated with 5-Aza-CdR at 0.5, 1, and 5 μM for 72 h and then cultured for this assay. As shown in Fig. 3A, dose dependent DNA damage was observed by the 72 h treatment. Compared with the untreated Lovo cells, 5-Aza-CdR even at a low concentration (0.5 μM) induced DNA damage, as indicated by the presence of cells with the comet tail (14.7%). More comet cells and longer DNA tail length showed more extensive DNA damage. These 5-Aza-CdR induced DNA damaging features were observed more frequently in Lovo cells treated with a higher concentration of 5-Aza-CdR than that with a lower concentration of 5-Aza-CdR (Fig. 3A).

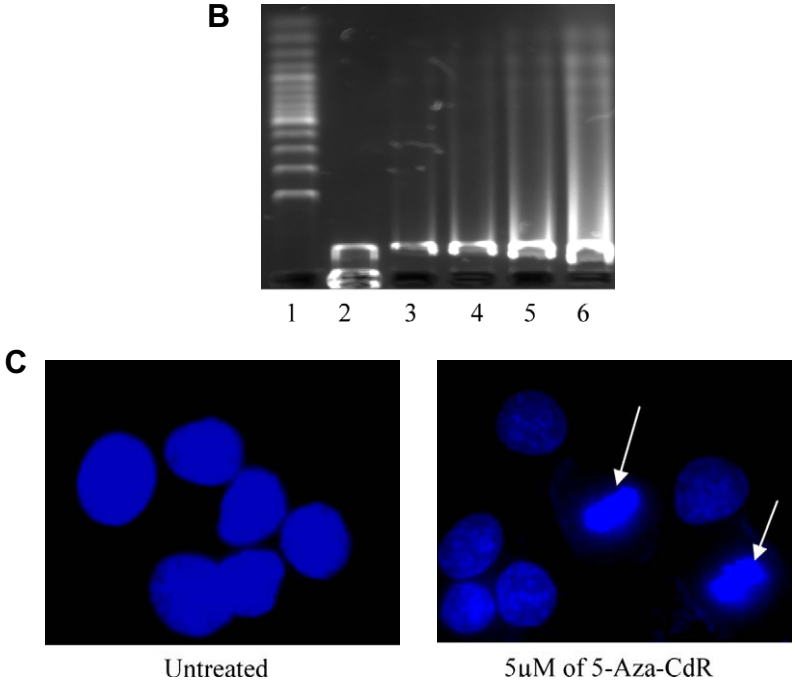


Fig. 2. (*continued*)

### *3.4. Activation of caspase 3 in Lovo cells exposed to 5-Aza-CdR*

To analyze whether caspase was responsible for the recruitment of apoptotic pathways by 5-Aza-CdR, we performed a colorimetric enzyme assay to examine the caspase 3 activity and Western blotting to detect the procaspase 3 expression. Here, caspase 3 enzymatic activity was increased in the cells after exposure to 5 μM of 5-Aza-CdR, but it was not significant with the untreated Lovo cells. Also, no marked change could be examined among the cells treated with 0, 0.5 and 1 μM of 5-Aza-CdR (Fig. 3B). Similar results were obtained using the Western blot analysis indicating that 5 μM of 5-Aza-CdR decreased the expression of procaspase 3 (Fig. 3C).

Next, we further confirmed the role played by caspases in 5-Aza-CdR induced apoptosis. Lovo cells were pretreated with the pan-caspase inhibitor, z-VAD-fmk (50 μM), and then treated with 5-Aza-CdR for 48 h. z-VAD-fmk only partially inhibited 5-Aza-CdR induced cell apoptosis (Fig. 2A), suggesting that the 5-Aza-CdR induced apoptosis was independent of the caspase pathway.

### *3.5. Dose response of 5-Aza-CdR on DNA methyltransferases and RUNX3*

Since 5-Aza-CdR has been an inhibitor of DNA methyltransferases and additional evidence suggested a link between them, we analyzed the effects of 5-Aza-CdR on DNA methyltransferase 1, 3a, and 3b in Lovo cells. As can be seen in Fig. 4A, treatment of cells with an increasing concentration of 5-Aza-CdR (0.5–5 μM) resulted in dose dependent depletion of DNMT3a and DNMT3b. DNMT3b was affected to a larger extent, followed by DNMT3a, which showed a decrease of mRNA only at higher 5-Aza-CdR doses (1 μM). The level of DNMT1, however, remained unchanged in the presence of 5-Aza-CdR (Fig. 4A).

Recently, increasing investigations have demonstrated that RUNX3 plays a critical role in growth inhibition as well as induction of apoptosis in carcinogenesis. To address the association of expression of the RUNX3 gene with cytotoxicity toward 5-Aza-CdR, Lovo cells were exposed to 0.5, 1 and 5 μM of 5-Aza-CdR for 72 h to detect the expression of RUNX3. As shown in Fig. 4B, we observed that the RUNX3 mRNA level was higher in the presence of 5-Aza-CdR at 0.5 μM than the untreated cells. Treatment of the cells with 1 μM of 5-Aza-CdR caused further increase in the RUNX3 expression and this peaked at 5 μM, indicating a dose dependent trend. Similarly, the Western blotting assay showed a dose dependent enhancement of protein expression of RUNX3 caused by the 5-Aza-CdR treatment.

## 4. Discussion

The field of epigenetic therapy in cancer has raised considerable interest over the last few years. This is due to the fact that aberrant methylation of multiple promoters which are associated with CpG islands is very prevalent in tumors including colorectal cancer [10]. A number of studies have reported that 5-Aza-CdR treatment decreased cell proliferation and induced apoptosis in tumors, but their exact in vivo mechanism remains unclear [11,12]. In our investigation, we first examined the effects of 5-Aza-CdR on RUNX3 and DNA methyltransferase in connection with its ability to induce cytotoxicity against Lovo cells.

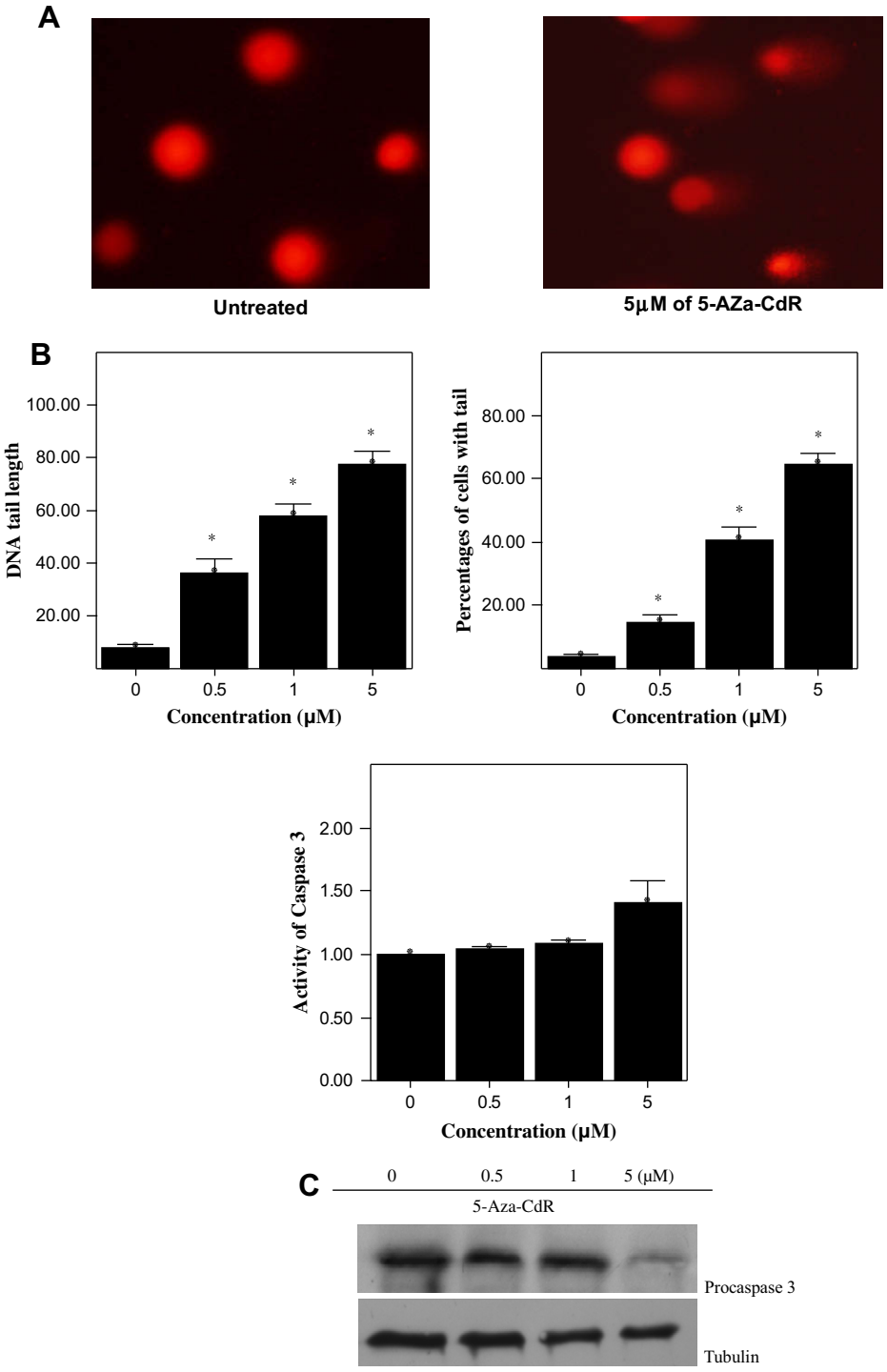


Fig. 3. Change of caspase 3 and DNA damage caused by 5-Aza-CdR in Lovo cells. (A) Detection of 5-Aza-CdR DNA damage by the comet assay. The more the cells with comet tail (%) or the longer the DNA tail length, the more significant the damage in Lovo cells. DNA damage was characterized by the percentage of cells with comet tail/100 cells (%) and the comet tail length (from the center of the DNA head to the end of the DNA tail). Representative pictures are shown. (B) Induction of caspase 3 activity in Lovo cells was determined using a colorimetric method after treatment with 5-Aza-CdR for 72 h as indicated. (C) Lovo cells were exposed to 0.5, 1 and 5 μM of 5-Aza-CdR for 72 h. The expression of procaspase 3 was examined by Western blotting. Blots were reprobed with β-tubulin antibody for loading control. Results represented the average of three independent experiments. $^*P < 0.01$, as compared with the untreated cells. Columns, mean of triplicate cultures; bars, SD.

From our results, we found that 5-Aza-CdR significantly inhibited Lovo cell growth in a dose and duration dependent manner. Regarding the mechanism underlying 5-Aza-CdR inhibiting cell growth, we detected that the induction of apoptosis resulted from DNA damage, while apoptosis activation was independent of the caspase pathway. This was not in line with Gomyo's report which indicated 5-Aza-CdR triggered caspase dependent apoptosis [13], providing

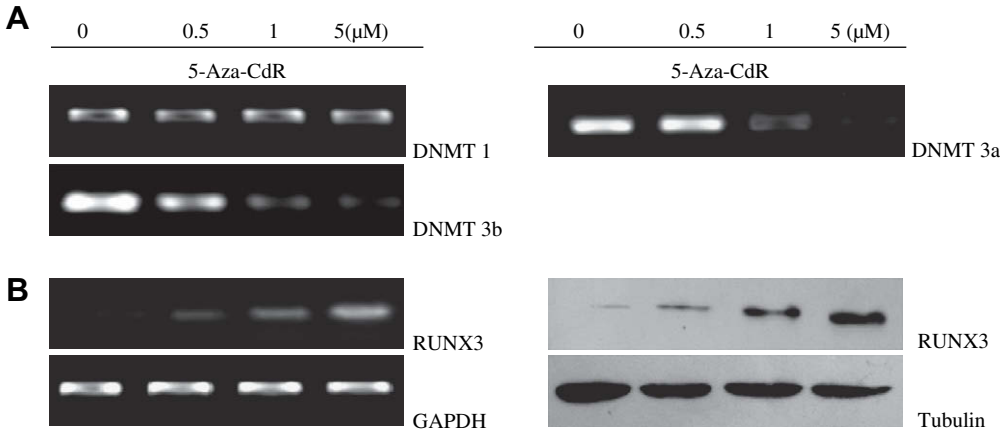


Fig. 4. Change of RUNX3 and DNA methyltransferases induced by 5-Aza-CdR. After exposure to 5-Aza-CdR for 72 h, Lovo cells were observed to have a dose dependent decrease in the levels of DNMT3a and DNMT3b, whereas no change was observed in the level of DNMT1 (A). (B) The expression of RUNX3 was reactivated following the addition of 0.5 μM of 5-Aza-CdR, which peaked at 5 μM. GAPDH and β-tubulin act as an internal control.

different apoptotic pathways rather than caspase may be recruited by 5-Aza-CdR [14,15]. The finding involving this does however warrant further investigation.

In mammals, global DNA methylation is catalysed mainly by three DNA methyltransferases: DNMT1, DNMT3a, and DNMT3b. DNMT1 has a high preference for hemimethylated DNA and is essential for maintaining methylation patterns during DNA replication, and thus is called the maintenance DNA methyltransferase [16]. DNMT3a and DNMT3b, on the other hand, are responsible for the wave of de novo methylation. It has been reported that DNMT1 is highly expressed in various cancer cells [17,18]. In addition, notable increases in the expression of de novo DNA methyltransferase are also reported in diverse cancer cells and cell lines [19,20]. Therefore, the abnormal expression of de novo DNA methyltransferases may serve as a marker for cancer cells, as well as a potential target for future cancer therapy. In vitro studies on the mechanism of action of 5-Aza-CdR indicated that the interaction of cytosine methyltransferases with the 5-Aza-CdR substituted DNA in the presence of S-adenosyl-methionine results in the irreversible binding of cysteine in the catalytic center of the enzyme to the 6-position of the cytidine ring [8]. Consequently, 5-Aza-CdR treated cells are depleted of active DNA MTase through sequestration of the enzyme to azacytosine residues in DNA, resulting in genome-wide demethylation. In the process of DNA demethylation, it is reasonable to suspect that DNMT3a and DNMT3b respond much more to the inhibitory effects of 5-Aza-CdR residues incorporated into DNA since they are randomly incorporated in place of cytidine, and, unlike DNMT1, DNMT3a and DNMT3b, are capable of methylating cytidine residues that are not in the CpG islands [21]. Our finding further proved the speculation in that 5-Aza-CdR caused a marked down-regulation of DNMT3b and DNMT3a mRNA levels, in contrast to a null effect on DNMT1, which was inconsistent with Palii's report that DNMT1-deficient cells demonstrated profound defects in response to 5-Aza-CdR induced DNA damage [22]. Oka's investigation that DNMT3a and DNMT3b null ES cells were highly resistant to 5-Aza-CdR when compared to wild type cells, however, emphasized the vital role of DNMT3a and DNMT3b de novo DNA methyltransferases in response to the 5-Aza-CdR treatment [23]. Therefore, our research is not far sufficient to establish the above hypothesis since we only measured the mRNA level of DNA methyltransferases rather than protein and activity. 5-Aza-CdR functions to covalently trap the enzyme, not to degrade it. More studies involving the correlation between DNA MTases and 5-Aza-CdR are warranted in the future.

The human runt-related transcription factor 3 (RUNX3) gene is part of the runt-domain family of transcription factors that act as master regulators of gene expression in major developmental pathways [24]. More recently, RUNX3 functions as a candidate tumor suppressor gene for cancer, because the gastric mucosa in RUNX3$^{-/-}$ mice undergoes hyperplasia due to reduced sensitivity of gastric epithelial cells to transforming growth factor (TGF)-β growth inhibitory activity and TGF-β-mediated apoptosis [25]. In colorectal cancer, Tan et al reported that the silence of RUNX3 gene expression was mainly due to promoter hypermethylation rather than gene mutation or something else [26]. Increasingly, it has well established that 5-Aza-CdR has been characterized as a demethylation agent that can reactivate expression of cancer-related hypermethylated genes and it has been linked to involve in tumor suppression in vitro. In line with Nagahama's work that enhancement of RUNX3 expression by transfection resulted in growth inhibition and apoptosis [27], we observed that 5-Aza-CdR restored the RUNX3 expression in a dose dependent fashion and coincided with 5-Aza-CdR induced cell apoptosis. Re-expression of RUNX3 occurred as a consequence of promoter demethylation derived from the depletion of DNMT3b and DNMT3a mRNA levels caused by 5-Aza-CdR treatment. Together, these data supported the notion that RUNX3 was a relevant target for the methylation dependent efficacy of 5-Aza-CdR in colorectal cancer Lovo cells.

In conclusion, our results show for the first time that 5-Aza-CdR, a cytosine analogue designed to inhibit DNA methyltransferases, abolishes colorectal cancer Lovo cell growth advantages via cell apoptosis induced by DNA damage. The

up-regulation of RUNX3 targeted by promoter demethylation resulting from the deletion of DNMT3a and DNMT3b play an active role in the cytotoxicity of 5-Aza-CdR against Lovo cells. To our knowledge, this is the first demonstration of RUNX3 functioning as a relevant target for methyltransferase dependent efficacy of 5-Aza-CdR in colorectal cancer.

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