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5-Aza-2'-deoxycytidine may influence the proliferation and apoptosis of cervical cancer cells via demethylation in a dose- and time-dependent manner

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ABSTRACT. The methylation of tumor suppressor genes has been shown to be involved in many human cancers. 5-Aza-2'-deoxycytidine (5-Aza-CdR) can reactivate the expression of methylated tumor suppressor genes. In our study, 2 human cervical cancer cell lines, HeLa and SiHa, were treated with different concentrations (20, 10, 5, and 2.5 μM) of 5-Aza-CdR for 24, 48, and 72 h. After incubation, cells were analyzed by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay and flow cytometry. The expression of *RASSF1A* and *APAF-1* was detected by RT-PCR. 5-Aza-CdR inhibited the growth of HeLa and SiHa cells at different concentrations. The strongest inhibition and apoptosis rates were obtained after incubation for 72 h (5.63 ± 1.38 and

8.24 ± 2.40%, respectively). No significant difference in the expression of *RASSF1A* was found upon drug treatment, while *APAF-1* expression increased in HeLa cells after treatment (0.790 ± 0.056%). Our results suggest that the tumor-suppressive effect of 5-Aza-CdR may result from the reactivation of silenced *APAF-1* through demethylation.

Key words: 5-Aza-2'-deoxycytidine; Cervical cancer; *APAF-1*; DNA methylation; *RASSF1A*

INTRODUCTION

Cervical cancer is the second most common cancer in women worldwide (Sankaranarayanan and Ferlay, 2006). Recent studies have revealed the genetic and epigenetic changes involved in cellular malignancy. Epigenetic changes modify the expression of genes without altering their DNA sequences. DNA methylation is the most common DNA modification in eukaryocytes. The hypermethylation of tumor suppressor gene promoters inhibits their transcription and inactivates these genes. Aberrant tumor suppressor gene methylation has been identified in many human cancers (Montenegro et al., 2012; Ozdemir et al., 2012; Menschikowski et al., 2012).

Ras association domain family 1A (*RASSF1A*) is a tumor suppressor gene that has been observed to be highly methylated in many malignant human tumors. Apoptotic protease activating factor-1 (*APAF-1*), the human analog of the *Caenorhabditis elegans ced-4* gene, is a critical factor in activating the caspase cascade. Inactivation of *APAF-1* may be due to hypermethylation of several genes.

5-Aza-2'-deoxycytidine (5-Aza-CdR) is a pyrimidine nucleoside analog that can integrate into DNA and block methylation by forming a covalent complex with DNA methyltransferase. It can reactivate the expression of methylated tumor suppressor genes and restore their anti-tumorigenic activities (Hassler et al., 2012; Chu et al., 2012). In this study, we investigated the effects of 5-Aza-CdR on cervical carcinoma cells in an attempt to provide new insights into cervical cancer treatment.

MATERIAL AND METHODS

Cell culture

The human cervical adenocarcinoma cell line HeLa and squamous cancer cell line SiHa were purchased from American Type Culture Collection and maintained in RPMI 1640 medium (Life Technologies, USA) supplemented with 10% fetal bovine serum (Invitrogen, USA). They were treated with 5-Aza-CdR (Sigma, USA) at different concentrations (2.5, 5, 10, and 20 μM) for 24, 48, and 72 h.

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay

Cell survival was assessed using the MTT assay (Sigma). MTT was added at a final concentration of 0.5 mg/mL 72 h after chemical intervention with 5-Aza-CdR, and the cells were incubated for 4 h at 37°C and dissolved in 150 μL DMSO (Sigma). Optical density values were read at 570 nm (A_{570}) using an Easy Reader 340 AT (EAR400 SLT-Lab. Instruments, Research

Triangle Park, NC, USA). Each experiment was carried out in triplicate and repeated 3 times.

Annexin-V analysis

Cell pellets were resuspended in Annexin-V-FLUOS staining solution (LightCycler, Roche Molecular Biochemicals, Mannheim, Germany) and incubated for 15 min at room temperature. Samples were then analyzed on an FSCAN flow cytometer (Hershey Medical Center Core Facility).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted using the TRIzol reagent (Invitrogen), and RT-PCR was performed using the PrimerScript RT-PCR kit (TaKaRa Biotechnology, China) according to manufacturer instructions on the DNA Engine Peltier Thermal Cycler (PTC-200, Bio-Rad Laboratories, Inc., China) with specific primers.

Primer sequences for detection were as follows: *RASSF1A* - forward: 5'-TTGGGTGAC CTCTTGTACCC-3', reverse: 5'-TGGCACTGTAGAGAGAAACCAA-3', and *APAF-1* - forward: 5'-TTGCTGCCCTTCTCCATGAT-3', reverse: 5'-TCCCAACTGAAACCCAATGC-3'. *β-actin* (forward: 5'-GTCCACCTTCCAGCAGATGT-3', reverse: 5'-CACCTTCACCGTTCCAGTTT-3') was used as an endogenous control. These primers yielded 497-, 285-, and 245-bp products, respectively.

The PCR cycling conditions were as follows: initial hold at 65°C for 5 min, 42°C for 30 min for cDNA synthesis, followed by amplification for 30 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min. All reactions were performed in triplicate, and a negative control lacking cDNA was included. PCR products were then separated on 1.5% agarose gels containing ethidium bromide and visualized by UV transillumination.

Statistical analysis

All statistical analyses were carried out using the SPSS for Windows version 13.0 software. All results are reported as means ± SE from at least 3 separate experiments. The differences between groups were analyzed using the Student *t*-test when only 2 groups were compared or by one-way analysis of variance for all other analyses. All tests were 2-sided. Differences were considered to be statistically significant at $P < 0.05$.

RESULTS

The effects of 5-Aza-CdR derivatives on DNA methylation and the expression of *RASSF1A* and *APAF-1* were examined after exposure at different concentrations (20, 10, 5, 2.5 μM) for 24, 48, and 72 h.

Cell proliferation

Cell proliferation was suppressed in a concentration-dependent manner in HeLa cells (Figure 1A). The 20 μM treatment group showed increasing growth inhibition rates with time.

The other 3 treatment groups reached maximum growth inhibition rates after exposure for 48 h, followed by a slight decrease from 48 to 72 h. These findings suggest that a high 5-Aza-CdR concentration may more effectively suppress HeLa growth. Compared to treatment with other doses, the inhibition rate was lower in SiHa cells upon treatment with 2.5 µM drug (Figure 1B). These results suggest that there may be an optimum dose for suppressing tumor cell proliferation. After 72 h, all SiHa groups showed similar inhibition rates, suggesting that a low-dose treatment could achieve optimal inhibition by prolonging treatment course.

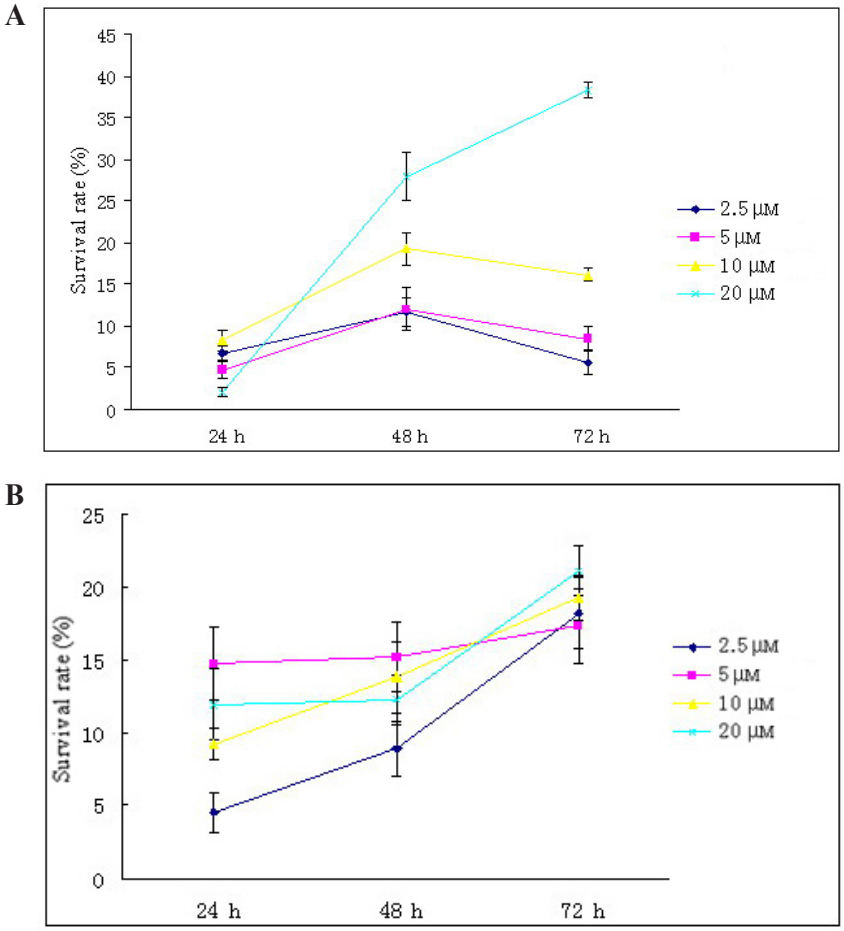

Figure 1. Growth inhibitory after exposure to 5-Aza-CdR. **A.** HeLa; **B.** SiHa.

Cell apoptosis

The apoptosis rate of HeLa cells increased significantly after exposure to 5 µM 5-Aza-CdR for 72 h as determined by flow cytometric analysis (Figure 2), while the apoptosis rate of SiHa cells increased significantly upon treatment with 20 µM 5-Aza-CdR.

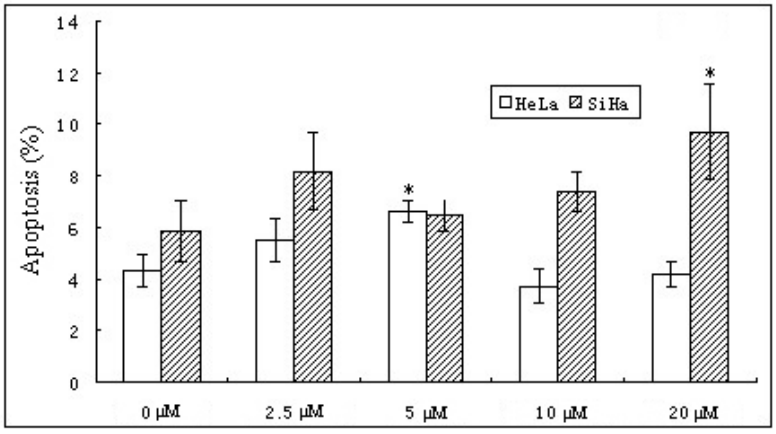

Figure 2. Flow cytometric analysis results.

RASSF1A and *APAF-1* expression

The expression of *RASSF1A* and *APAF-1* mRNA could be detected in HeLa and SiHa cells before 5-Aza-CdR treatment. After treatment with 5-Aza-CdR, the expression of *RASSF1A* did not change significantly. The expression of *APAF-1* mRNA increased in HeLa cells after treatment with 20 μM 5-Aza-CdR, while its expression was not significantly different in SiHa cells before and after treatment with 5-Aza-CdR (Figure 3A and B).

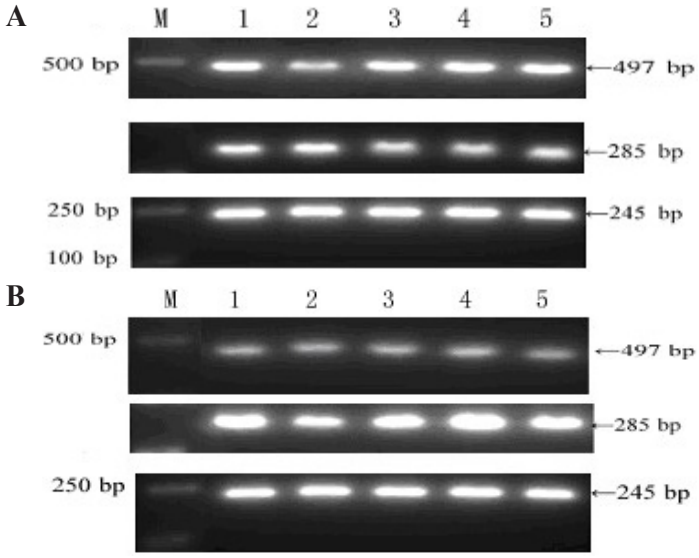

Figure 3. Expression of *RASSF1A* and *APAF-1* mRNA by RT-PCR. **A.** HeLa; **B.** SiHa. *Lane M* = molecular maker 2000; *lane 1* = 20 μM 5-Aza-CdR; *lane 2* = 10 μM 5-Aza-CdR; *lane 3* = 5 μM 5-Aza-CdR; *lane 4* = 2.5 μM 5-Aza-CdR; *lane 5* = 0 μM 5-Aza-CdR.

DISCUSSION

5-Aza-CdR is a pyrimidine nucleoside analog that forms a covalent complex with DNMT during DNA replication. No heritable growth inhibition was found treated with varying doses of arabinofuranosyl cytidine, which does not inhibit DNA methylation (Wu et al., 2006). In comparison, after treatment with 5-Aza-CdR, methylation-silenced growth-regulatory genes were reactivated and cervical cells CaSki, C-33A, HeLa, and SiHa showed heritable growth inhibition, demonstrating that 5-Aza-CdR inhibits tumor cell proliferation by demethylating and reactivating genes silenced by methylation (Wu et al., 2006). We hypothesized that 5-Aza-CdR may suppress cervical carcinoma cell proliferation and induce cell apoptosis by changing the methylation status of some genes.

RASSF1A is located on human chromosome 3 at p21.3. The *RASSF1A* promoter has been reported to be hypermethylated in 10% of squamous cell carcinomas, in 21% of adenosquamous carcinomas, and in 24% of cervical adenocarcinomas. Promoter hypermethylation has been discovered in various human tumors, including small cell lung, breast, bladder, prostate, gastric, and renal cell carcinomas (Ferguson et al., 1997; Dammann et al., 2001; Dreijerink et al., 2001; Lee et al., 2001; Burbee et al., 2001; Kwong et al., 2002; Kuzmin et al., 2002; Liu et al., 2002). Vos et al. (2000) suggested that *RASSF1* binds Ras in a GTP-dependent manner. Kuzmin et al. (2003) observed the methylation of the *RASSF1A* promoter in 6% of normal cervical tissues and in 10% of squamous carcinomas, 21% of adenosquamous carcinomas, and 24% of cervical adenocarcinomas. Moreover, Kuzmin et al. (2003) found that *RASSF1A* mRNA was expressed in 6 HPV-positive cervical carcinoma cell lines (ME 180, MS751, SiHa, C-4I, HeLa, and CaSki) whereas it was not expressed in 2 HPV-negative cell lines (C-33A and HT-3). This is most likely because HPV infection reduces *RASSF1A* inactivation. Consistent with this, our analyses showed that there was no increase in *RASSF1A* mRNA levels in HeLa or SiHa cells treated with 5-Aza-CdR.

APAF-1 is located on human chromosome 12q23. Its hypermethylation-based silencing has been confirmed in various malignant human tumors. This hypermethylation can be reversed with demethylating agents (Christoph et al., 2006). In our study, 5-Aza-CdR-treated HeLa cells showed a higher apoptosis rate than untreated HeLa cells. The expression of *APAF-1* is correlated with cancer cell apoptosis (Andreev et al., 2012; Niimi et al., 2012; Melzer et al., 2012). Presumably, 5-Aza-CdR reduces *APAF-1* methylation and restores its expression, thus reactivating its functions. In comparison, *APAF-1* gene expression in SiHa cells appeared unchanged after treatment. Whether *APAF-1* expression is related to HPV infection needs to be clarified by future studies.

In summary, the present study shows that 5-Aza-CdR can suppress *in vitro* proliferation of cervical carcinoma cells and induce their apoptosis. These results provide experimental support for and new insights into novel therapies for cervical cancer. However, the present study is limited to *in vitro* experiments. Therefore, further studies will be necessary to better understand the effects of 5-Aza-CdR on cervical cancer.

Conflict of interest

The authors declare no conflict of interest.

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