Synergistic effects of the combination of 5-Aza-CdR and suberoylanilide hydroxamic acid on the anticancer property of pancreatic cancer

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Abstract. Despite increasing advances in the diagnosis and treatment for pancreatic cancer, the mortality rate remains high world-wide. There is an urgent need for new therapies to improve survival and quality of life for pancreatic cancer patient. Epigenetic therapeutic agents such as 5-Aza-CdR and suberoylanilide hydroxamic acid (SAHA) have shown therapeutic effects for human cancers. We evaluated the efficacy of 5-Aza-CdR or SAHA and their combination as potential therapies for pancreatic cancer *in vitro*. Treatment with 5-Aza-CdR or SAHA inhibited pancreatic cancer cell proliferation, migration and induced cell arrest. However, 5-Aza-CdR alone can not induce cell apoptosis. Combination of the two agents enhanced the proliferation and migration inhibition, and induced more cells to G2 arrest and increased the cell apoptosis proportion. Furthermore, combination treatment with SAHA and 5-Aza-CdR significantly increased expression of TP53 and P16. The possible mechanism might be that the two agents inhibited the PI3K/AKT/PTEN signaling pathway. In conclusion, these data demonstrate a potential role for epigenetic modifier drugs for the management of pancreatic cancer.

Introduction

Pancreatic cancer is a lethal disease. It is the fourth most common cause of cancer-related death in USA with a 5-year survival rate of <5% (1). This extremely malignant tumor type exhibits rapid progression without the presentation of obvious symptoms, and as a consequence it is advanced in the majority of cases at diagnosis (2,3). At that time, surgical intervention was not suitable for 80% of the patients (4,5). Current chemo- and radio-therapy have also met with limited success. Novel diagnostic and therapeutic strategies are urgently needed to improve prognosis for pancreatic cancer patients.

Epigenetic therapeutic agents, such as pan-HDAC inhibitors and DNA methyltransferase (DNMTs) inhibitors, have showed efficacy in the treatment of cutaneous T-cell lymphoma (CTCL) (6), myelodysplastic syndrome (MDS) (7,8), breast cancers (9), ovarian cancers (10). 5-Aza-CdR is currently one of the most commonly used demethylation nucleoside analogues (11). It mainly inhibits DNMT expression under a low concentration. It was approved by the Food and Drug Administration (FDA) to be mainly used for the treatment of blood system tumors. Suberoylanilide hydroxamic acid (SAHA) is an HDAC inhibitor that has the permeability to cross the blood-brain-barrier and to cause biological responses in the mouse brain, therefore, making it as a preferred candidate drug for testing in gliomas (12).

Comprehensive treatment is the main trend for cancers. Drug combinations are currently an important strategy for antitumor treatment. The aim of this study was to investigate the demethylation efficacy of 5-Aza-CdR in combination with SAHA on pancreatic cancer cells. It was demonstrated that 5-Aza-CdR combined with SAHA inhibited cell proliferation, migration and invasion, and induced cell cycle arrest and apoptosis, through upregulation tumor suppressor genes p16 and p53 and inhibiting PI3K/AKT/PTEN signaling pathway. This finding might provide a new strategy for the clinical treatment of pancreatic cancer.

Materials and methods

Cell lines and reagents. Pancreatic cancer cells lines AsPC-1 and SW1990 were all obtained from the Chinese Academy of Sciences Cell Bank (Shanghai, China). AsPC-1 were cultured in RPMI-1640 (Gibco) supplemented with 10% FBS, grown

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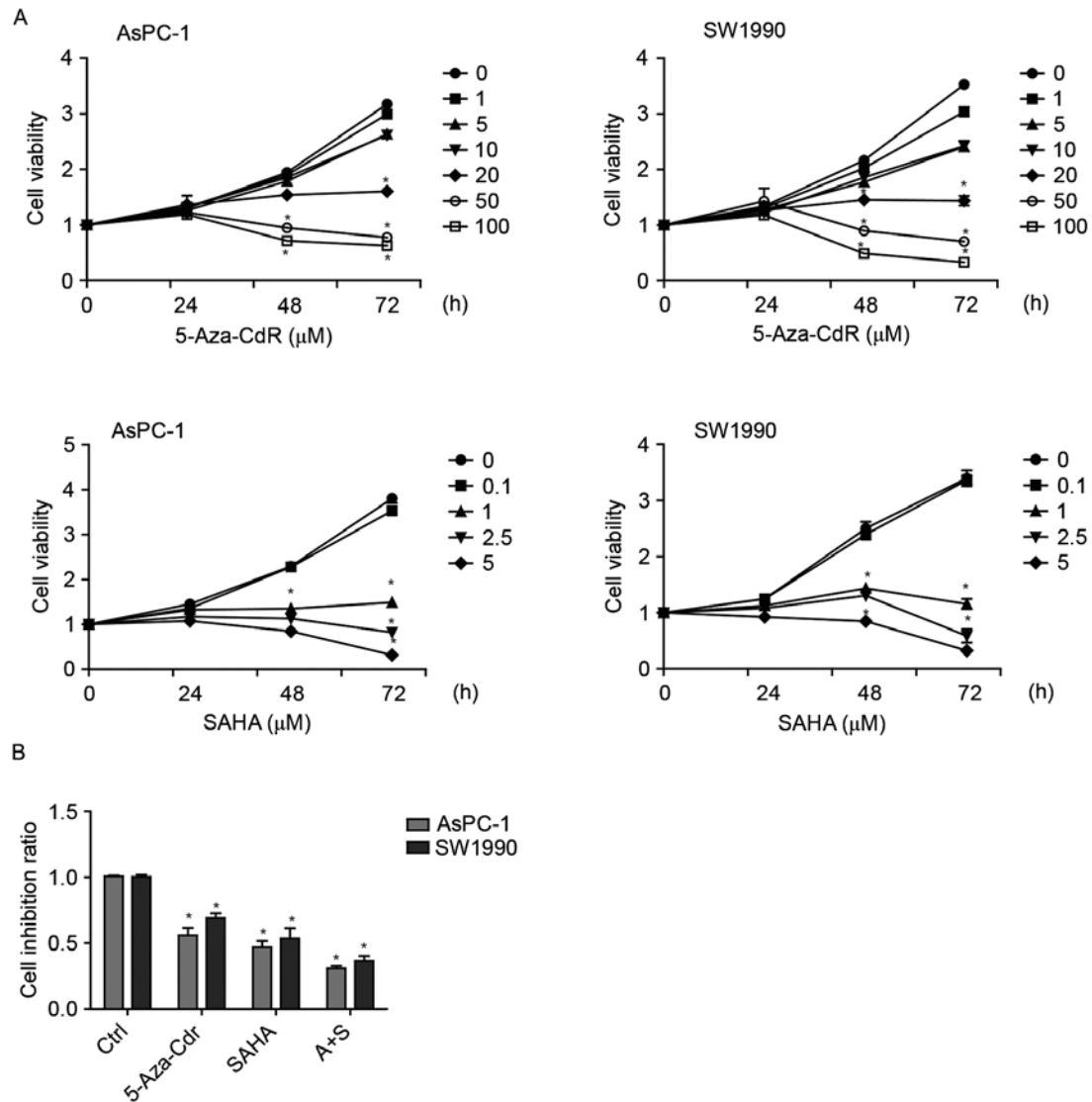E-mail: jiao_f@outlook.com

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Figure 1. Dose response curves for the effect of 5-Aza-CdR/SAHA on cell viability. (A) Treatment with different concentration of 5-Aza-CdR or SAHA on two cell lines. (B) Cells were treated with 50 μM AZA and 1 μM SAHA for 72 h before cell viability was determined. Data are shown as the mean ± SEM for 3 independent experiments, each performed in triplicates. Significance levels are represented as *P<0.05.

in 5% CO_2 saturated humidity at 37°C. SW1990 cells were cultured in L-15 medium (Gibco) supplemented with 10% FBS.

5-Aza-CdR and SAHA were purchased from Sigma (Selleck). RPMI-1640 and Dulbecco's modified Eagle's medium were obtained from Invitrogen (Carlsbad, CA, USA). The Annexin/PI apoptosis analysis kit was from BD Biosciences (San Diego, CA, USA). Total RNA was isolated from the cultured cells by using miniBEST universal RNA extraction kit and amplified using SYBR green RT-PCR mix (Takara Bio, Inc.).

Cell proliferation assay. SRB method was used to test cell proliferation with the treatment of 5-Aza-CdR and SAHA. Cells were seeded in 96-well microtiter tissue culture plates and cultured for 24 h. Then we added indicated concentration of 5-Aza-CdR and SAHA and cultured for 24, 48 and 72 h, respectively. At the end of the treatment, cells were fixed with 10% w/v of trichloroacetic acid (100 μl) for 1 h at 4°C. The plates were then washed and air-dried. Samples were

stained with 100 μl of SRB solution (in 0.4% w/v in acetic acid) for 20 min at room temperature. The plates were then washed with acetic acid (1%) and air-dried. Tris-base (10 mM, 100 μl, pH 10.0) was added to each well for solubilization. Optical density (OD) values were measured at 540 nm with a reference wavelength of 630 nm using microtiter plate reader (VERSMax).

Cell migration and cell invasion assay. The cell migratory potential was evaluated using Transwell assay. Briefly, Transwell assay was conducted using specialized MilliCell chambers (Millipore, Bedford, MA, USA). The inserts contained an 8-μm pore size polycarbonate membrane. FBS-containing medium (10%) was placed in the lower chambers to act as a chemo-attractant. Then, $1x10^5$ AsPC-1, SW1990 harvested from treatment group in a 100-μl volume of serum-free medium were placed in the upper chambers and incubated at 37°C for <24 h. Then they were fixed and stained by 0.1% crystal violet staining solution for 15 min. Cells on the upper surface of membrane were scraped off with cotton
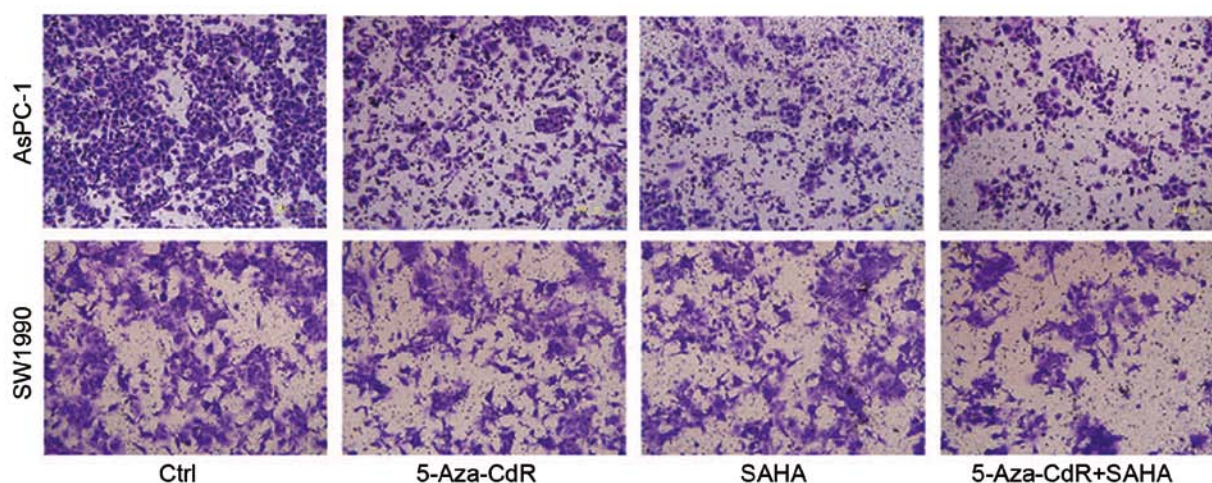
Figure 2. Treatment of 5-Aza-CdR/SAHA in pancreatic cancer cells inhibits cell migration.

swabs and counted under a microscope in five randomly selected fields at a magnification of x200 after dried.

Flow cytometry analysis of cell cycle and apoptosis. For cell apoptosis analysis, cells treated with single or two reagents were harvested at 70-80% confluence and incubated with reagent containing Annexin V-FITC and propidium iodide (BD Biosciences) for 15 min in darkness at room temperature. Apoptotic cells were analyzed using FACSCaliber flow cytometer (BD Biosciences).

Western blot analysis. The cells were treated with 5-Aza-CdR and SAHA, either alone or in combination for 72 h. The control cells were treated with 0.1% DMSO only. Total proteins were extracted from the cells using precooling lysis buffer, and the liquid was collected. After centrifugation at 120,000 x g for 5 min at 4˚C, the supernatant was collected and the protein concentration was determined using the BCA protein assay kit according to the manufacturer's instructions. The protein lysates (20 μg/lane) were separated on 10% SDS polyacrylamide gel and transferred onto a nitrocellulose membrane. Each membrane was blocked with 5% BSA and then incubated with the indicated primary antibodies against P16, TP53, p-AKT, AKT, PTEN and β-actin overnight at 4˚C. Subsequently, the membrane was incubated with the secondary antibodies for 1 h at room temperature.

Statistical analysis. The results were repeated in at least three separate experiments. The data are expressed as the means ± SD. Statistical comparisons were carried out using one-way analysis of variance, which revealed significant differences between groups. Statistical analyses were carried out using SPSS version 17.0 software (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

5-Aza-CdR and SAHA inhibits cell proliferation. To investigate the effect of 5-Aza-CdR and SAHA on cell growth, AsPC-1 and SW1990 cells were cultured with 5-Aza-CdR or/and SAHA for 24, 48 and 72 h. According to SRB assay, 5-Aza-CdR and SAHA reduced proliferation of the pancreatic cell lines in either time- or dose-dependent manner (Fig. 1A). Additionally, there was an enhanced additive effect of 5-Aza-CdR and SAHA on the proliferative inhibition of pancreatic cancer cells (Fig. 1B).

5-Aza-CdR and SAHA inhibit cell migration. We used a Transwell assay to examine the effects of 5-Aza-CdR and SAHA on pancreatic cancer cell migration. The number of 5-Aza-CdR or SAHA treated cells were remarkably reduced as compared to control group, indicating decreased migratory abilities following the treatment. The combination of both reagents inhibited cell migrated ablity the most (Fig. 2).

5-Aza-CdR and SAHA induce cell arrest and promote cell apoptosis. We performed flow cytometric analysis to assess whether cell cycle and apoptosis were altered. We observed that the cells were arrested in G2/M phase with the treatment of both 5-Aza-CdR and SAHA, and combination of two reagents induced more cells in G2 arrest (Fig. 3A). The proportion ratio of each period is also shown (Fig. 3B). SAHA alone induced cell apoptosis, but 5-Aza-CdR alone could not. Interestingly, combination of 5-Aza-CdR and SAHA remarkably increased apoptosis (Fig. 4).

The mRNA and protein expression of p16, p53 and Rb in pancreatic cancer cells after the exposure to 5-Aza-CdR or/and SAHA. In order to demonstrate whether 5-Aza-CdR and SAHA have an effect at the transcriptional and protein level, PCR and WB was used to detect the levels of tumor-suppressor genes P16 and TP53. After the treatment with 5-Aza-CdR or/and SAHA, the mRNA and protein expression levels of P16 and TP53 were upregulated in treated groups compared to those observed in the control. Their combination was more significantly enhanced when compared with the levels following treatment with 5-Aza-CdR or SAHA alone (Fig. 5A and B).

5-Aza-CdR or/and SAHA inhibit the PI3K/AKT/PTEN signaling pathway. PI3K/AKT signaling pathway has been reported to be activated in pancreatic cancer (13). Here,
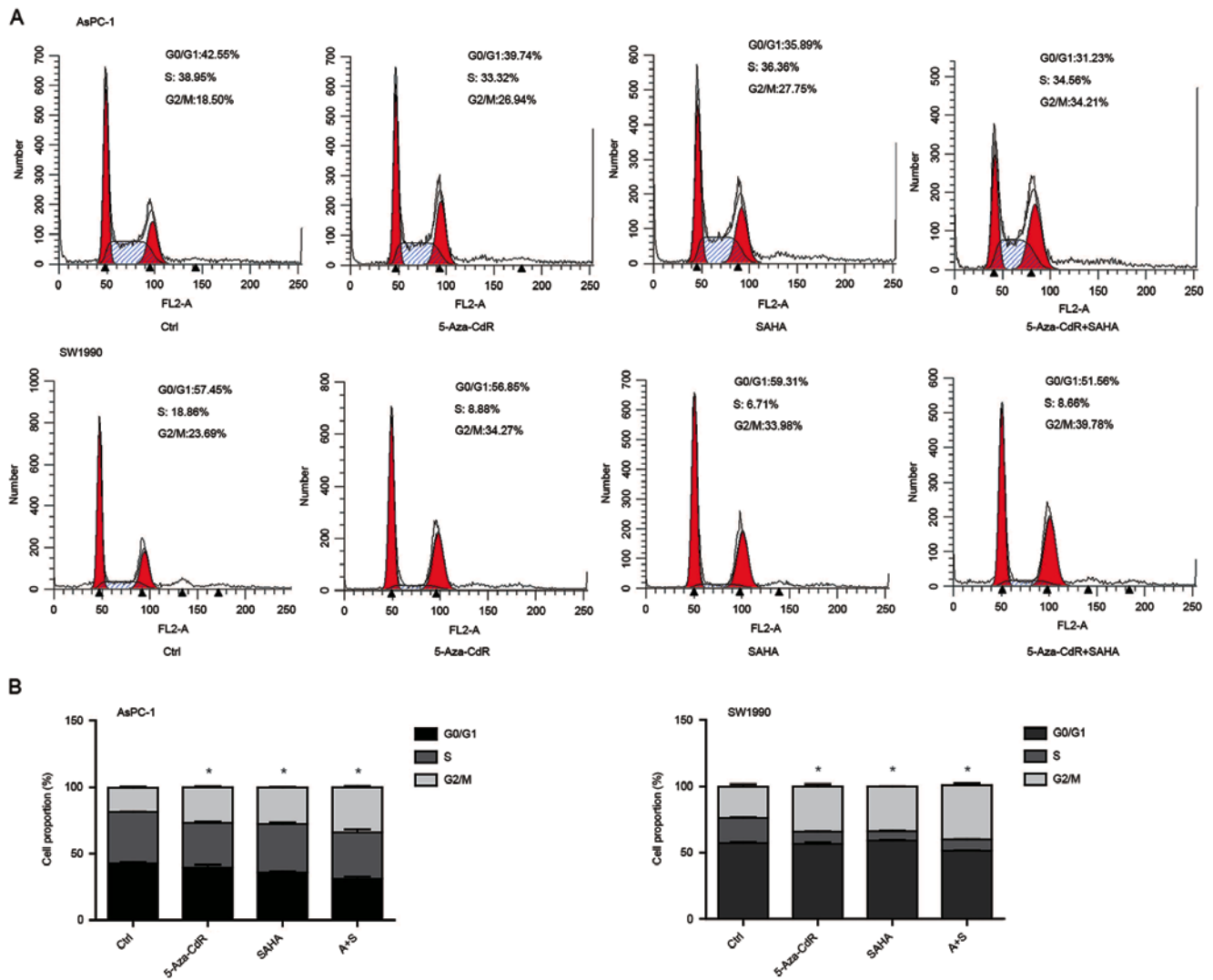
Figure 3. Flow cytometric analysis of cell cycle treated with 5-Aza-CdR/SAHA. (A) Flow cytometric analysis of cell treated with 5-Aza-CdR/SAHA to detect the cell cycle. (B) The ratio of cell cycle arrest in each group. *P<0.05.
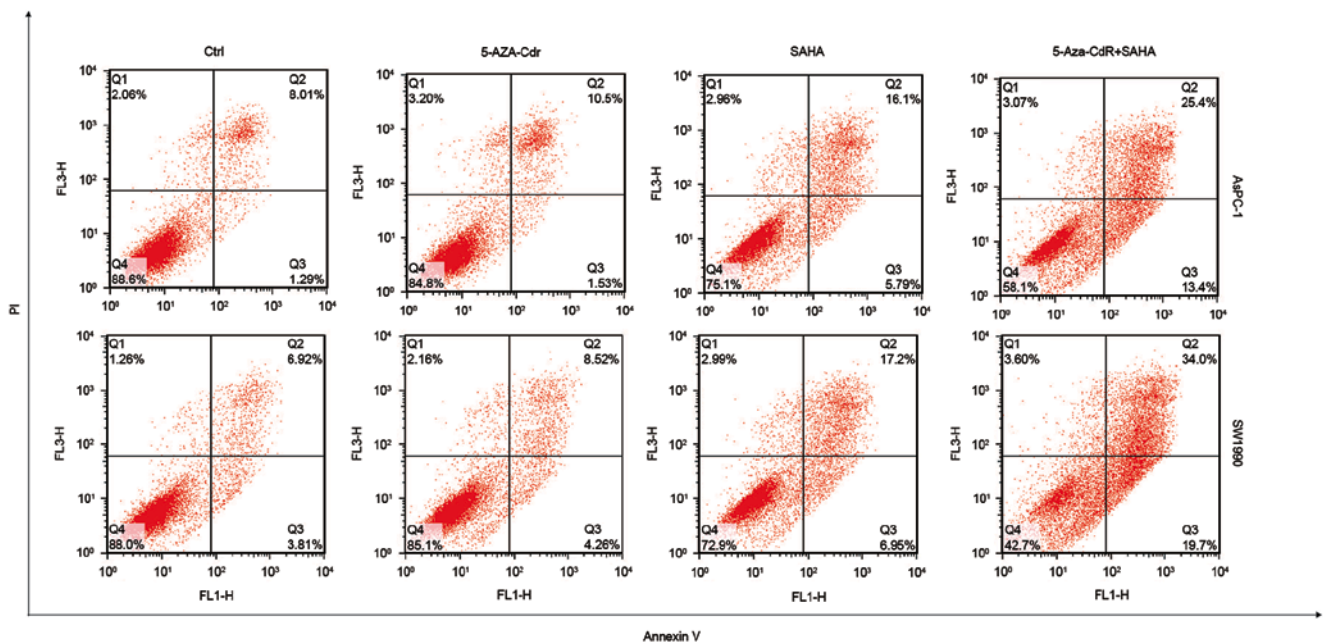

Figure 4. Flow cytometric analysis of cell apoptosis treated with 5-Aza-CdR/SAHA.
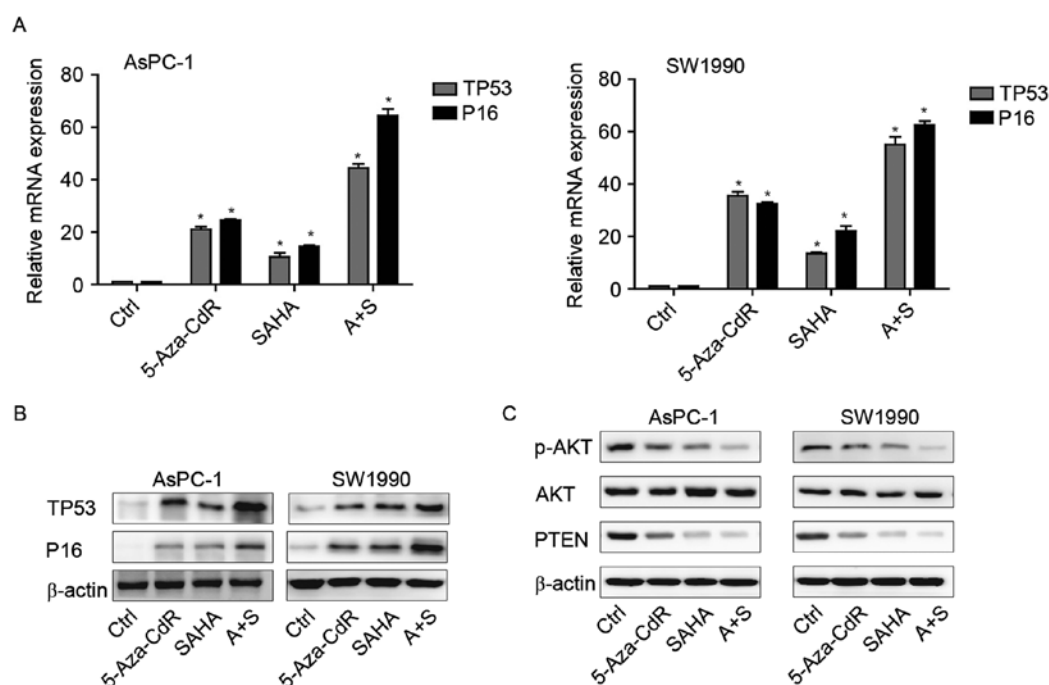
Figure 5. mRNA and protein level of TP53 and P16, and PI3K/AKT/PTEN pathway. (A) The mRNA level of TP53 and P16 after treated with 5-Aza-CdR/SAHA. (B) The protein level of TP53 and P16 after treated with 5-Aza-CdR/SAHA. (C) The protein level of PI3K/AKT/PTEN after treated with 5-Aza-CdR/SAHA. *P<0.05.

we investigated whether these two agents could affect this pathways, leading to pancreatic cancer cell inhibition. Our findings showed that either alone or combined, they could downregulate p-AKT and PTEN expression in both cell types (Fig. 5C).

Discussion

Pancreatic cancer is a world-wide problem that is hard to conquer. This is because its unique anatomic location and rapid progress. Gemcitabine has been the standard systemic therapy for the palliative treatment of pancreatic cancer over the last decade, although the 1-year survival rate of <20% remains unsatisfactory (14-17). Many studies have shown epigenetic modifications associated with cancer (18,19). Epigenetic modification has been shown as promising antitumor effect in T cell lymphoma (20), acute myeloid leukemia (21,22), breast cancers (9), and some other malignant tumors (23), however, with limited efficacy of limited drugs. Therefore, novel therapies are urgently needed to better inhibit pancreatic cancer cell proliferation and prolong overall survival time of the patients. At these circumstances, we consider that some epigenetic inhibitors might take effect since epigenetic modification is involved in the pancreatic cancer progression (24). Here, we reported two drugs, 5-Aza-CdR and SAHA, and aimed to define their combination effects on pancreatic cancer cells.

5-Aza-CdR was first characterized 30 years ago and it functions as a mechanism-dependent suicide inhibitor of DNA methyltransferases, with which genes silenced by hypermethylation can be reactivated (25). SAHA is a pan-HDAC inhibitor, and the antitumor effects of SAHA have been reported in chronic myelogenous leukemia (26), lung (27),

pancreatic (28), liver (29), cervical (30), head and neck (31), breast (32) and ovarian cancers (33). Our results showed that 5-Aza-CdR or SAHA could inhibit pancreatic cancer cell proliferation in a dose- and time-dependent manner. Then we further investigated the possible mechanism of its inhibition. We detected cell migration, invasion, the cell cycle and apoptosis. We found that 5-Aza-CdR or SAHA alone could decrease the migration of pancreatic cancer cells and induced cell cycle arrest. SAHA increased cell apoptosis, however, 5-Aza-CdR could not induce cell apoptosis. Combination of two drugs had stronger inhibition effect than each one alone. Specially, combination of 5-Aza-CdR and SAHA enhanced apoptosis, though 5-Aza-CdR had no effects alone. TP53 has been reported with high methylation and promoter in different cancers, such as mutiple myeloma (34), esophageal squamous cell cancer (35), lung cancer (36), and breast cancer (37). The gene encoding p16 is mutated or downregulated in several cancer cells (38-40). However, breast carcinoma progression has been related to overexpression of p16 (41), and this has been reported in head and neck squamous carcinoma (42), in prostate carcinoma (43). Here, we detected TP53 and p16 mRNA and protein expression after treatment of 5-Aza-CdR and SAHA. We found both expressions were upregulated after treatment. Besides, the two drugs inhibited the PI3K/AKT/PTEN signaling pathway, leading to inhibition of cell proliferation.

In conclusion, this study illustrated that treatment with epigenetic agents decreased cell proliferation and induced cell death in pancreatic cancer cells, while we also demonstrated that epigenetic agents were able to upregulate TP53, P16 expression and inhibited the I3K/AKT/PTEN signaling pathway. These data suggest that epigenetic therapy has the potential to delay pancreatic progression, and may

have potential application in the management of pancreatic cancer.

Acknowledgements

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