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Original Article

An Effective Concentration of 5-Aza-CdR to Induce Cell Death and Apoptosis in Human Pancreatic Cancer Cell Line through Reactivating RASSF1A and Up-Regulation of Bax Genes

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What's Known

- Promoter hyper-methylation of tumor suppressor genes is a common event that occurs in cancer. Since methylation is a reversible modification, agents capable of reversing an abnormal methylation status (e.g. 5-Aza-CdR) should help to combat cancer.
- Evaluation of the epigenetic effect of 5-Aza-CdR on solid tumor has been limited and further investigation is required.

What's New

- 5-Aza-CdR can limit the proliferation of human pancreatic cancer cell line (PANC-1) through epigenetic reactivation of RASSF1A and consequently up-regulation of Bax in a time- and dose-dependent manner.

Abstract

Background: Promoter hyper-methylation of tumor suppressor genes is a common event that occurs in cancer. As methylation is a reversible modification, agents capable of reversing an abnormal methylation status should help to combat cancer. 5-Aza-CdR is a DNA methyl-transferase inhibitor. The present study aimed to evaluate the effect of 5-Aza-CdR on the proliferation of human pancreatic cancer cell line (PANC-1) and the expression of RASSF1A and Bax genes.

Methods: PANC-1 cells were cultured and treated with 5 and 10 µM/L of 5-Aza-CdR for 24, 48, 72, and 96 hours and the percentages of cell viability and apoptosis were measured by MTT and flow cytometry. RASSF1A gene promoter methylation was assessed by methyl-specific primer-PCR (MSP-PCR) and the expression of RASSF1A and Bax genes was measured using quantitative real-time PCR (qPCR). All quantitative data are presented as mean±SD (standard deviation). The one-way analysis of variance (ANOVA) with the LSD post hoc test was performed for statistical analysis using the SPSS software package, version 16.0.

Results: 3-[4,5-dimethythiaziazol-2yl]-2,5-diphenyl tetrazoliumbr omide (MTT) assay revealed that 5-Aza-CdR significantly inhibit the growth and proliferation of PANC-1. The flow cytometry results showed over 40% and 70% of early and late apoptotic cells after treatment with 5 and 10 µm/L of 5-Aza-CdR, respectively. MSP-PCR data indicated that the treatment of cells with 10 µm/L 5-Aza-CdR resulted in partial demethylation of RASSF1A gene promoter. qPCR results showed significant re-expression of RASSF1A and up-regulation of Bax genes after 96 hours treatment of cells with 10 µm/L 5-Aza-CdR versus control cells ($P<0.01$).

Conclusion: The result demonstrated that 5 and 10 µM of 5-Aza-CdR induce cell death and apoptosis by epigenetic reactivation of RASSF1A and up-regulation of Bax genes.

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Keywords ● Decitabine ● Pancreatic Neoplasms ● DNA modification methylases ● Methylation

Introduction

Pancreatic cancer (PC) is known as “silent killer” because there are no clear symptoms in the early stage of the disease. Specific

symptoms usually develop until the later stages of the disease. For this reason, pancreatic cancer is the eighth and the ninth leading causes of cancer-related death in men and women, respectively.[1-3] About 5-10% of patients with pancreatic cancer have almost a family history and inherited mutations in few genes, including BRCA2, p16/CDKN2A, PRSS1, STK11, hMLH1, and FANCC that account for less than 20% of familial clustering.[4] However, similar to most other cancers, PC is a multifactorial disease that occurs in a multi-stage process in which genetic and epigenetic factors control its development.

Methylation is the major epigenetic regulation of genes in which a methyl residue is added on cytosine in CpG dinucleotide catalyzed by a DNA methyltransferases (DNMTs). Although the CpG islands are concentrated mostly in the gene promoter, approximately 5% of these promoters are methylated. However, this figure is far higher in cancer and changes in DNA methylation is one of the cancer hallmarks.[5] On the other hand, the alteration of DNA methylation has been reported to be related to up-regulation of DNMTs and whole genome methyl analysis have shown hypomethylation and hypermethylation in pancreatic cancer.[6]

Different mechanisms, including point mutation, gene deletion, and hypermethylation are involved in the inactivation of tumor suppressor genes (TSGs). Promoter hypermethylation of Ras association domain family 1A (RASSF1A) gene is one of the frequent events reported in a large variety of cancers. RASSF1A is known as a significant tumor suppressor and controls the growth of cells through the inhibition of RAS pathway.[7] Lack of RASSF1A expression has been reported in different tumors.[8,9] It also induces cell cycle arrest, apoptosis, genomic, and microtubule instability and its ectopic expression in different cell lines of cancers has been indicated that suppresses the tumor growth.[10-14] Apoptosis is a physiological event that is a critical homeostasis regulation in normal tissue.[12] Several bio-molecules are involved in this process, including BCL-2 family. Bax gene is the first pro-apoptotic member of BCL-2 family, which acts as the heart of intrinsic apoptosis pathway. Bax is inserted tightly within the outer mitochondrial membrane and involved in promoting death during apoptosis. Bax and/or Bak proteins assemble and induces penetration in the outer membrane of mitochondria to release apoptotic components such as cytochrome C.[15] The ability of cells to escape from apoptosis allows tumors to develop metastasis and chemotherapy resistance. Therefore, loss of function of key genes such as RASSF1A through methylation can lead to loss of vital function of cells including apoptosis and cell growth control. Bax expression, as a pro-apoptotic biomarker, was measured for confirming the effect of 5-AZA-CdR on apoptosis through epigenetic reactivation of RASSF1A.

DNA methylation and histone modification are the two major characteristics of epigenetic events, nevertheless, they are reversible.[16] Hence, using agents with the capability of epigenetic reversion can be used to re-express the important gene silenced via methylation. 5-Aza-2′-deoxycytidine (5-AZA-CdR, decitabine) is a demethylating agent that its pharmacological effects were first reported for the treatment of leukemia in 1968.[17] However, approving 5-AZA-CdR potential as a therapeutic agent for cancer treatment took a long time.[18,19] On the other hand, there are limited reports on the clinical activity of 5-AZA-CdR on solid tumors.[17]

5-Aza-CdR makes the transcriptional activation by promoter demethylation and DNMT inactivation.[20] Therefore, we encouraged to design the current study to examine the sufficient dose of 5-Aza-cdr on PANC-1, epigenetic reversion of RASSF1A, and up-regulation of Bax. In doing so, appropriated time and concentration of 5-Aza-cdr as an epigenetic treatment could be introduced to control cancers and improve its prognosis.

Materials and Methods

Cell Line and Cell Culture

PANC-1 was provided from the national cell bank, Pasteur Institute of Iran (Tehran, Iran). The cells were cultured in T25 culture flask containing high glucose DMEM (Sigma, USA) supplemented with 10% fetal bovine serum (FBS) (Sigma, USA) and 1% penicillin-streptomycin (Sigma, USA) at 37 °C in a humidified atmosphere containing 5% CO_2. When cells got to their logarithmic phase of growth (80% confluent cells), 10^5 cells were transferred to 24-well culture plates. Drug treatment was performed 24 hours after cell seeding.[12]

5-Aza-CdR Treatment

A stock solution of 5-Aza-CdR (50 mM) (Sigma, USA) was dissolved in DMSO and stored at -80 °C. The stock was diluted in cell culture media at different concentrations and IC_{50} was determined. Each culture was treated with different experimental dosages for 24, 48, 72, and 96 hours and treated cells were then harvested for further analysis. Culture without intervention with 5-Aza-CdR was considered as the control group.

MTT Assay

MTT assay was carried out to determine the viability of treated PANC-1 cells by 5-Aza-CdR according to previously reported instruction.[21] 5-Aza-CdR was dissolved in PBS at the final concentration of 5 mg/ml (82.5 µM). Then, the stock solution was added to the culture medium at a dilution of 1:10. The cells were incubated at 37 °C for 4 hours. In the next step, the medium was aspirated and 400 µl of DMSO was added to the extract. The cell viability rate was calculated as the percentage of MTT absorption by a microplate reader (Hiperion MPR 4, Germany) at the wavelength of 570 nm.

DNA Extraction, Bisulfite Treatment, and Methylation Specific PCR (MSP)

Initially, the isolation of genomic DNA was accomplished by PrimePrep™ Genomic DNA isolation kit (GeNet Bio, Korea) according to manufacturer's instruction. Sodium bisulfate treatment of the extracted DNA was performed using the EpiTect Bisulfite kit (Qiagen, Germany) according to manufacturer's instruction. Next, the PCR reactions were carried out in thermal cycler EP-Gradient PCR (Eppendorf, Germany) according to the previous study.[11]

Quantitative Real-Time Polymerase Chain Reaction (qPCR)

Total RNA isolation from each cell group was performed by RNeasy Mini kit (Qiagen, USA). RNA samples were treated with RNase-free DNase (Qiagen, USA) to eliminate any possible contamination with genomic DNA. RNA concentration was measured using BioPhotometer (Eppendorf, Germany). 5 µl of total RNA (200 ng/µl) was reverse-transcribed to cDNA by RevertAid™ kit (Fermentas, EU) according to the manufacturer's instruction. qPCR was carried out using the Maxima SYBR Green/ROX qPCR master mix kit (Fermentas, EU). The primers for amplifying the target regions were designed using AlleleID software (Primer Biosoft, USA) with the following sequences: RASSF1A (forward 5'-TCATCTGGGGCGTCGTG-3', reverse 5'-CGTTCGTGTCCCGCTCC-3'), Bax (forward 5'-GACCAGCATGACAGATTTCTACCA-3', reverse 5'-AACTGAG ACTAA GGCA GAAGATG-3'), and the reference gene ACTB (forward 5'-GTT GTCGACGACGAGCG-3', reverse 5'-GCA CAGAGCCTCGCCTT-3'). qPCR reactions were carried out in StepOnePlus™ quantitative real-time PCR detection system (Applied Biosystems, USA). The PCR amplification conditions consisted of 10 minutes at 95 °C followed by 40 cycles of denaturation at 95 °C for 15 seconds and annealing and extension for 1 minute at 60 °C. The gene expression ratios were calculated by the $2^{-\Delta\Delta Ct}$ method. These experiments were carried out in triplicates and the mean Ct put into expression analysis.

Flow Cytometric Analysis

The percentage of the apoptotic cell was evaluated by Annexin-V-FLUOS staining kit (Roche). About 10^6 cells of each sample were washed in PBS and re-suspended in 100 µL of Annexin-V-FLUOS labeling solution. Cells were then incubated at room temperature for 10-15 minutes. Finally, the apoptotic cells were counted by FACScan flow cytometry (Becton Dickinson, Heidelberg, Germany). Annexin-V-FITC can discriminate between apoptotic and necrotic cells. In apoptotic cells, the phospholipid phosphatidylserine is exposed on the outer surface of plasmalemma, leading to binding of Annexin-V and apoptotic cells stain green. However, necrotic cells take up propidium iodide and stain orange/green. These experiments were performed in triplicates intermittently.

Statistical Analysis

All the quantitative data are presented as mean±SD (standard deviation). The one-way analysis of variance (ANOVA) with the LSD post hoc test was performed to determine statistical significance among different groups using the SPSS software package, version 16.0. $P<0.05$ was accepted as a significant level.

Results

MTT Assay

Dose-response curves for 5-Aza-CdR treatment were designed based on MTT assay results to determine IC_{50}. Different concentrations of 5-Aza-CdR were tested during 24, 48, 72, 96 hours. No significant anti-cancer effect was observed during the first three days, but cell viability was significantly decreased after 4 days of treatment (96 h). The survival rate of cells after 96 hours of treatment with concentrations of 5 and 10 µM was 55% and 45%, respectively, and determined as IC_{50} (table 1). Morphological change of treated cells is illustrated in figure 1.

MSP-PCR

The findings revealed that treatment with 10 µM 5-Aza-CdR for 4 days induced partial demethylation of RASSF1A gene in PANC-1 cell line.[11]

Real-Time PCR

The result showed no significant re-expression and up-regulation of RASSF1A and Bax after

Table 1: The percentages of PANC-1 cell viability after treatment with 5-Aza-CdR in different dosages (1, 5, 10, 15, 20, 25, and 30 µM) and at different times (24, 48, 72, and 96 hours)

	Cell viability after 24 h treatment (%)	Cell viability after 48 h treatment (%)	Cell viability after 72 h treatment (%)	Cell viability after 96 h treatment (%)
5-Aza-CdR (1 µM)	100	100	100	100
5-Aza-CdR (5 µM)	100	96	92	55
5-Aza-CdR (10 µM)	98	84	78	45
5-Aza-CdR (15 µM)	95	80	74	36
5-Aza-CdR (20 µM)	95	78	75	32
5-Aza-CdR (25 µM)	86	75	72	28
5-Aza-CdR (30 µM)	88	73	70	22

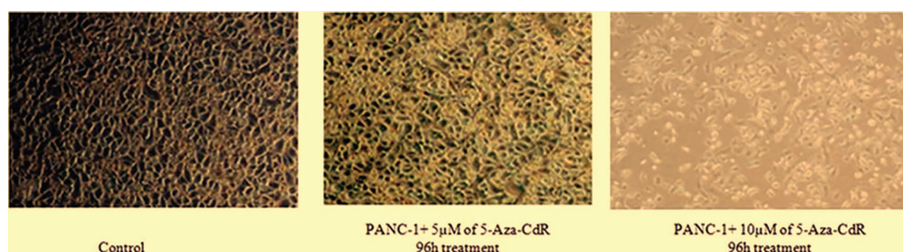

Figure 1: The morphologic view of PANC-1 after treatment with 5 and 10 µm of 5-Aza-CdR (×100). Clearly, the percentage of survived cells has significantly decreased after 96 hours of treatment with 10 µm of 5-Aza-CdR.

treatment of cells with 5 µM (P=0.087), while RASSF1A re-expression (P=0.034) and Bax up-regulation (P=0.042) were significant after treatment with 10 µM of 5-Aza-CdR compared to the untreated group (figure 2).

Flow Cytometry

An apoptosis assessment was done using the PI/Annexin-V dot plots as follow: Annexin-V and PI negative cells were known as normal and undamaged cells, apoptotic cells were those stained with annexin-V but negative for PI, and necrotic cells were those with both annexin-V and PI positive. Compared to the control (73%), the percentage of PANC-1 cell viability reduced to 54% and 28% in the presence of 5 µmol and 10 µmol after 96 hours of treatment, respectively (figure 3).

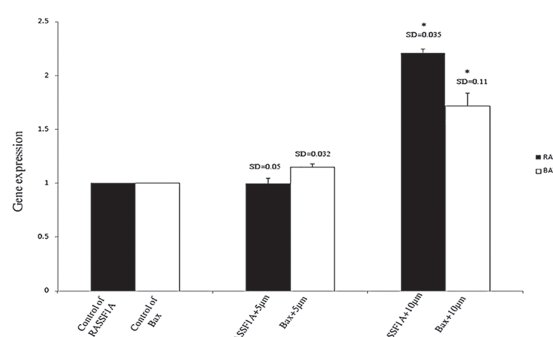

Figure 2: 5-Aza-CdR treatment in PANC-1 (5 and 10 µM for 96 h). 10 µm of 5-Aza-CdR epigenetically induced re-expression of RASSF1A (P=0.042) and subsequently up-regulation of Bax genes (P=0.034). However, in a concentration of 5 µm, the expression ratio of these genes was not significantly different in comparison with controls (P=0.784).

Discussion

In the present study, the time- and dose-dependent epigenetic effects of 5-Aza-CdR on PANC-1 were evaluated. Our results showed that 5-Aza-CdR has an inhibitory effect on PANC-1 growth after 4 days through the re-expression of RASSF1A and up-regulation of Bax, as the key players involved in apoptosis. It was observed that 10 µM of 5-Aza-CdR was a more effective concentration on PANC-1.

DNA methylation is a major part of epigenetic events that has a significant role in the cellular process, including genomic imprinting, tissue-specific gene expression, chromosomal inactivation, and tumor specific gene expression.[22] This event is catalyzed and maintained by DNMTs enzymes. Inactivation of TSGs is closely related to hypermethylation of CpG islands of the promoter region. On the other hand, the overexpression of DNMTs is a reason for gene silencing and cancer induction.[23] Fortunately, epigenetic events are reversible through DNMTs inhibition, hence, epigenetic reversion has attracted attention in drug discovery in cancer treatment studies.[24] 5-Aza-CdR activity in inhibition of DNMTs has previously been reported and its therapeutic use in low-dose was encouraged in myelodysplastic syndrome (MDS) and acute myelogenous leukemia (AML). However, the evaluation of its effects on solid

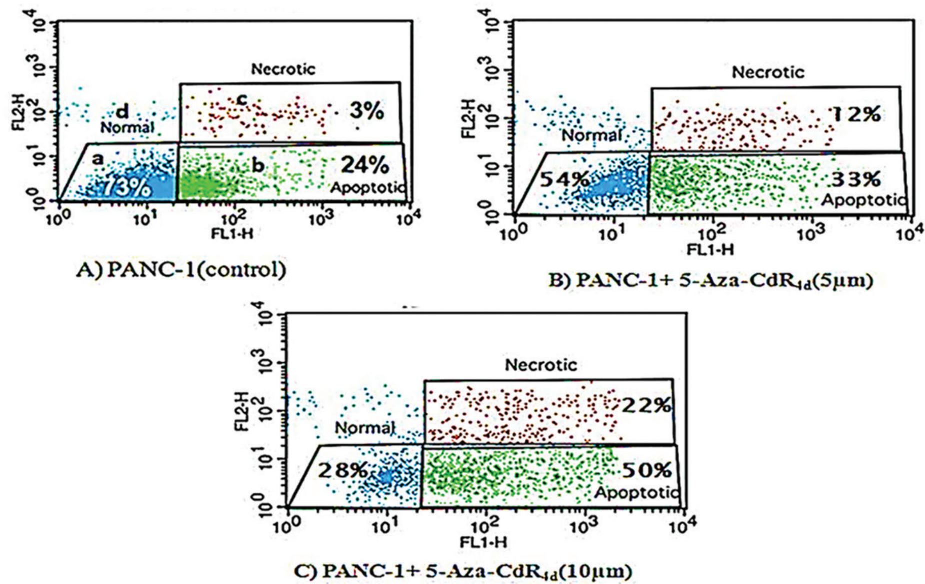

Figure 3: (A) Untreated PANC-1 (control), (B) PANC-1 treated with 5 µM of 5-AzaCdR, (C) PANC-1 treated with 10 µM of 5-Aza-CdR. The (a) quadrant indicates viable cells, which are negative for annexin-V and PI staining. The (b) quadrant shows apoptotic cells that take up annexin-V and stain green, while negative for PI staining. The (c) quadrant represents late apoptotic cells that are annexin-V and PI positive. The (d) quadrant represents necrotic cells, which are annexin-V negative and PI positive staining.

tumors has been limited which requires more investigations.[25,26]

An abnormal pattern of DNA methylation is observed in human neoplasia, although the promoter region of tumor suppressor genes in normal cells is un-methylated.[27] Therefore, any alterations of gene expression after 5-Aza-CdR treatment in normal cells are unrelated to 5-Aza-CdR DNMT inhibitory effects.[28] On the other hand, Qiu demonstrated that 5-Aza-CdR did not show any synergistic effect on the growth inhibition of normal fibroblast in combination with irradiation, while radiotherapy alone induced normal cell necrosis.[29] Furthermore, Fandy explained that 5-Aza-CdR incorporated only into DNA whereas 5-AC incorporates in RNA, interferes with protein translation, and induces specific cytotoxicity. This character of 5-Aza-CdR has made it more efficient on tumor cells and tissue in current clinical trials.[27]

Many studies have demonstrated that RASSF1A is expressed in all normal tissues, while none or low expression of this gene has been reported in many cancers, including the lung, pancreas, liver, and biliary tract cancers.[30,31] Some studies have shown that RASSF1A does not act through enzymatic activity, but functions as an underlying factor for other complex signaling pathways. It has two promoters; the shorter promoter has a length of 737 bp containing about 85 C and G dinucleotides and found in RASSF1A, RASSF1D, RASSF1E, RASSF1F, and RASSF1G genes. The longer promoter consists of 1364 bp and contains 139 CpG that acts as the promoter of RASSF1C and RASSF1B genes. Among this family, A and C isoforms have been biologically analyzed and little data are available about B, D, E, F, G, and H isoforms.[32]

It is reported that RASSF1A regulates the G1-S phase of cell cycle by controlling the level of cyclin D1. Inhibition of JNK pathway has been found to be the reason for this regulation.[33] It is also believed that RASSF1 family is a member of pro-apoptosis proteins and activates apoptosis through a variety of intracellular pathways.[34] In the current study, the demethylation effect and molecular mechanisms of a DNMT enzyme inhibitor, known as 5-Aza-CdR, on the PANC-1 cell line showed the same results as Pfeifer; that concentrations of 5 and 10 µM were more effective.[35]

Treatment with 5-Aza-CdR during the initial three days did not induce cell death while cell growth inhibition in comparison with the control group was significant on the fourth day after treatment (figure 3). This finding was partially consistent with the results reported by Stresemann et al.[36] Epigenetic reversion has previously been reported in the first and second days of treatment; however, the time dependency of 5-Aza-CdR has not been mentioned.[37-39] Therefore, 5-Aza-CdR probably needs more incubation (i.e. at least more than three days) to be an effective epigenetic therapy.

Our results also showed that the treatment of PNAC-1 cells with 5-Aza-CdR leads to partial

demethylation of RASSF1A gene promoter. After treatment with 5-Aza-CdR, unmethylated bands were observed in the MSP assay. The results of MSP were confirmed by measuring the gene expression ratio of RASSF1A before and after treatment by qPCR in which significantly increased expression value was seen after treatment (figure 2). This is in accordance with the studies conducted by Zuo, Agathangglous, and Shen.[23,32,37] Therefore, RASSF1A promoter hypermethylation seems to be an important occurrence in the pathogenesis of cancer (particularly pancreatic cancer) and its targeted epigenetic therapy can open a promising window to cancer treatment.[38]

As mentioned, RASSF1A gene has several functions and its inactivation is expected to affect various aspects of tumor biology. Vos reported that RASSF family proteins probably act as mediators for inducing some Ras-related growth inhibition and apoptosis. Through MOAP-1, RASSF1A activates Bcl-2 family proteins, including Bax and consequently intrinsic pathway of apoptosis.[39] In the present study, the correlation of RASSF1A re-expression by 5-Aza-CdR with cell apoptosis was investigated by measuring Bax gene expression ratio. Consistent with previous studies,[39] Bax gene expression was not significant (P=0.087) in 5 µM concentration, whereas its expression increased significantly (P=0.042) in 10 µM concentration compared with the control group (figure 2). Conversely, we have previously demonstrated the apoptotic effect of disulfiram (DSF) and Bax up-regulation after 24 hours of treatment without RASSF1A re-expression.[12] Furthermore, the flow cytometry data confirmed the activation of apoptosis pathway in treated cells and cell viability had a decreasing trend. The percentage of viable cells in untreated, 5 µM treated, and 10 µm treated groups was 73%, 54%, and 28%, respectively (figure 3). However, it would have been better to investigate the expression of RASSF1A and Bax at the protein level before and after treatment with 5-Aza-CdR. In addition, detailed tracking of signaling pathway activated by the reactivated RASSF1A could guide us to a better understanding of the molecular profile of the survived cells.

Conclusion

Taken together, 5-Aza-CdR was found to have a partial demethylation effect and epigenetically reverses the expression of silenced RASSAF1A in PNAC-1 cell line. It was also found that the epigenetic reversion potential of 5-Aza-CdR is time- and dose-depending and did not occur before day 4. Furthermore, the result showed a correlation between Bax up-regulation and RASSF1A re-expression. Discovering agents with demethylation effect could help cancer treatment.

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Conflict of Interest: None declared.

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