Combination Effect of Epigenetic Regulation and Ionizing Radiation in Colorectal Cancer Cells

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Abstract
Exposure of cells to ionizing radiation (IR) induces, not only, activation of multiple signaling pathways that play critical roles in cell fate determination, but also alteration of molecular pathways involved in cell death or survival. Recently, DNA methylation has been established as a critical epigenetic process involved in the regulation of gene expression in cancer cells, suggesting that DNA methylation inhibition may be an effective cancer treatment strategy. Because alterations of gene expression by DNA methylation have been considered to influence radiosensitivity, we investigated the effect of a DNA methyltransferase inhibitor, 5-aza-2′-deoxycytidine (5-aza-dC), on radiosensitivity. In addition, we investigated the underlying cellular mechanisms of combination treatments of ionizing irradiation (IR) and 5-aza-dC in human colon cancer cells. Colon cancer cell lines were initially tested for radiation sensitivity by IR in vitro and were treated with two different doses of 5-aza-dC. Survival of these cell lines was measured using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and clonogenic assays. The effects of 5-aza-dC alone with irradiation on cell growth, cell cycle distribution, apoptosis, and apoptosis-related gene expression were examined. Combination irradiation treatment with 5-aza-dC significantly decreased growth activity compared with irradiation treatment alone or with 5-aza-dC treatment alone. The percentage of HCT116 cells in the sub-G1 phase and their apoptotic rate was increased when cells were treated with irradiation in combination with 5-aza-dC compared with either treatment alone. These observations were strongly supported by increased caspase activity, increased comet tails using comet assays, and increased protein levels of apoptosis-associated molecules (caspase 3/9, cleaved PARP). Our data demonstrated that 5-aza-dC enhanced radiosensitivity in colon cancer cells, and the combination effects of 5-aza-dC with radiation showed greater cellular effects than that of single treatment, suggesting that the combination of 5-aza-dC and radiation has the potential to become a clinical strategy for the treatment of cancer.

Citation: Kim J-G, Bae J-H, Kim J-A, Heo K, Yang K, et al. (2014) Combination Effect of Epigenetic Regulation and Ionizing Radiation in Colorectal Cancer Cells. PLoS ONE 9(8): e105405. doi:10.1371/journal.pone.0105405  
Editor: Hiromu Suzuki, Sapporo Medical University, Japan  
Received May 25, 2014; Accepted July 21, 2014; Published August 19, 2014  
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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper and its Supporting information files.  
Funding: This study was supported by a National R&D Program (50596-2014) through the Dongnam Institute of Radiological & Medical Sciences funded by the Korean Ministry of Education, Science, and Technology. This work was also supported by the National Research Foundation (NRF) and Ministry of Science, ICT and Future Planning (MSIP), Korean government, through its National Nuclear Technology Program (2013M2A2A7043665). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.  
Competing Interests: The authors have declared that no competing interests exist. * Email: kmyang@dirams.re.kr (KMY); jmyi@dirams.re.kr (JMY)

Introduction
Epigenetics is the study of inheritable changes in gene expression or cellular phenotype caused by mechanisms other than changes in the underlying DNA sequences [1]. The epigenetic regulation of gene expression is mediated by mechanisms such as DNA methylation, modifications of histones, and positioning of the nucleosome along the DNA. Typically, DNA hypermethylation plays a critical role in the inactivation of genes involved in cell cycle regulation, DNA repair, apoptosis, cell signaling, transcription, and other cellular processes [2].

Aberrations in DNA methylation are frequently observed in many different cancer types [3,4]. In particular, silencing of tumor suppressor genes or other cancer-related genes by aberrant DNA hypermethylation in promoter or regulatory regions contributes to tumorigenesis [5,6]. Unlike genetic alterations, epigenetic events, including DNA methylation, are reversible, making epigenetic regulation extremely interesting from the point of view of developing new approaches to therapy. DNA hypermethylation can be reversed by DNA-demethylating agents. In addition, DNA methyltransferase (DNMT) inhibitors can restore the expression of genes silenced by DNA methylation. In recent years, the DNMT inhibitor, 5-aza-2′-deoxycytidine (5-aza-dC), has been shown to have anticancer activities in patients with leukemia, myelodysplastic syndrome, and several solid tumors [7,8]. Although a single epigenetic therapy has not shown significant reactions against most solid tumors [9], preclinical studies suggest that a combination of epigenetic modifiers, such as DNMT inhibitors or histone deacetylase inhibitors, may be effective. In addition, combination of these epigenetic modifiers with conventional chemotherapeutics may also be effective. Therefore, these types of combinatorial therapies are being examined in clinical trials [10,11]. However, few reports have investigated radiosensitivity associated with exposure to 5-aza-dC [12–15]. Recently, there has been growing interest in strategies using substances that regulate cellular
radiosensitivity to increase tumor radiosensitivity. Therefore, in this study, we report the therapeutic potential of combining 5-aza-dC with ionizing radiation (IR) to increase radiosensitivity in colorectal carcinoma cells and examine the cellular mechanisms underlying these effects.

**Materials and Methods**

**Cell culture and 5-aza-dC treatment**

The human colorectal carcinoma cell lines: HCT116, SW480, Colo320, and RKO, which were obtained from the American Type Culture Collection (ATCC, VA, USA), as well as a double-knockout cells (DKO) for DNA methyltransferase-1 and DNA methyltransferase-3b in HCT116 cell line [16], which retains <5% genomic DNA methylation, were cultured at 37°C with 20% O2 and 5% CO2. The HCT116, DKO, and SW480 cells were maintained in McCoy's 5A medium (WelGENE, Daegu, Korea). Colo320 cells were maintained in RPMI medium (WelGENE). 5-aza-dC (0.5 or 1 μM; Sigma-Aldrich, St. Louis, MO, USA) once daily for 3 days (Figure S1).

**Ionizing irradiation (IR) exposure**

Cells treated with 5-aza-dC for 3 days or non-treated control cells were exposed to gamma rays from a 137Cs-ray source (Eckert & Ziegler, Berlin, Germany) at a dose rate of 2.6 Gy/min. Following irradiation at doses of 2, 5, and 10 Gy, the cells were incubated for 3 days at 37°C, 20% O2, and 5% CO2.

**Clonogenic assay**

HCT116, SW480, RKO, Colo320, and DKO cells were seeded in 6-well plates (5000 cells/well) and treated with 5-aza-dC and IR. Cells were cultured for 2 weeks. The culture medium was replaced with fresh medium every 2 days. The colonies were fixed and stained with 1.25% crystal violet, washed extensively to remove excess dye, and imaged using a MultiXpress C9250ND scanner (SAMSUMG, Seoul, Korea). Colonies with >50 cells/colony were counted using the Image-Pro Plus 7.0 software (Media Cybernetics, Rockville, MD, USA).

**Cell proliferation and cell viability assays**

Cell proliferation was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells (2×10^3 cells/well) were seeded in 6-well plates and incubated at 37°C. After 48 h, cells were washed twice with phosphate-buffered saline (PBS), and 5 mg/mL MTT was added to PBS in each well. After removing the MTT solution, a solubilization solution (dimethyl sulfoxide/ethanol, 1:1) was added to each well to dissolve the formazan crystals. The absorbance at 570 nm was measured using a Paradigm microplate reader (Beckman Coulter, Fullerton, CA, USA). HCT116 cells treated with 5-aza-dC and/or irradiation were seeded at a concentration of 3×10^5 cells/well in 6-well plates. After 24, 48, 72 and 96 h, the cells were harvested, diluted with a Trypan blue working solution, and counted to generate a growth curve.

**Tumor growth analysis**

For in vivo evaluation of tumor growth, we used a subcutaneous tumor-bearing SCID mouse xenograft model generated by infecting cells. Female C.B-17 SCID mice were purchased from the Central Lab. Animals (Seoul, Korea). Six-week-old female mice were divided into experimental groups (n = 3 in each group). Mice were injected subcutaneously into the right sides of the dorsal area with 5×10^6 cells diluted in 100 μL of PBS. The tumor volumes were measured once per week. Tumor volume was estimated using the following equation: (short axis)^2 x (long axis) × 0.5.

**Flow cytometric analysis**

Cell cycle analysis was conducted using propidium iodide (PI). Cells were trypsinized, washed with PBS, and fixed in 75% ethanol at 4°C for 1 h. Prior to analysis, cells were washed again with PBS, suspended in a cold PI solution with 1 mg/mL RNase, and incubated in the dark for 30 min at room temperature. Flow cytometry analysis was performed using a FACScan instrument (BD FACSAria; BD Biosciences, San Jose, CA, USA). Apoptosis of treated cells was assessed using an Annexin V/FITC Apoptosis Detection Kit (BD Biosciences). Briefly, all of the cells were seeded and treated in 100-mm dishes. Subsequently, the cells were washed twice with cold PBS. The cells were stained with phycocerythrin (PE) annexin V and 7-amino-actinomycin and incubated for 15 min in the dark. After staining, binding buffer was added to the cells, which were analyzed using the FACScan instrument. FACS Diva software (BD Biosciences) was used for data analysis.

**Caspase 3/7 activity assay**

For the caspase 3/7 activity assay, the cells (5×10^5 cells) were seeded with 100 μL of McCoy's 5A medium into a 96-well plate. After 24 h of incubation, 100 μL of the Caspase-Glu 3/7 assay kit (Promega, Madison, WI, USA) substrate and buffer mixture were added to each well. The cells and solutions were gently mixed for 30 min and incubated at room temperature for 1 h in the dark. Fluorescence activity was measured using a luminometer (ATTO, Tokyo, Japan). All of the assays were repeated three times and contained negative controls.

**DNA damage assay**

DNA damage was determined using the OxiSelect Comet Assay Kit (Cell Biolabs, San Diego, CA, USA). 5-aza-dC- and/or IR-treated HCT116 cells (1×10^6) were mixed with low-melting-point agarose (1:10 ratio), and then the comet slide was filled with 75 μL of the mixture. The slides were incubated at 4°C for 30 min and then immersed in lysis buffer at 4°C in the dark for 1 h. The lysis buffer was then aspirated from slides, and slides were immersed in alkaline solution at 4°C in the dark for 30 min. Next, to eliminate the alkaline solution, slides were stored in Tris-acetate-EDTA (TAE) buffer for 5 min. The slides were placed in a horizontal electrophoresis chamber and subjected to electrophoresis with TAE buffer at 25 V for 20 min. The slides were dried and stained with DNA dye (CELL Biolabs). Comet tails were detected under a fluorescent microscope (Nikon, Tokyo, Japan).

**Western blot analysis**

Cells were lysed in lysis buffer, and total cell lysates containing equal amounts of proteins were loaded onto 4–12% gels for sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes (GE Healthcare Life Sciences, Piscatway, NJ USA). The membranes were blocked with 5% milk dissolved in PBS containing 0.02% Tween-20 and incubated overnight at 4°C with specific primary antibodies. The membranes were subsequently incubated with specific horseradish peroxidase conjugated secondary antibodies. Protein bands were visualized using a Fusion FX5 system (Vilber
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|   | HCT116 |   | SW480 |   | DKO |
|---|--------|---|--------|---|-----|
| mock | ![Image](image1.png) | 2Gy | ![Image](image2.png) | 5Gy | ![Image](image3.png) | 10Gy |
| 0.5μM | ![Image](image4.png) | 0.5μM+2Gy | ![Image](image5.png) | 0.5μM+5Gy | ![Image](image6.png) | 0.5μM+10Gy |
| 1μM | ![Image](image7.png) | 1μM+2Gy | ![Image](image8.png) | 1μM+5Gy | ![Image](image9.png) | 1μM+10Gy |

| Colo320 |   | RKO |
|--------|---|-----|
| mock | ![Image](image10.png) | 2Gy | ![Image](image11.png) | 5Gy | ![Image](image12.png) | 10Gy |
| 0.5μM | ![Image](image13.png) | 0.5μM+2Gy | ![Image](image14.png) | 0.5μM+5Gy | ![Image](image15.png) | 0.5μM+10Gy |
| 1μM | ![Image](image16.png) | 1μM+2Gy | ![Image](image17.png) | 1μM+5Gy | ![Image](image18.png) | 1μM+10Gy |

**B** HCT116

- survival fraction vs. radiation dose (Gy)
- control vs. 0.5 μM 5-aza-dC vs. 1 μM 5-aza-dC vs. DKO

**C** SW480

- survival fraction vs. radiation dose (Gy)
- control vs. 0.5 μM 5-aza-dC vs. 1 μM 5-aza-dC

**D** Colo320

- survival fraction vs. radiation dose (Gy)
- control vs. 0.5 μM 5-aza-dC vs. 1 μM 5-aza-dC

**E** RKO

- survival fraction vs. radiation dose (Gy)
- control vs. 0.5 μM 5-aza-dC vs. 1 μM 5-aza-dC
Lourmat, Eberhardzell, Germany). The following primary antibodies were used: anti-cleaved caspase 3 (Cell Signaling Technology, Danvers, MA, USA), anti-cleaved caspase 9 (Cell Signaling Technology), anti-cleaved PARP1 (Cell Signaling Technology), anti-survivin (Abcam, Cambridge, MA, USA), anti-p-p33 (Santa Cruz Biotechnology, CA, USA), and anti-α-actin (Sigma-Aldrich).

Statistical analysis
The results were presented as means ± standard deviation. Statistical analyses were performed using Student’s t-test. A P-value less than 0.05 was considered statistically significant.

Ethic Statement
All animal protocols used in the current study were reviewed and approved by the Institutional Animal Care and Use Committee at Dongnam Institute of Radiological & Medical Sciences (DIRAMS-IACUC-11-005).

Results
Effects of 5-aza-dC on the radiosensitivity of colorectal cancer cell lines
To determine whether 5-aza-dC enhances the cellular sensitivity to IR, the colon cancer cell lines HCT116, SW480, RKO, Colo320, and DKO were exposed to 5-aza-dC at two different doses (0.5 μM and 1 μM) for 72 h before being exposed to three different IR doses (2 Gy, 5 Gy, and 10 Gy) (Figure S1). Next, a clonogenic assay was performed showing that 5-aza-dC could radiosensitize all of the colon cancer cell lines tested, and the combination of 5-aza-dC and IR was superior to treatment with 5-aza-dC alone. DKO cells, genetically inhibit DNMT1 and 3b, showed also growth suppression by IR with dose dependent manner (Figure 1A).

Radiation survival curves were generated for each cell line treated with 5-aza-dC and IR to understand whether irradiation can be sensitized by 5-aza-dC in colon cancer cell lines (Figure 1B-E). Using the dose required to generate a survival fraction (SF) of 0.5 as a reference, the dose enhancement rates (DERs) were estimated. Cells treated with 5-aza-dC for 72 h before irradiation showed an increase in radiosensitivity, with a DER of: 1.19 (0.5 μM 5-aza-dC and 1.41 (1 μM 5-aza-dC) in HCT116 cells, 1.23 (0.5 μM 5-aza-dC) and 1.42 (1 μM 5-aza-dC) in SW480 cells, 1.23 (0.5 μM 5-aza-dC) and 1.28 (1 μM 5-aza-dC) in Colo320 cells, and 1.14 (0.5 μM 5-aza-dC) and 1.31 (1 μM 5-aza-dC) in RKO cells (Figure 1B-E). Our data suggested that a lower dose of 5-aza-dC could radiosensitize colorectal cancer cells. Because 1 μM 5-aza-dC or 10 Gy of IR are cytotoxic, relatively low doses of 5-aza-dC (0.5 μM) and IR (2 Gy and 5 Gy) were chosen for further studies to examine the effects of combining a DNMT inhibitor, 5-aza-dC and IR.

The combination of 5-aza-dC and IR induced growth suppression in colon cancer cells
We analyzed cell proliferation in HCT116 cells treated with 5-aza-dC or IR alone or with the combination of 5-aza-dC and IR. The growth curves showed that treatment with a combination of 5-aza-dC and IR (2 Gy and 5 Gy) resulted in statistically significant growth inhibition in HCT116 and SW480 cells at each time point examined (24, 48, 72, and 96 h) compared with that in control cells, in cells treated only with 5-aza-dC, or in cells treated only with IR (2 Gy and 5 Gy) (Figure 2A; P<0.05). In addition, we carried out the MTT assay in both HCT116 and SW480 cells that had been treated with or without 5-aza-dC (0.5 μM) and then irradiated them with 2, 5, and 10 Gy, respectively. The absorbance from the assay performed on cells treated with a combination of 5-aza-dC and IR was significantly lower (P<0.05) than that from cells treated with 5-aza-dC or IR alone (Figure 2B). We also performed growth curve analysis and the MTT assay in DKO cells, which also showed a decrease of growth suppression as well as proliferation, depending on the radiation dose (Figure 2A and B). Thus, these results indicate that the effects of 5-aza-dC and IR on growth inhibition are additive. Based on our in vitro data of significant growth inhibition in cells treated with the combination of 5-aza-dC and IR, we were interested in determining whether these effects could be observed in vivo. To this end, HCT116 cells were exposed to 5-aza-dC (0.5 μM) for 72 h before exposure to IR (2 Gy and 5 Gy), and then were subcutaneously injected into SCID mice. Figure 2C showed significantly delayed tumor growth with the combination treatment of 5-aza-dC and IR (2 Gy and 5 Gy) compared with single treatment with either 5-aza-dC or IR. In addition, the volume of the xenografts from cells treated with both 5-aza-dC and IR were reduced compared with those from cells treated with either 5-aza-dC or IR alone.

To elucidate the mechanisms of growth inhibition by 5-aza-dC, IR, and the combination treatment, we used flow cytometry analysis to determine whether growth inhibition was associated with cell cycle changes. Cell cycle distribution analysis showed that the proportion of treated cells in the G1, S, and G2-M phases was not different from the control cells except that there was an increase in the proportion of cells in the sub-G1 phase, suggesting an increase in apoptosis (Figure 3). The proportion of HCT116 cells treated with 5-aza-dC or IR (2 Gy) alone in the sub-G1 phase of cells was eight fold (5-aza-dC), two fold (IR, 2 Gy), and four fold (5 Gy) greater than that of control cells and over eight fold greater in cells treated with 5-aza-dC and IR than in control cells. Interestingly, unlike HCT116 cells, SW480 cells showed G2/M arrest after IR alone (2 Gy and 5 Gy) compared with controls [2-Gy G2/M fraction: 46.77% (vs. 28.03% in control cells); 5 Gy G2/M fraction: 58.88% (vs. 22.03% in control cells)]. However, the sub-G1 phase of cells treated with 5-aza-dC or IR (2 Gy) alone was six fold (5-aza-dC), two fold (2 Gy), and seven fold (5 Gy) greater than control cells and an over 19-fold increase in cells treated with 5-aza-dC and IR than in control cells. We also confirmed that the sub-G1 phase of cells irradiated with 2 Gy or...
In both analyses, DKO cells were in apoptosis caused by the combination treatment of 5-aza-dC and aza-dC alone (Figure 4B). These results correspond with increased (2 Gy and 5 Gy) compared with those in cells treated with IR or 5-aza-dC and IR, either alone or in combination, the activities of caspases 3 and 7, which are key effectors for apoptosis in mammalian cells [18]. In both cell extracts treated with 5-aza-dC caspases 3 and 7, which are key effectors for apoptosis in mammalian cells [18]. In both cell extracts treated with 5-aza-dC and IR compared with IR (3.4-fold for 2 Gy or 2.4-fold for 5 Gy) or 5-aza-dC (>1.5-fold) alone in SW480 cells (Figure 4A). Furthermore, we investigated the cellular mechanisms underlying the apoptotic effects of the combination of 5-aza-dC and IR. One of the most common signaling cascades involved in apoptosis is the activation of the highly apoptosis specific family of caspases, which, once activated, initiate cell death by cleaving and activating effector caspases driving apoptosis [17]. To determine whether caspases mediated the effects of 5-aza-dC and IR, we measured the activities of caspases 3 and 7, which are key effectors for apoptosis in mammalian cells [18]. In both cell extracts treated with 5-aza-dC and IR, either alone or in combination, the activities of caspases 5 and 7 were greatly increased in cells treated with 5-aza-dC and IR (2 Gy and 5 Gy) compared with those in cells treated with IR or 5-aza-dC alone (Figure 4B). These results correspond with increased in apoptosis caused by the combination treatment of 5-aza-dC and IR and is shown in Figure 4A. In both analyses, DKO cells were also shown increasing the level of apoptosis by IR. These results are strongly supported by longer comet tails observed in the comet assay, indicating greater amount of cellular DNA damage in cells treated with a combination of 5-aza-dC and IR compared with control cells or cells treated with 5-aza-dC or IR alone (Figure 4C and D).

Caspases 3 and 9 are also known to be involved in radiation-induced apoptosis [19]. Thus, we used western blotting to confirm the activation levels of caspases 3 and 9 in cells treated with 5-aza-dC and/or IR. Figure 5 shows higher levels of activated caspases 3 and 9 in HCT116, DKO, and SW480 cells treated with 5-aza-dC or IR alone compared with those in control cells. Interestingly, western blotting, in the three, tested colon cancer cell lines, revealed that the protein levels of caspases 3 and 9 were also increased in cells treated with a combination of 5-aza-dC and IR compared with cells treated with either agent alone or in controls cells. The level of cleaved PARP1, which is another key effector of apoptosis induction [20,21], was also increased in cells treated with 5-aza-dC or IR alone compared with control cells, and even more increased in cells treated with a combination of 5-aza-dC and IR. By contrast, the protein expression levels of survivin were decreased in cells treated with 5-aza-dC and IR alone and in combination compared with those in control cells. In addition, we tested the level of p53 as well. Interestingly, the p53 expression level increased in cells treated with a combination of 5-aza-dC and IR than in cells treated with 5-aza-dC alone. This data is agreement with previous report which is cells expressing wild type of p53 are better sensitive IR than mutant type of p53 [14]. These protein expression patterns were confirmed in irradiated DKO cells. Taken together, our data suggest that the combination of 5-aza-dC and IR synergistically induces a higher level of apoptosis.

Figure 2. Growth inhibition in colon cancer cell lines in response to treatment with 5-aza-dC and irradiation. (A) Cell growth curves obtained using 0.5 μM 5-aza-dC and two different radiation doses (2 Gy and 5 Gy) in colon cancer cells (HCT116, DKO and SW480) and (B) MTT assays in colon cancer cells treated with 5-aza-dC (0.5 μM) and/or irradiation (2 Gy and 5 Gy). Data are expressed as the mean ± standard deviation of triplicate experiments. (C) Tumor growth following 5-aza-dC (0.5 μM) treatment and/or irradiation (2 Gy and 5 Gy) in SCID mice. HCT116 cells (5×10⁶ cells) that had been treated with 5-aza-dC (0.5 μM) and irradiated (2 Gy and 5 Gy) were injected subcutaneously into SCID mice (n = 4), and the average tumor size was measured once weekly for 7 weeks. P-values were calculated using Student’s t-test. *P<0.05; **P<0.01.

doi:10.1371/journal.pone.0105405.g002

Figure 3. Cell cycle distributions of colon cancer cell lines in response to 5-aza-dC and irradiation exposure. Colon cancer cells (HCT116, DKO and SW480) treated with 5-aza-dC (0.5 μM) and/or irradiation (2 Gy and 5 Gy) were stained with propidium iodide and analyzed using a FACS flow cytometer. Columns show the proportion of cells in each cell cycle phase. Black column, sub-G1 phase; bright gray column, G1 phase; dark gray column, S phase; white column, G2-M phase.

doi:10.1371/journal.pone.0105405.g003
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A. Annexin V assay

B. Caspase-3/7 activity

C. Images showing cell morphology

D. Percent of Positive cells (%)
compared with single treatment using 5-aza-dC or IR in several colon cancer cells.

Discussion

IR exposure results in the simultaneous activation or downregulation of multiple signaling pathways that play critical roles in cell type-specific control of survival or death. IR is a well-known genotoxic agent and human carcinogen that induces cellular damage through direct and indirect mechanisms [22]. Recently, many studies have focused on the molecules and processes that influence the response of cells to IR. Many different types of molecules are known to increase radiosensitivity by affecting cell cycle checkpoints, DNA repair, gene transcription, and apoptosis. The most recent studies have suggested that epigenetic mechanisms such as histone modification and DNA methylation are associated with gene silencing and may be involved in regulating radiosensitivity in cancer cells. A number of previous studies have reported that several histone deacetylase inhibitors are cytotoxic and can sensitize tumor cells to radiotherapy. However, scant information is available regarding the effects of DNMT inhibitors on radiosensitization [13,23]. Moreover, 5-aza-dC has been demonstrated to have little activity in solid tumors as a single agent [24,25], and little is known concerning the molecular or cellular mechanisms underlying the radiosensitivity induced by epigenetic inhibitors. Combining epigenetic drugs with radiotherapy is particularly interesting, in this context, and has demonstrated improved efficacy both in vitro and in vivo in several solid tumors [13–15,26]. In the present study, we investigated the cellular effects of the DNMT inhibitor, 5-aza-dC, and IR, both alone and in combination, on colon cancer cells. We found that 5-aza-dC demonstrated additive effects on growth inhibition combined with IR, suggesting that 5-aza-dC might be a useful radiation sensitizer in colon cancer treatment. One thing that we need to consider further based on our results, DKO cells, genetic model system for inhibition of DNA methyltransferase, do not seem to have more strong growth suppressive effect with IR than using pharmacological model system that we treated 5-aza-dC with IR.

Since our results indicate that this effect is mediated by the induction of apoptosis, apoptosis has been previously regarded as a potential mechanism for radiosensitization. Several different results have been reported regarding the radiosensitizing effects of DNMT inhibitors. Dote et al. previously reported that the combination of IR and zebularine did not significantly increase apoptosis [13]. By contrast, Qiu et al. demonstrated that 5-aza-dC induced radiosensitization in certain gastric cancer cell lines and caused an increase in apoptosis, which was accompanied by enhanced expression of p53, RASSF1, and DAPK gene families [14]. In our study, 5-aza-dC increased the level of apoptosis in HCT116 and SW480 cells, a finding that was consistent with...
results from Qiu et al. Very interestingly, Qiu et al. also suggested that gastric cancer cell lines expressing wild-type p53 are more sensitive to combination therapy with IR and 5-aza-dC compared with those expressing mutant p53. The HCT116 cell line used in this study expresses wild-type p53, and we observed that the p53 level increased in response to 5-aza-dC treatment both with and without IR. In addition, the p53 expression level increased in cells treated with a combination of 5-aza-dC and IR than in cells treated with 5-aza-dC alone. However, unlike in HCT116 cells, no effect was shown on the p53 level in SW480 cells, which expresses mutant type p53 (Figure 5). p53 has been classically described as a mediator of IR cytotoxicity and acts by promoting either cell cycle arrest or apoptosis [27,28]. Previous studies have reported that 5-aza-dC induces p53 expression, which is associated with inhibition of cell proliferation in wild-type p53 cells but not in mutant p53 cells in prostate cancer [29,30]. However, because only a few cell lines and p53-associated molecules were examined in these studies, further research into the possible association of 5-aza-dC with p53 is necessary. Further investigation is also required to identify additional epigenetic alterations associated with radiosensitivity. There are limited studies on the role of DNA methylation in resistance to IR. One previous study indicated that treatment with 5-aza-dC causes global hypomethylation, which has a radiosensitizing effect [23]. Therefore, definitive studies should be conducted to determine whether IR has an effect on site- or gene-specific DNA methylation in cancer (manuscript in preparation).

In this study, cell cycle analysis was not significantly altered by single treatment with 5-aza-dC or IR, as well as by the combination treatment with both, except for the proportion of cells in the apoptotic sub-G1 phase. The proportion of cells in the G2/M phase detected in HCT116 and SW480 cells was not significantly changed by 5-aza-dC alone, a finding that is in agreement with other previous studies [31,32]. Although there is accumulation of the G2/M phase by treatment with radiation alone in SW480, a discrete apoptotic sub-G1 peak appeared in the combined treatment. Thus, it is likely that, after combination treatment with 5-aza-dC and IR, 5-aza-dC may modify the apoptotic portion of cells to some extent.

Other groups have previously reported that 5-aza-dC enhanced radiosensitivity in various cancer types [14,23,26]. In the present study, a low dose of 5-aza-dC (0.5 μM) was used in combination with IR. As shown in Figure 1, although we tested treatment using 1 μM 5-aza-dC in combination with different doses of IR, we observed that a lower dose of 5-aza-dC (0.5 μM) and a nontoxic level of IR (2 Gy) are sufficient to induce anticancer effects in colorectal cancer cell lines. Although Schuebel et al. reported that 5 μM 5-aza-dC was required to reactivate the expression of most silenced genes [33], we found that 0.5 μM 5-aza-dC can reactivate most of the key genes regulated by promoter DNA methylation in HCT116 cells (data not shown).

In conclusion, a lower dose of 5-aza-dC can act as a radiation sensitizer to induce apoptosis in combination with IR. We have confirmed that treatment of HCT116 and SW480 cells with a combination of 5-aza-dC and IR increased the level of apoptosis and contributed to an anticancer effect. Our results strongly suggest that combining 5-aza-dC and IR may be a potential cancer treatment strategy.

Acknowledgments

This study was supported by a National R&D Program (50596–2014) through the Dongnam Institute of Radiological & Medical Sciences funded by the Korean Ministry of Education, Science, and Technology. This work was also supported by the National Research Foundation (NRF) and Ministry of Science, ICT and Future Planning (MSIP), Korean government, through its National Nuclear Technology Program (2013M2A2A7043665). We would like to thank Khadijah Mitchell at the Johns Hopkins School of Medicine for the critical reading of this manuscript and providing language help.

Author Contributions

Conceived and designed the experiments: JGK JHB KMY JMY. Performed the experiments: JGK JHB KMY. Analyzed the data: JGK JHB JAK. Contributed reagents/materials/analysis tools: KH KMY. Contributed to the writing of the manuscript: JGK JHB JAK. Contributed to the writing of the manuscript: JGK JMY. Contributed to the writing of the manuscript: JGK JMY.

References

1. Esteller M (2008) Epigenetics in Cancer. New England Journal of Medicine 358: 1148–1159.
2. Kulis M, Esteller M (2010) 2′-DNA Methylation and Cancer. In: Zdenko H, Toshikazu U, editors. Advances in Genetics: Academic Press. pp. 27–56.
3. Jones PA, Laird PW (1999) Cancer-epigenetics comes of age. Nat Genet 21: 163–167.
4. Jones PA, Baylin SB (2007) The Epigenomics of Cancer. Cell 128: 683–692.
5. Herman JG, Baylin SB (2000) Gene Silencing in Cancer in Association with Promoter Hypermethylation. New England Journal of Medicine 349: 2042–2054.
6. Baylin SB, Jones PA (2011) A decade of exploring the cancer epigenome — biological and translational implications. Nature Reviews Cancer 11: 726–734.
7. Kaminakas E, Farrell A, Abraham S, Baird A, Hsieh LS, et al. (2005) Approval Summary: Azacitidine for Treatment of Myelodysplastic Syndrome Subtypes. Clinical Cancer Research 11: 3604–3608.
8. Yoo CB, Jones PA (2006) Epigenetic therapy of cancer: past, present and future. Nat Rev Drug Discov 5: 37–50.
9. Plimack ER, Stewart DJ, Isa JP (2007) Combining Epigenetic and Cytotoxic Therapy in the Treatment of Solid Tumors. Journal of Clinical Oncology 25: 4519–4521.
10. Kelly TK, De Carvalho DD, Jones PA (2010) Epigenetic modifications as therapeutic targets. Nat Biotechnol 28: 1090–1097.
11. Kristenzen LS, Nielsen HM, Hansen LL (2009) Epigenetics and cancer treatment. European Journal of Pharmacology 625: 131–142.
12. Camphausen K, Tolfo P (2007) Inhibition of Histone Deacetylation: A Strategy for Tumor Radiosensitization. Journal of Clinical Oncology 25: 4051–4056.
13. Dote H, Cerna D, Burgan WE, Carter DJ, Cerra MA, et al. (2005) Enhancement of In vitro and In vivo Tumor Cell Radiosensitivity by the DNA Methyltransferase Inhibitor Zebularine. Clinical Cancer Research 11: 4571–4579.
14. Qiu H, Yashiro M, Shinto O, Matsuzaki T, Hirakawa K (2009) DNA methyltransferase inhibitor 5-aza-CdR enhances the radiosensitivity of gastric cancer cells. Cancer Science 100: 111–118.
15. De Schutter H, Kimpe M, Ibeaert S, Nuyts S (2009) A Systematic Assessment of Radiation Dose Enhancement by 5-Aza-2′-Deoxycytidine and Histone Deacetylase Inhibitors in Head-and-Neck Squamous Cell Carcinoma. International Journal of Radiation Oncology Biology Physics 73: 904–912.
16. Rhee J, Bachman KE, Park BH, Jair KW, Yes RW, et al. (2002) DNMT1 and DNMT3b cooperate to silence genes in human cancer cells. Nature 416: 552–556.
17. Li J, Yuan J (2008) Caspases in apoptosis and beyond. Oncogene 27: 6194–6206.
18. Lakhani SA, Masud A, Kuida K, Porter GA Jr, Booth CJ, et al. (2006) Caspases 3 and 7: key mediators of mitochondrial events of apoptosis. Science 311: 847–851.
19. Zhang Y, Dimtchev A, Dritschilo A, Jung M (2001) Ionizing Radiation-induced Apoptosis in Ataxia-Telangiectasia Fibroblasts: Roles of caspase-9 and cellular inhibitor of apoptosis protein-1. Journal of Biological Chemistry 276: 28842–28848.
20. Kaufmann SH, Desnoyers S, Ottaviano Y, Davidson NE, Poirier GG (1993) Specific Proteolytic Cleavage of Poly(ADP-ribose) Polymerase: An Early Marker of Chemotherapy-induced Apoptosis. Cancer Research 53: 3976–3985.
21. Tewari M, Quan LT, O’Rourke K, Desnoyers S, Zeng Z, et al. (1995) Yama/CPP32 is a mammalian homolog of CED-3, a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. Cell 81: 801–809.
22. Goodhead DT (1994) Initial events in the cellular effects of ionizing radiations: clustered damage in DNA. International journal of radiation biology 65: 7–17.
23. Hofstetter B, Niemierko A, Forrer C, Benhattar J, Albertini V, et al. (2010) Impact of Genomic Methylation on Radiation Sensitivity of Colorectal Carcinoma. International Journal of Radiation Oncology Biology Physics 76: 1512–1519.
24. Abele R, Dodion P, Brustuch U, Gandersen S, Smyth J, et al. (1987) The EORTC Early Clinical Trials Cooperative Group experience with 5-aza-2’-deoxycytidine (NSC 127716) in patients with colo-rectal, head and neck, renal carcinomas and malignant melanomas. European Journal of cancer & clinical oncology 23: 1921–1924.
25. van Groeningen CJ, Levy A, O’Brien AMP, Gall HE, Pinedo HM (1986) Phase I and Pharmacokinetic Study of 5-Aza-2’-deoxycytidine (NSC 127716) in Cancer Patients. Cancer Research 46: 4831–4836.
26. Wang L, Zhang Y, Li R, Chen Y, Pan X, et al. (2013) 5-Aza-2’-Deoxycytidine Enhances the Radiosensitivity of Breast Cancer Cells Cancer Biotherapy & Radiopharmaceuticals 28: 34–44.
27. Kuerbitz SJ, Plunkett BS, Walsh WV, Kastan MB (1992) Wild-type p53 is a cell cycle checkpoint determinant following irradiation. Proceedings of the National Academy of Sciences 89: 7491–7495.
28. Lowe SW, Schmitt EM, Smith SW, Osborne BA, Jacks T (1993) p53 is required for radiation-induced apoptosis in mouse thymocytes. Nature 362: 847–849.
29. Karpf AR, Moore BC, Ririe TO, Jones DA (2001) Activation of the p53 DNA Damage Response Pathway after Inhibition of DNA Methyltransferase by 5-Aza-2’-deoxycytidine. Molecular Pharmacology 59: 751–757.
30. Pulakuri SM, Rao JS (2005) Activation of p53/p21Waf1/Cip1 pathway by 5-aza-2’-deoxycytidine inhibits cell proliferation, induces pro-apoptotic genes and mitogen-activated protein kinases in human prostate cancer cells. international Journal of Oncology 26: 863–871.
31. Kim HJ, Kim JH, Chie EK, Young PD, Kim IA, et al. (2012) DNMT (DNA methyltransferase) inhibitors radiosensitize human cancer cells by suppressing DNA repair activity. Radiat Oncol 7: 39.
32. Doe H, Cerna D, Burgan WE, Carter DJ, Cerra MA, et al. (2005) Enhancement of in vitro and in vivo tumor cell radiosensitivity by the DNA methyltransferase inhibitor zebularine. Clinical Cancer Research 11: 4571–4579.
33. Schubel KE, Chen W, Cope L, Gleckner SC, Suzuki H, et al. (2007) Comparing the DNA Hypermethylation with Gene Mutations in Human Colorectal Cancer. PLoS Genet 3: e157.