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Original Article

Anacardic acid sensitizes prostate cancer cells to radiation therapy by regulating H2AX expression

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Abstract: Anacardic acid (6-pentadecylsalicylic acid, AA), a natural compound isolated from the traditional medicine Amphipterygiumadstringens, has been reported as potential antitumor agents in various cancers including prostate cancer (PC). However, the effects and mechanism of AA on the radiosensitivity of prostate cancer remains unknown. The results indicated that AA exhibited strong antitumor activity in PC cell lines, either as a single agentor in combination with radiation. AA significantly induced the downregulation of H2AX and p-H2AX expression, increase of cell apoptosis and decreasing of cell invasion, which were reversed by overexpressed H2AX. These results suggest that AA sensitize prostate cancer cells to radiation therapy by repressing H2AX expression.

Keywords: Anacardic acid, prostate cancer, H2AX, radio therapy

Introduction

Prostate cancer (PC) as the most common type of cancer is the second leading cause of cancer death among men in the developed world [1]. The standard treatments of PC include surgery, chemotherapy and radiotherapy. Radiotherapy is generally used as preoperative and postoperative treatment that provides high biochemical control, low risk of complications, minimal duration of treatment, and out-patient treatment opportunity. However, intrinsic tumor radioresistance accounts for the high recurrence, leading to accelerated disease progression and death [2, 3]. Therefore, identification of reliable radio sensitizers to PC cells would be urgently desirable. Targeted biological therapies that selectively interfere with cancer cell growth signals may improve patient survival by enhancing the effects of radiation with little damage to normal tissue.

Anacardic acid (2-hydroxy-6-pentadecylbenzoic acid, AA) is a constituent of the shell of the cashew-nut (Anacardiumoccidentale) [4], and is a dietary component found in cashew apple (Anacardium occidentale) and ginkgo (Ginkgo biloba) leaves and fruits. Studies show that AA acts as a mitochondrial uncoupler of oxidative phosphorylation [5] and as a traditional medicine for treatment of gastric ulcers, gastritis and stomach cancers [6]. Recently, AA showed certain antitumor activities in prostate cancer, lung carcinoma, ovarian cancer and breast carcinoma, and is thought to exert its action via various mechanisms [7-9]. AA also sensitizes HeLa cells to ionizing radiation by inhibition of histone acetyl transferase activity [10]. However, the effects and mechanism of AA on the radio therapy of prostate cancer remain unknown.

Materials and methods

Reagent, cell culture and antibodies

A 50-mmol/L solution of AA (Merck, Germany) was added in dimethylsulfoxide. AA was prepared in dilution with culture medium when necessary.

Human Prostate Cancer Cell Lines DU145 and PC3 were purchased from Yinrun (Changsha, China). And cultured in Dulbecco's modified Eagle's Medium (DMEM) and F12KM medium supplemented respectively with 50 U/ml penicillin, 50 mg/ml streptomycin (Gibco), and 10% fetal bovine serum (FBS, Sijiqing Biological Engineering Materials) at 37°C in an atmosphere of 5% CO_2.

CCK-8 assay

Logarithmically growing cells were counted and seeded in 96-well plates at 2000 cells per well, in triplicate and incubated overnight. The next morning, all plates were aspirated and fresh medium were added in a final volume of 200 μl with increasing concentrations of AA (0, 5, 25, and 125 μmol/L). Following drug addition, the plates were incubated for 5 days. Cell growth inhibition was examined by CCK-8 assay (Best Bio, China). 10 μl CCK-8 labeling reagent was added to each well with 100 μl fresh medium, the plates were incubated at 37°C for 4 h. The absorbance of each well was measured at 450 nm using Thermo Scientific Varioskan Flash (Thermo Scientific, Finland). Percentage of viable cells = (OD_{450} of treated sampleOD_{450} of blank sample)/(OD_{450} of control sample-OD_{450} of blank sample)×100. The results shown were mean values of 3 independent experiments.

Clonogenic assay

Clonogenic assay was used to evaluate the effect of BIIB021 in combination with radiation. Cells were trypsinized to generate a single cell suspension and seeded in 6-well plates at 1500 cells per well. After allowing cells to attach, AA was added about 125 μmol/L. For AA in combination with radiation, 24 h after adding AA, cells were irradiated with a single dose of 2, 4, 6, 8 Gy from amedicallinear accelerator (varian23EX, USA). Four hours after radiation, all plates were aspirated and fresh medium were added. 14 days after seeding, colonies were stained with crystalviolet, and the number of colonies containing at least 50 cells was counted. The colony survival fraction was calculated for each treatment and data were presented as log plot. The results shown were mean value of 3 independent experiments with triplicate setting in each experiment.

Flow cytometry analysis of apoptosis

To test if cells were undergo apoptosis, Eca109 and Eca9706 cells were plated and exposed to either 1 μM AA or 6 Gy radiation for 24 h. For combination, cells were treated with 1 μM AA for 24 h, and then treated with a single dose of 6 Gy of radiation. Cells were collected 24 h after radiation without washing (both floating and attached cells were collected by centrifugation). Apoptosis analysis was performed according to the manufacturer's instructions (Annexin V-FITC Apoptosis detection kit; BestBio, China). Approximately 1×10^5 cells were incubated with FITC-conjugated annexin V in the presence of PI and then analyzed by flow cytometry (FACS, Becton Dickson). Annexin V positive PI negative cells scored as early apoptotic, Annexin V positive PI positive cells corresponded to late apoptotic cells. Cell cycle distribution was measured before and after the same treated as above. Cells were digested by trypsin, washed with PBS, fixed with 75% cold ethanol at 4°C overnight and dyed with PI, and then analyzed by flow cytometry.

Cell invasion assay

The capability of cell invasion was examined by transwell invasion assay. Cells were cultivated to 80% confluence on the 12-well plates. Then, we observed the procedures of cellular growth at 72 h. All the experiments were repeated in triplicate. The transwell migration chambers were used to evaluate cell invasion. Then cells invading cells across the membrane were counted under a light microscope.

Western blot analysis

Whole cell extracts were prepared with a cell lysis reagent (Sigma-Aldrich, St. Louis, MO, USA) according to the manual, and then, the protein was quantified by a BCA assay (Pierce, Rockford, IL, USA). Then, the protein samples were separated by SDS-PAGE (10%) and detected by Western blot using polyclonal (rabbit) anti-H2AX antibody (Santa Cruz Bio-technology, Santa Cruz, CA, USA). Goat anti-rabbit IgG (Pierce, Rockford, IL, USA) secondary antibody conjugated to horse radish peroxidase and ECL detection systems (SuperSignal West Femto, Pierce) were used for detection.

Statistical analysis

All data were shown as mean ± SD. Statistical significance was assessed by T-test for two-group comparison. Differences with P value <0.05 were considered statistically significant.

Results

AA inhibited the proliferation and survival of PC cell lines

To investigate the effects of AA on PC3 cells, we detect the influence of different doses of AA on

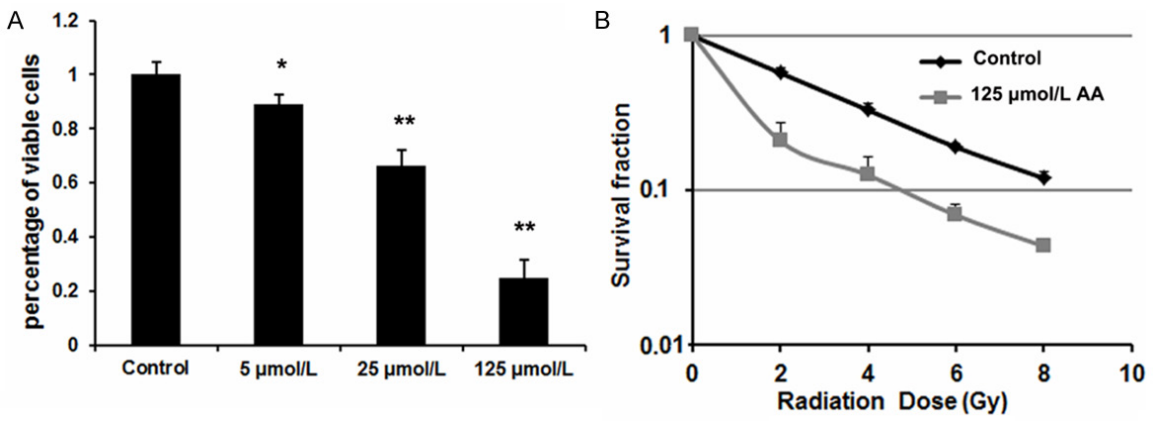

Figure 1. A. AA inhibited the growth of PC3 cells in a dose-dependent manner. *P<0.05 versus control group. B. AA1 radio sensitized PC3 cell lines. PC3 cells were irradiated at a single dose of 0, 2, 4, 6 or 8 Gy in the presence or absence of AA (IC25 concentration for each cell line). Data were showed as mean ± SD of three experiments. P<0.05 for each radiation dose in combination with AA versus each radiation dose alone for both of the two cell lines.

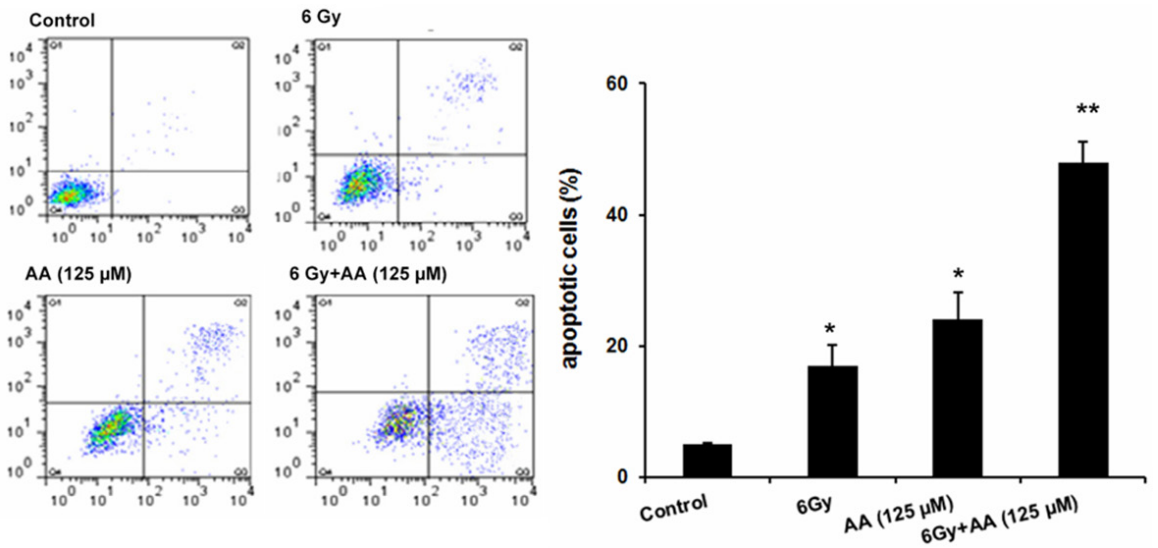

Figure 2. AA induced PC cell death via apoptosis. Apoptosis for all treatments of PC3 cells. Combination of radiation and AA induced significant apoptosis in PC cell line. Bargraphs represent the total apoptosis of all conditions. The total amount of apoptosis was the results of early apoptosis plus late apoptosis. Data were shown as mean ± SD of three experiments. *P<0.05 versus control or radiation alone; **P<0.05 versus control or other treatment alone.

PC3 cells using the CCK-8 assay. The cells were treated with 0, 5, 25, and 125 μmol/L AA for 24 h. As showed in **Figure 1**, PC3 cell lines displayed a dose-dependent reduction in cell proliferation. 5 μmol/L AA treatment inhibits 12% cell proliferation and reached the peak at the concentration of 125 μmol/L.

AA can radio sensitize PC cells

Studies have indicated that AA may radio sensitize tumor cells [10, 11].

We performed clonogenic cell survival assays to address the same issue in PC3 cell lines. PC3 cells were treated with AA for 24 h followed by a single dose of radiation. The impact of radiation alone or combined with AA was showed a survival curves (**Figure 1B**). We found that cells pretreated with AA priorto radiation observed a significant growth inhibition in PC3 (P=0.002 at 2 Gy, P=0.0033 at 4 Gy, P=0.0041 at 6 Gy and P=0.0028 at 8 Gy). These results indicated that AA maybe used as radiosensitizer in the radio therapy of PC patients to improve the anti-

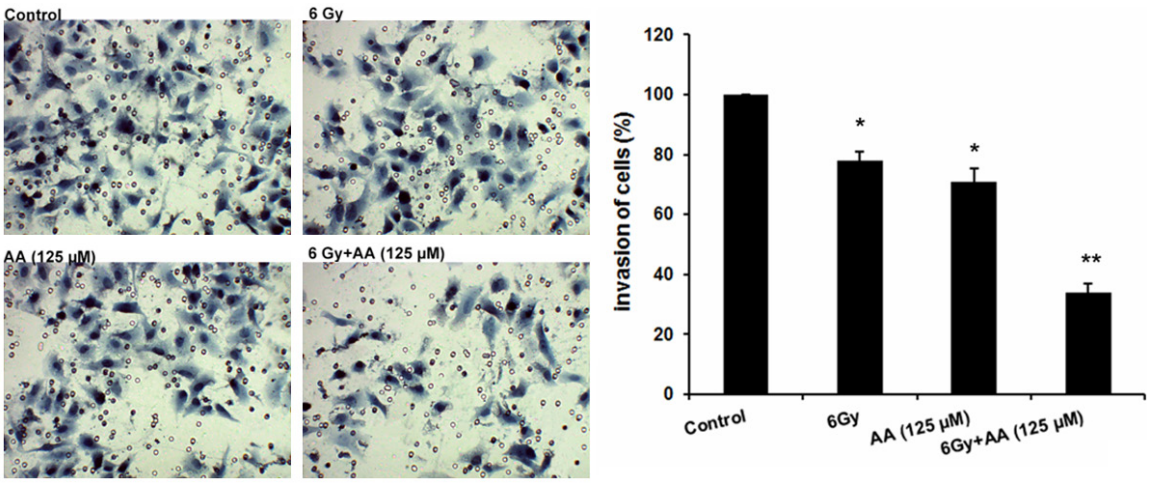

Figure 3. AA sensitized PC cells to radiation by inhibiting cell invasion. Invasion for all treatments of PC3 cells. Combination of radiation and AA induced significant invasion in PC cell line. Bargraphs represent the total apoptosis of all conditions. Data were shown as mean ± SD of three experiments. *P<0.05 versus control or radiation alone; **P<0.05 versus control or other treatment alone.

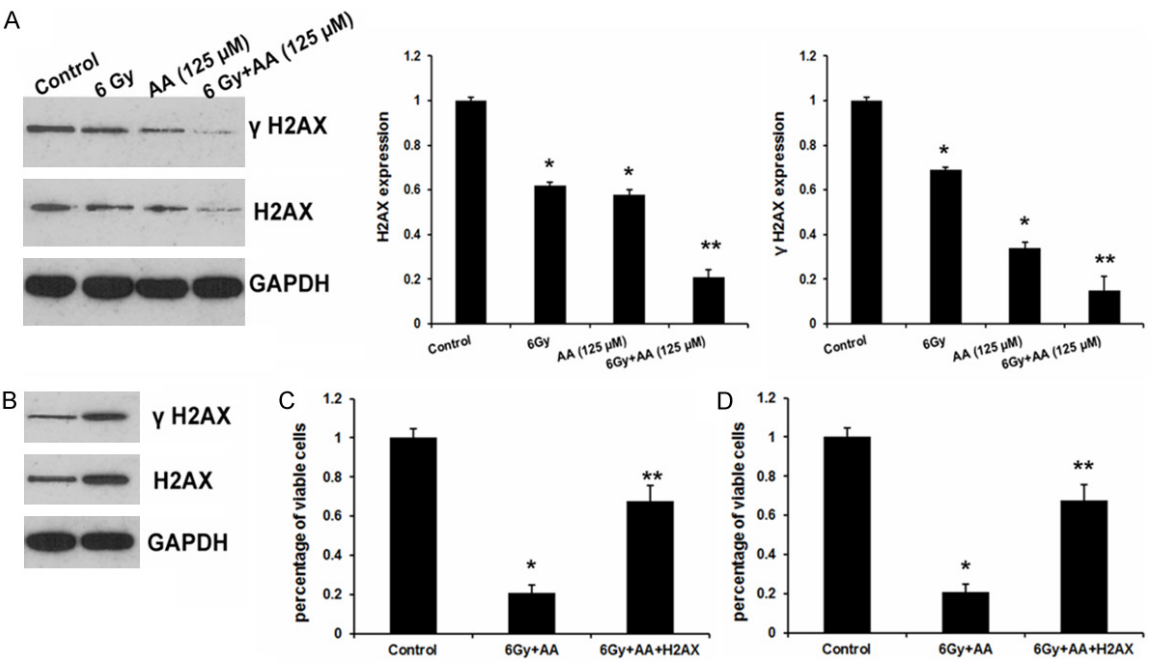

Figure 4. H2AX involved AA-induced radio sensitivity in PC cells. (A) The effects of AA and radiation on H2AX expression and γ-H2AX expression. Data were showed as mean ± SD of three experiments. *P<0.05 versus control or radiation alone; **P<0.05 versus control or other treatment alone. (B) H2AX overexpression in PC cells. (C) H2AX involved proliferation and (D), H2AX involved invasion of PC cells. Data were showed as mean ± SD of three experiments. *P<0.05 versus control; **P<0.05 versus AA and radiation treatment group.

tumor effect of radiation, especially the patients who are insensitive to radiation.

AA sensitized PC cells to radiation by increasing apoptotic cell death

Apoptosis is a mode of cell death in response to radiation. Annexin V stain followed by flow cytometry analysis was performed for PC3 cells treated with radiation (6 Gy) or AA (125 μM) alone or the combination. As shown in **Figure 2**, radiation alone only induced a small amount of cell apoptotic in PC3 cells (P=0.024) compared with control. Apoptosis rate for PC3 cells was approximately 26% when treated with AA alone. When AA was combined with radiation, the

apoptosis rate increased to 48% for PC3, significantly higher rate compared with radiation or AA treatment alone ($P<0.05$). These results indicated that AA inhibited cell growth and enhanced radiation effect by inducing apoptosis.

AA sensitized PC cells to radiation by inhibiting cell invasion in PC cell lines

To further reveal the effects of AA on radio sensitivity of PC, we analyzed the invasion ability of PC3 cells using transwell invasion assay. As shown in **Figure 3**, radiation alone or AA treatment alone inhibited cell invasion in PC3 cells compared with control. When AA was combined with radiation, the invasion ability greatly decreased, compared with radiation or AA treatment alone ($P<0.05$). These results indicated that AA inhibited cell invasion and enhanced radiation effect by repressing cell invasion.

H2AX involved AA-induced radio sensitivity in PC cells

High expression of phospho-H2AX predicts a poor prognosis in various types of cancers [12]. Here, we found that AA or radiation evidently repressed H2AX and p-H2AX expression in PC3 cells (**Figure 4A**).

To further confirm the function of H2AX in PC cell proliferation and invasion, we successfully engineered H2AX overexpression in PC3 cells (**Figure 4B**). Gamma-H2AX (γ-H2AX) is a phosphorylated H2AX which exists nearby double-strand DNA breaks and is required for DNA damage repair [13]. As shown in **Figure 4B**, H2AX overexpression results in increased γ-H2AX level and restored the cell growth (**Figure 4C**) compared with AA and radiation group. Moreover, both cell invasion assays exhibited a similar effect as well (**Figure 4D**). In conclusion, these results indicated that H2AX involved AA-induced radio sensitivity in PC cells.

Discussion

Drugs from plants have become an important resource for discovery of anticancer drugs [10]. Although AA has already been reported to have significant anti-proliferative effect on a variety of cancer cells [7, 9, 14], its mechanism of action in prostate cancer is still unclear. In this study, we report that AA inhibits PC3 cell proliferation in dose-and time-dependent manners. Based on these results, we investigated the molecular mechanism of action of AA on the suppression of tumor growth. Apoptosis is a form of programmed cell death that is characterized by a variety of morphological features, including cell shrinkage, chromatin condensation, and chromosomal DNA fragmentation. In this study, we found that AA induces apoptosis in PC3 cells, as evident by Annexin V/PI assay with flow cytometry. Furthermore, transwell invasion assay revealed that AA inhibited cell invasion and enhanced radiation effect by repressing cell invasion.

DNA damage response (DDR) is an important cellular guard that protects genetic material from a constant barrage of genotoxic agents. Increased DNA damage is often associated with growth inhibition, which is also the case for cancer cells [15, 16]. Phosphorylation of the H2AX histone (p-H2AX) is an early indicator of DNA double-strand breaks and resulting DDR [17]. When DNA double-strand breaks occur, a PI3-like kinase and DNA-dependent protein kinases become activated and phosphorylate H2AX on acarboxylserine residue (Ser139) to generate γ-H2AX [17-19]. The overexpression of p-H2AX has been observed in multiple types of cancer, including, breast cancer, lung cancer, cervical cancer, renal cancer and bladder cancer [12, 20, 21]. Recently, p-H2AX has been used as a biomarker for cancer, as abiodosimeter for drug development and radiation exposure, and for clinical trials, for cancer chemo- and radiotherapy [22, 23]. Furthermore, emerging uses for p-H2AX include detection of toxic environmental agents and chronic inflammation [24]. In this study, we found that radiation and AA treatment decreased the H2AX and γ-H2AX expression levels in PC3 cells. Furthermore, overexpressed H2AX reversed cell growth and invasion mediated by radiation, which suggests that AA-induced radio sensitivity by repressing H2AX protein expression.

In summary, we have demonstrated the antitumor activity of AA in vitro, which is at least partially associated with H2AX-mediated DDR.

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Disclosure of conflict of interest

None.

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