Genistein and AG1024 synergistically increase the radiosensitivity of prostate cancer cells

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Abstract. Radiosensitivity of prostate cancer (PCa) cells promotes the curative treatment for PCa. The present study was designed to investigate the synergistic effect of genistein and AG1024 on the radiosensitivity of PCa cells. The optimal X-irradiation dose (4 Gy) and genistein concentration (30 μM) were selected by using the CCK-8 assay. Before X-irradiation (4 Gy), PC3 and DU145 cells were treated with genistein (30 μM), AG1024 (10 μM) and their combination. All treatments significantly reduced cell proliferation and enhanced cell apoptosis. Using flow cytometric analysis, we found that genistein arrested the cell cycle at S phase and AG1024 arrested the cell cycle at G2/M phase. Genistein treatment suppressed the homologous recombination (HRR) and the non-homologous end joining (NHEJ) pathways by inhibiting the expression of Rad51 and Ku70, and AG1024 treatment only inhibited the NHEJ pathway via the inactivation of Ku70 as detected by western blot analysis. Moreover, the combination treatment with genistein and AG1024 more effectively radiosensitized PCa cells than single treatments by suppressing cell proliferation, enhancing cell apoptosis and inactivating the HRR and NHEJ pathways. *In vivo* experiments demonstrated that animals receiving the combination treatment with genistein and AG1024 displayed obviously decreased tumor volume compared with animals treated with single treatment with either genistein or AG1024. We conclude that the combination of genistein (30 μM) and AG1024 (10 μM) exhibited a synergistic effect on the radiosensitivity of PCa cells by suppressing the HRR and NHEJ pathways.

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Introduction

Radiotherapy is an important curative treatment option for patients with metastatic tumors such as prostate cancer (PCa) (1,2). High tumor recurrence and therapy resistance rate in patients with PCa (>30%) suggest that cellular radioresistance is a major obstacle and challenge to effective radiotherapy for PCa (3,4). Improved radiotherapy modalities and treatments for PCa are warranted and being assessed in pre-clinical applications.

Radiotherapy usually induces severe damage in genomic DNA, including DNA single-strand breaks (SSBs) and double-strand breaks (DSBs) (5). In higher eukaryotes, DNA-DSBs are repaired by two major DNA repair pathways, the homologous recombination (HRR) and the non-homologous end joining (NHEJ) pathways (9). Cell cycle arrest, apoptosis and autophagy are typical impairments induced by radiotherapy (6-8). In response to DNA damage, cell cycles are mainly interfered by genotoxic stress at two checkpoints: G1/S and G2/M transitions (9). DNA damage could be monitored by characterizations of inhibited DNA replication at G1/S checkpoint and damaged chromosome segregation at G2/M checkpoint (9,10). Irradiation-mediated genotoxic stress is caused by sustained DNA damage that overloads DNA repair capacity by the HRR and NHEJ pathways (5). This DNA repair failure finally results in increased cell apoptosis, cell cycle arrest and autophagy, thus, inducing radiosensitivity.

Radiosensitivity of tumor cells could be achieved by targeting signalings, gene transcriptions and non-coding RNAs (microRNAs and lncRNAs) which ultimately regulate cell cycle progression, apoptosis and induce cell cycle checkpoint defects (4,6,11-14). Two important tyrosine kinase receptors, insulin-like growth factor 1 receptor (IGF1R) and epidermal growth factor receptor (EGFR), play essential roles in cancer development and cell cycle progression, invasion and radiosensitivity via the activation of downstream pathways (5,15). IGF1R gene silencing was found to enhance the sensitivity of human PCa cells to DNA-damaging agents (16).

P13K/Akt/mTOR signaling is responsive to various stresses and plays a critical role in cell cycle, apoptosis, autophagy, invasion, metastasis, tumorigenesis and radiosensitivity of tumor cells (5,17-19). Genistein is a tyrosine-specific protein kinases inhibitor, topoisomerase II poison, which inactivates

EGFR, IGF1R and Akt-mediated signalling (20-23). It was reported that genistein inhibited DSB repair through the inactivation of DNA-dependent protein kinases (DNA-PKs) and led to NHEJ and HRR incompleteness (24). Tyrosine kinase inhibitor (tyrphostin) AG1024 is a specific IGF1R inhibitor, which has been reported to radiosensitize PCa cells and breast cancer cells (5,18). However, less information is known about the radiosensitivity of PCa cells to the combination treatment with AG1024 and genistein.

In the present study, we investigated the synergistic effect of combination treatment with AG1024 and genistein on the radiosensitivity of PCa cells. Before X-irradiation, PC3 and DU145 PCa cells were treated with genistein, AG1024 and their combination. The radiosensitivity of PCa cells was evaluated by changes in DNA damage, cell proliferation, apoptosis, cell cycle distribution and the inactivation of the NHEJ and HRR pathways. The synergistic effect of AG1024 and genistein on the sensitivity of PCa cells to radiotherapy is discussed. This study provides new insights into the compensation/synergic effect on NHEJ and HRR pathways for DNA damage repair by AG1024 and genistein.

Materials and methods

Cell lines, culture conditions and treatments. The human PCa cell lines PC3 and DU145 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in RPMI-1640 medium (Gibco-BRL; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Gibco-BRL; Thermo Fisher Scientific) at 37˚C under 5% CO_2 in a humidified incubator. For cell treatment, PC3 and DU145 cells were cultured in RPMI-1640 medium supplemented with genistein (purity >98%, Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and AG1024 (Sigma-Aldrich; Merck KGaA) at series concentrations. Genistein (0, 10, 20, 30, 50 and 100 μM) and AG1024 (10 μM) (5) were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich; Merck KGaA). X-ray irradiation was delivered using an X-6 MV photon linear accelerator (Varian Associates Inc., Palo Alto, CA, USA) at room temperature with a dose rate of 2 Gy/min (25). Cells treated with DMSO were considered as negative control for irradiation or drug treatment.

Cell viability and in vitro cytotoxicity assay. The optimal concentration of genistein and optimal dose of X-ray irradiation were selected using a Cell Counting Kit-8 (CCK-8) assay kit (Sigma-Aldrich; Merck KGaA). The cytotoxicity of genistein and X-irradiation to human PCa cells was assessed using cell viability analysis. Cells were placed into 96-well plates, pretreated with DMSO, genistein, AG1024 and combination of genistein and AG1024 for 1 h, followed by X-irradiation. Then, cells were incubated in RPMI-1640 medium for another 24 h in conditions as above. Ten microliters of CCK-8 solution per well was added into the cell culture for 2 h, and then the cell culture was subjected to a microplate spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA) for determination of the optical density (OD_{450nm}).

Soft agar colony formation analysis. Monolayer clonogenic assay was performed to investigate the effect of genistein, AG1024 and the combination on colony formation ability. Cells were placed into 6-well plates and grown to log phase, followed by transferring to medium containing DMSO, genistein, AG1024 and genistein plus AG1024. After a 1-h incubation, the cells were irradiated with 4 Gy of X-irradiation and incubated in RPMI-1640 medium for 24 h. Then, the cells were treated with trypsin (Gibco-BRL; Thermo Fisher Scientific, Inc.), transferred to a 6-cm diameter tissue culture dish with fresh RPMI-1640 medium and incubated for 12 days. Medium was replaced every third day. For colony detection, dishes were washed with phosphate-buffered saline (PBS) for two times, fixed with methanol at 4˚C for 15 min, and stained with Giemsa (Sigma-Aldrich; Merck KGaA) for 30 min. Colony counting was performed on clearly visible colonies (colony with >50 cells or diameter>50 μm) using a light microscope (BX51; Olympus Optical Co., Ltd., Tokyo, Japan). Each experiment was performed in triplicate.

Flow cytometric analysis. The effects of the combination treatment of genistein and AG1024 and single treatments on cell cycle distribution and apoptosis were detected using a flow cytometer (BD Biosciences, San Jose, CA, USA). For cell cycle analysis, cells under different treatments for 24 h were harvested (trypsin; Gibco-BRL; Thermo Fisher Scientific, Inc.), fixed (70% ethanol), stained with 50 μg/ml propidium iodide (PI) solution, and were subjected to flow cytometry. Cell cycle distribution at G0/G1, S and G2/M phase was analyzed. For cell apoptosis analysis, fixed cells were incubated with PI and Annexin V-FITC for 10 min in the dark, and then subjected to a BD FACSCalibur flow cytometer (BD Biosciences). Cells undergoing early apoptosis were considered as apoptotic cells (Annexin V-positive and PI-negative, Annexin V^+/PI^-). Each experiment was conducted in triplicate.

Immunofluorescence. Immunofluorescence analysis was performed to detect the γH2AX foci formation as described by Wang *et al* (5). In brief, $1x10^6$ cells/ml of PC3 and DU145 cells were seeded into 6-well plates with coverslips and were treated with different treatments combined with X-irradiation for 24 h. The cells were then fixed with 4% paraformaldehyde for 20 min, incubated with 0.2% Triton X-100 in PBS for 5 min, and coverslips were blocked with 5% bovine serum albumin (BSA; Gibco-BRL; Thermo Fisher Scientific, Inc.) for 30 min at room temperature. Then slips with fixed cells were incubated with specific primary antibody against phospho-histone γH2AX (1:500; cat. no. 2595; Cell Signaling Technology, Inc., Danvers, MA, USA) at 4˚C overnight, followed by incubation with Cy3-labelled goat anti-rabbit fluorescent secondary antibody (1:2,000; cat. no. 111-165-003; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 h at room temperature and 1 μg/ml DAPI (Invitrogen; Thermo Fisher Scientific, Inc.) for additional 10 min in the dark. Images were captured using an Olympus laser scanning confocal microscopy (LEXT 3100; Olympus Corp., Tokyo, Japan).

Western blot analysis. Cells were placed into 6-well plates and incubated using the different treatments as above. Cells were harvested at 24 h post X-irradiation. Cellular and nuclear

protein was isolated using RIPA buffer (Pierce Inc., Beijing, China). Proteins were prepared as described by Liu *et al* (26). Western blot analysis was performed according to the standard methods. Specific primary antibodies of anti-phospho (p)-IGF1R (Tyr1135), -IGF1R, -ATM, -ATM(Ser1981), -Bax, -Bcl2, -cleaved caspase-3, -Ku70, -Rad51, -DNA-PKcs and -GAPDH were purchased from Cell Signaling Technology, Inc. Primary antibody p-DNA-PKcs (Thr2609) was purchased from Santa Cruz Biotechnology Inc., (Santa Cruz, CA, USA; cat. no. sc-101664).

In vivo tumor radiation protocol. The *in vivo* subcutaneous mouse tumors were produced by subcutaneously injecting $5x10^6$ DU145 cells, mixed with BD Matrigel (BD Biosciences), into the flank of male nude mice (6-7 weeks old, 18-20 g, n=60) provided by the Experimental Animal Center of the Fourth Military Medical University (5). Animals were maintained with *ad libitum* access to food and water for 5 days at 25±1˚C in environmental chambers, with 40-50% humidity and 12 h light: 12 h dark cycle. A digital Vernier caliper was used for measuring tumor volume [V = 0.5 x tumor length (mm) x tumor width^2 (mm^2)]. Twenty days later, mice were randomly divided into four groups (n=15 in each group): the DMSO + IR (control) group received X-irradiation every three days for 5 times (15-day treatment course), with orally intubated with 200 mg/kg/day DMSO; the genistein + IR group received 100 mg/kg/day genistein, 100 mg/kg/day DMSO and X-irradiation for 5 times; the AG1024 + IR group received 100 mg/kg/day AG1024, 100 mg/kg/day DMSO and X-irradiation for 5 times; the Combination (genistein + AG1024) + IR group received 100 mg/kg/day genistein, 100 mg/kg/day AG1024, plus with X-irradiation for 5 times. The *in vivo* therapeutic efficacy of the different treatments on *in vivo* tumors was evaluated using changes in tumor volume and proliferation index (PI, $PI=V_{treatment}/V_{control}$) (5). Body weight (g) of experimental animals were recorded. Multiple nodes in one mouse were circled into one circle and the accumulated volume was calculated as above. All mice were sacrificed by anesthesia and the tumors were removed on day 15 after the 1st administration of genistein, AG1024 and the combination treatment. The animal experiment protocols were approved by the Ethics Committee of the Fourth Military Medical University (Xi'an, China).

Statistical analyses. Each cellular experiment was performed in triplicate. All quantitative data and continuous variables are expressed as mean ± standard deviation (SD). Statistical analysis was performed using the unpaired two-tailed Student's t-test in SPSS software 17.0 (SPSS, Inc., Chicago, IL, USA). GraphPad Prism 6 (Graphpad Software, Inc., San Diego, CA, USA) was used for plotting. Statistical significant P-value is given as $P \leq 0.05$.

Results

Cytotoxicity of irradiation and genistein in PCa cells. The frequently used concentration of AG1024 is 10 μM for cancer cells (5,18). In order to select suitable doses of X-irradiation and genistein, we treated PC3 and DU145 cells with different doses of X-irradiation (0, 2, 4, 6, 10 and 20 Gy) and genistein (0, 10, 20, 30, 50 and 100 μM). Cell cytotoxicity was detected using the CCK-8 assay. In comparison analysis, we found that cell viability was significantly inhibited by ≥4-Gy X-irradiation ($P<0.05$; Fig. 1A) and ≥10 μM genistein ($P<0.05$; Fig. 1B). Thus, we selected 4-Gy X-irradiation and 30 μM genistein as the optimal conditions for cell cytotoxicity in the PCa cells.

Combination of genistein and AG1024 enhances X-irradiation-reduced PC3 and DU145 cell proliferation. We pretreated PC3 and DU145 cells with genistein (30 μM), AG1024 (10 μM) and the combination before X-irradiation (4 Gy). Twenty-four hours later, we confirmed that the combination of genistein and AG1024 significantly decreased cell proliferation ($P<0.01$), followed by genistein (30 μM, $P<0.05$) and AG1024 (10 μM, $P<0.05$) as compared with that of the control cells (Fig. 1C). Using colony formation assay, we detected that the colony numbers of PC3 and DU145 cells were reduced by treatments of genistein (30 μM), AG1024 (10 μM) and the combination treatment ($P<0.05$; Fig. 1D). PC3 and DU145 cells pretreated with the combination of genistein (30 μM) and AG1024 (10 μM) before X-irradiation (4 Gy) showed the lowest frequency of colony formation ($P<0.01$), followed by cells pretreated with either genistein (30 μM) or AG1024 (10 μM) before X-irradiation (4 Gy, $P<0.05$; Fig. 1D). These results showed that the combination treatment of genistein and AG1024 and single treatments enhanced PCa cell cytotoxicity by X-irradiation. In addition, the combination of genistein and AG1024 indicated higher efficacy in the inhibition of cell proliferation than either genistein or AG1024 single treatment, suggesting a synergistic inhibition effect on PCa cell proliferation.

Combination treatment of genistein and AG1024 and single treatments induce cell cycle arrest. Cell cycle distribution analysis showed the percentages of cells at G0/G1, S and G2/M phases were significantly disturbed by genistein (30 μM), AG1024 (10 μM) and the combination treatment. As compared with the control (DMSO + irradiation), genistein (30 μM) treatment significantly decreased the percentages of cells at G0/G1 and G2/M phases ($P<0.05$), and increased the percentages of cells at S phases ($P<0.01$), revealing that genistein induced S cell cycle arrest (Fig. 2). In addition, we observed that AG1024 treatment induced G2/M cell cycle arrest in PC3 and DU145 cells. AG1024 treatment significantly increased the percentages of cells at the G2/M phases ($P<0.01$ for PC3 cells; $P<0.05$ for DU145 cells) and reduced the percentages of cells at G0/G1 and/or S phases ($P<0.05$).

Notably, the combination treatment with genistein (30 μM) and AG1024 (10 μM) significantly increased the percentages of cells at the S phase ($P<0.01$ for PC3 cells; $P<0.05$ for DU145 cells) and decreased the percentages of cells at G2/M phases ($P<0.01$ for PC3 cells; $P<0.05$ for DU145 cells). The percentages of cells at G0/G1 phases were concomitant with those of the control cells (DMSO + IR; $P>0.05$). Taken together, we found that genistein (30 μM) induced S cell cycle arrest, AG1024 (10 μM) induced G2/M cell cycle arrest, and the combination of genistein (30 μM) and AG1024 (10 μM) induced S cell cycle arrest. In addition, the combination treatment with genistein and AG1024 showed a synergistic inhibitory effect on chromosome segregation at the G2/M phase, but not DNA replication at the G0/G1 phase in PCa cells.

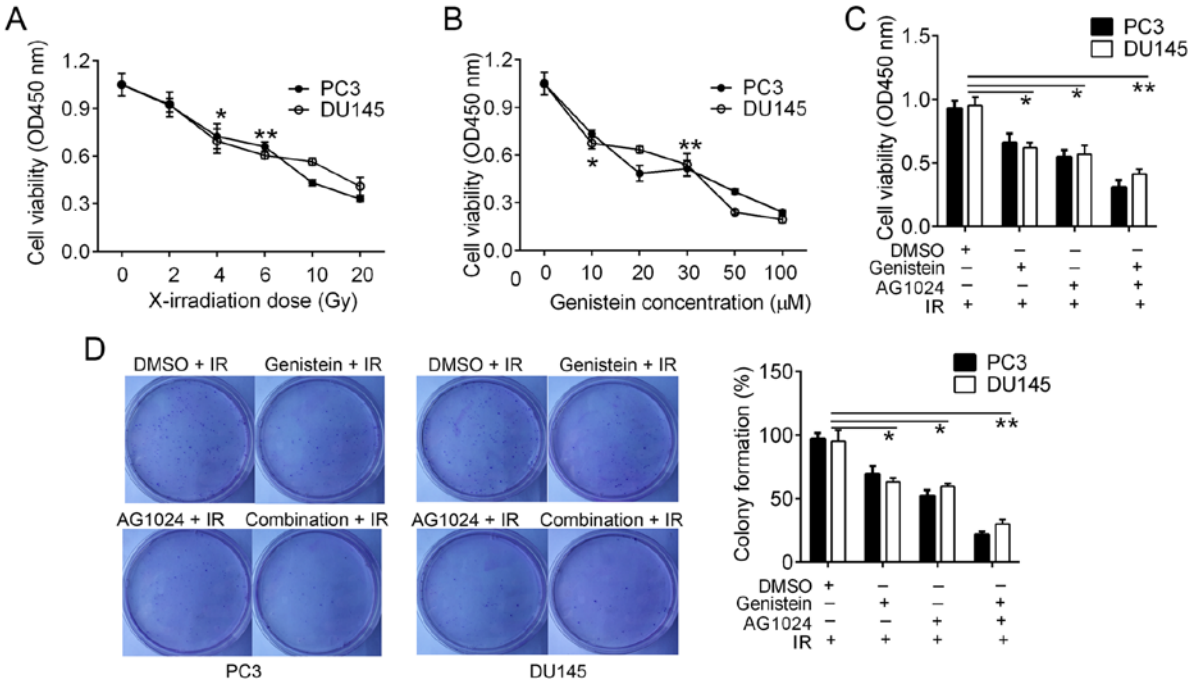

Figure 1. Effect of X-irradiation, genistein and the combination of genistein and AG1024 on PCa cell proliferation. (A and B) Cell viability in response to different doses of X-irradiation and genistein. The selected optimal condition was 4-Gy X-irradiation and 30 μM genistein. (C) Cell viability analysis with the single and combined treatment of genistein and AG1024. (D) Colony formation analysis. Combination treatment with genistein and AG1024 and single treatments revealed an inhibitory effect on cell proliferation. *P<0.05 vs. control (A and B, dose 0; C and D, DMSO). **P<0.01 vs. control (A and B, dose 0; C and D, DMSO). PCa, prostate cancer; IR, X-irradiation.

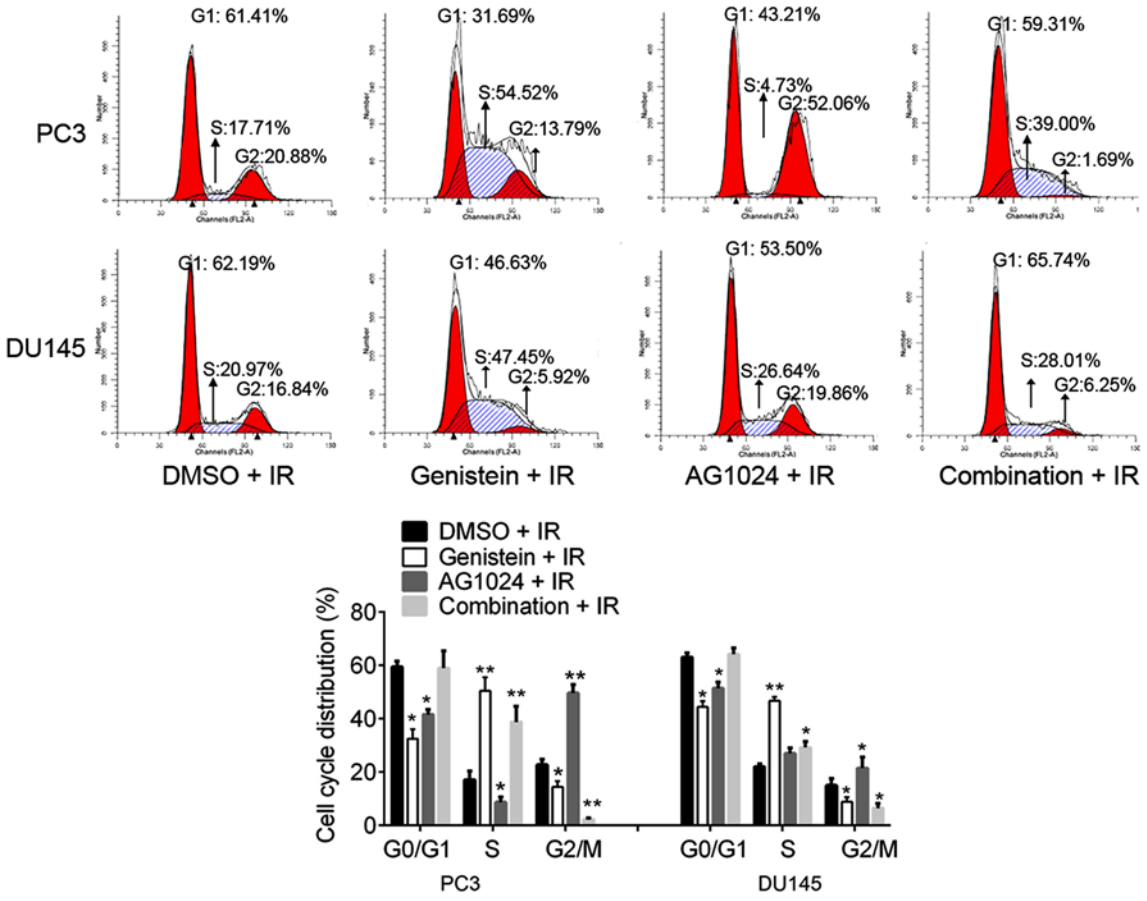

Figure 2. Cell cycle distribution by flow cytometric analysis. PC3 and DU145 cells were pretreated with genistein (30 μM), AG1024 (10 μM) and their combination before X-irradiation (4 Gy). After X-irradiation, cells were incubated for 24 h. Cell cycle arrest at S phase and G2/M phase was observed in genistein (30 μM) and AG1024 (10 μM) pretreated cells, respectively. The combination of genistein and AG1024 arrested cell cycle at the G2/M phase. *P<0.05 vs. control (DMSO+IR). **P<0.01 vs. control (DMSO+IR). IR, X-irradiation.

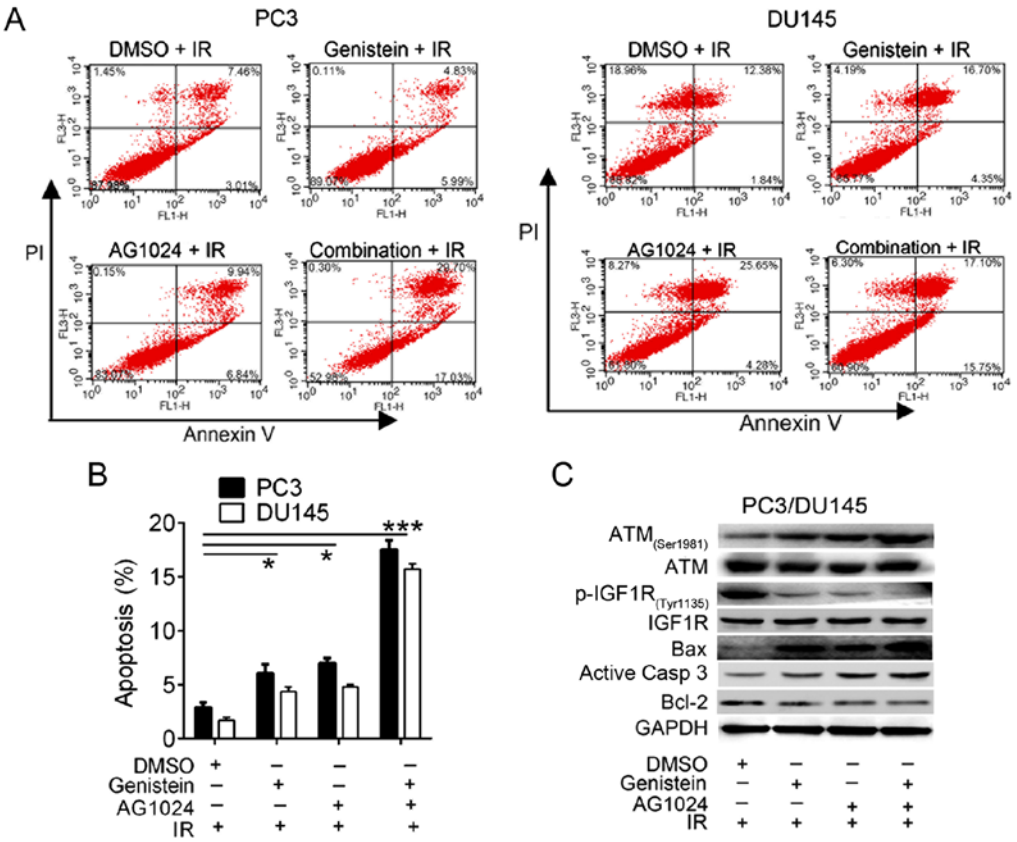

Figure 3. Analysis of cell apoptosis and expression of cell apoptosis-related proteins. (A and B) Cell apoptosis analysis was analyzed using Annexin V/PI flow cytometric analysis. (C) The expression of cell apoptosis-related proteins was detected using western blot analysis. *P<0.05 vs. control (DMSO + IR). ***P<0.001 vs. control (DMSO + IR). PI, propidium iodide; R, X-irradiation.

Combination treatment with genistein and AG1024 enhances X-irradiation-induced apoptosis in PC3 and DU145 cells. We then confirmed that the treatment of genistein (30 μM), AG1024 (10 μM) and their combination promote cell apoptosis using flow cytometric analysis. Significant enhancement of cell apoptosis was observed in cells treated with genistein (30 μM, P<0.05), AG1024 (10 μM, P<0.05), and both of them (P<0.01) combined with X-irradiation (Fig. 3A and B). These data demonstrated that genistein (30 μM) and/or AG1024 (10 μM) treatment enhanced X-irradiation-induced PCa cell apoptosis, and the combination treatment with genistein and AG1024 synergistically promoted cell radiosensitivity.

Using western blot analysis, we detected the expression of cell apoptosis-related proteins and pathway. In comparison with the control cells (DMSO + irradiation), the expression of ATM (Ser1981), Bax and active caspase-3 were increased, whereas the expression of p-IGF1R (Tyr1135) and Bcl-2 were decreased in PC3 and DU145 cells treated with genistein (30 μM) and/or AG1024 (10 μM) plus X-irradiation (Fig. 3C). Thus, we suggested that both genistein and AG1024 induced PCa cell apoptosis via the activation of apoptosis-related pathways, which may be associated with the inactivation of IGF1R. In addition, combination of genistein and AG1024 synergistically modulated the expression of these proteins, showing synergistic effect on irradiation-induced cell apoptosis of PCa cells.

Genistein and/or AG1024 enhances irradiation-induced DSB in PCa cells. We then revealed the increased γH2AX foci formation, a DSB marker, in genistein (30 μM) and/or AG1024 (10 μM) treated PCa cells (Fig. 4), suggesting the elevation of DNA damage by pretreatment with genistein, AG1024, and combination before X-irradiation. In PC3 and DU145 cells pretreated with different treatments before X-irradiation, the numbers of residual γH2AX foci were significantly elevated as compared with that in the control cells (P<0.05). Moreover, PCa cells treated with the combined treatment with genistein and AG1024 demonstrated a higher number of γH2AX foci than cells treated with single treatments of either genistein or AG1024, showing synergistic enhancement of γH2AX foci formation by genistein and AG1024.

Combination of genistein and AG1024 enhances radiosensitivity of PCa cells via the inactivation of HRR and NHEJ pathways. Using western blot analysis, we detected the decreased expression of NHEJ-related DNA repair proteins DNA-PKcs (Thr2609) and Ku70 in PCa cells pretreated with genistein (30 μM), AG1024 (10 μM) and their combination as compared with the control (Fig. 5). PCa cells pretreated with the combination of genistein and AG1024 before X-irradiation showed the lowest expression levels of nuclear DNA-PKcs and Ku70, followed by PCa cells pretreated with either genistein (30 μM) or AG1024 (10 μM). However, the expression of nuclear Rad51, an enzymatic component of HRR, was decreased by genistein alone and in combination with AG1024, but not AG1024 alone. These results revealed that genistein and the combination of genistein and AG1024

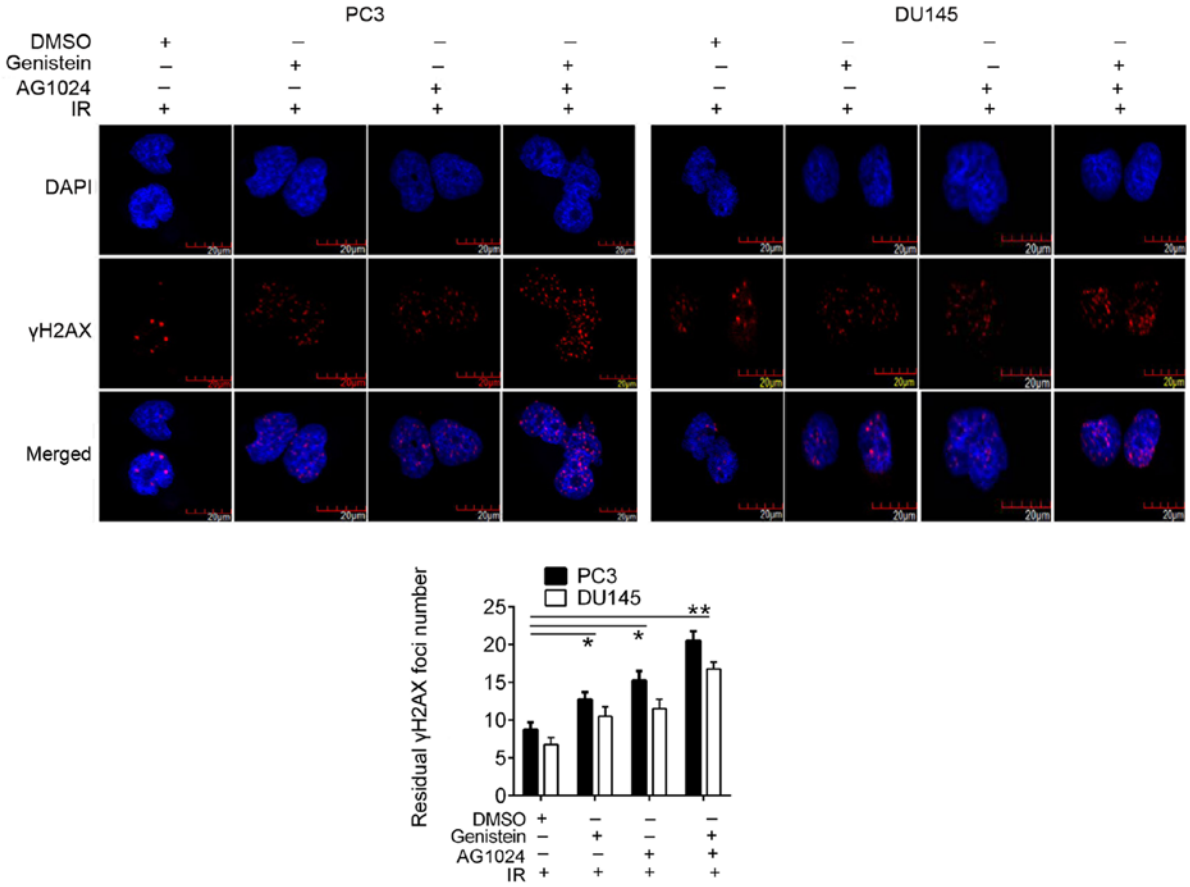

Figure 4. Analysis of γH2AX foci formation in treated PCa cells. γH2AX foci were indicated by purple foci in cells. More purple-colored foci were formed in cells pretreated with genistein (30 μM) and/or AG1024 (10 μM) before X-irradiation (4 Gy). *P<0.05 vs. control (DMSO+IR). **P<0.01 vs. control (DMSO+IR). PCa, prostate cancer; IR, X-irradiation.

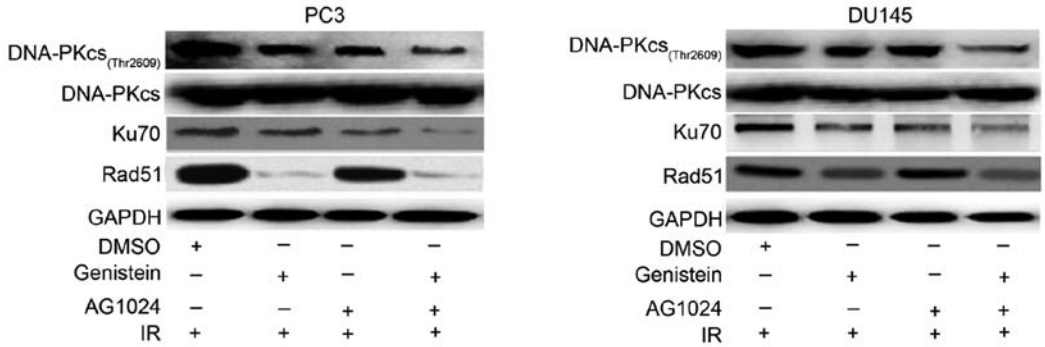

Figure 5. Expression of DNA repair-associated proteins. Expression of NHEJ-related DNA repair proteins DNA-PKcs (Thr2609), Ku70 and HRR-related protein Rad51 in PCa cells. Three proteins were nuclear proteins. DNA-PKcs (Thr2609) and Ku70 were inhibited by treatment of genistein (30 μM), AG1024 (10 μM), and their combination, whereas Rad51 was only influenced by genistein (30 μM). PCa, prostate cancer; IR, X-irradiation.

enhanced the radiosensitivity of PCa cells via the inhibition of both HRR and NHEJ pathways, while AG1024 promoted the radiosensitivity of PCa cells only by inactivating the NHEJ pathway.

Genistein and/or AG1024 enhance cancer radiotherapy in a preclinical model of PCa tumors. The *in vitro* cellular experiments demonstrated the single treatment with either genistein (30 μM) or AG1024 (10 μM) and the combination treatment radio-sensitized PCa cells to X-irradiation. To investigate the *in vivo* effect of genistein and AG1024 on cancer radiotherapy, we constructed the *in vivo* subcutaneous tumor model using DU145 cells. Before X-irradiation, mice were orally intubated with DMSO, genistein (100 mg/kg/day), AG1024 (100 mg/kg/day), and the combination of genistein and AG1024. The body weight of the mouse model increased from 18-20 g at the onset (6-7 weeks of age) to 20-24 g at the end of this study (11-12 weeks of age). At the day 15 post the 1st administration of genistein and/or AG1024, the tumor volume ranged from 1,697.20 mm^3 in control mice to 252.01 mm^3 in mice treated with the combination of genistein and AG1024 (Fig. 6A). No difference was observed in body

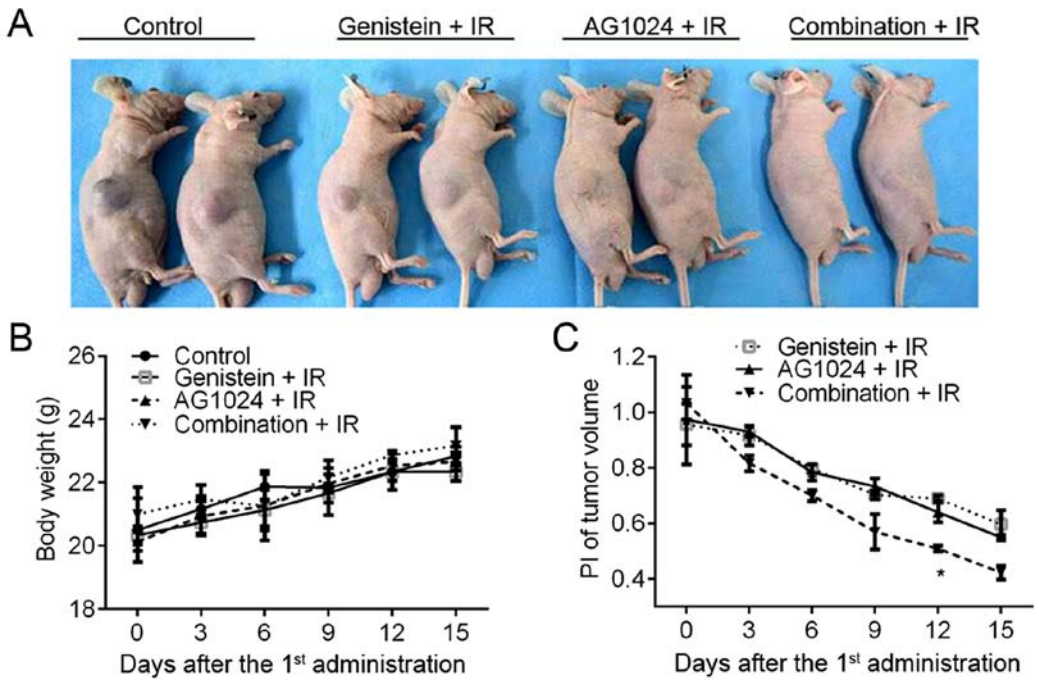

Figure 6. *In vivo* effect of genistein and AG1024 on PCa cells. Animals received 100 mg/kg/day genistein, 100 mg/kg/day AG1024, or their combination thm (genistein + AG1024) before X-irradiation for 15 days. (A) The image of xenograft tumor-bearing mice at day 15 post the 1st time of medicine treatment. (B) Body weight of the experimental animals. (C) Tumor volume (V, mm^3) change during treatment time was indicated by the proliferation index (PI, $PI=V_{treatment}/V_{control}$). Significant decrease in PI was observed in mice treated with the combination treatment at day 12 post treatment, compared with those in the other groups. *P<0.05 vs. genistein or AG1024. PCa, prostate cancer; IR, X-irradiation.

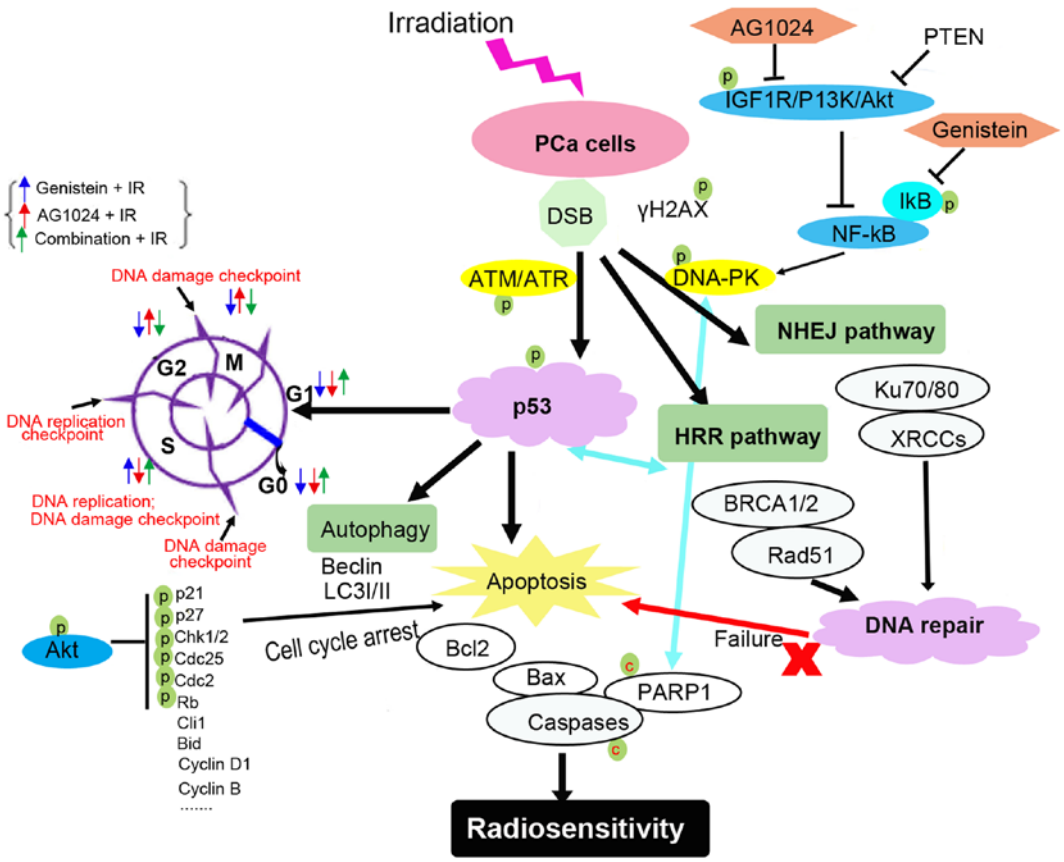

Figure 7. Schematic depiction of genistein and AG1024-mediated DNA-repair pathways related to radiosensitivity of PCa cells. The IGF1R/P13K/Akt and NF-κB pathways are associated with the AG1024 and genistein-mediated inhibition of DNA repair pathways, respectively. AG1024 and genistein promote cell apoptosis via ATM/ATR/p53-dependent downstream pathways or factors. AG1024 and genistein arrests cell cycle arrest at G2/M phase and S phase, respectively. The DNA repair capacity is controlled by HRR and NHEJ pathways in PCa cells. The failures of DNA repair and cell cycle arrest result in enhancement of cell apoptosis, thus, enhancing the radiosensitivity of tumor cells. PCa, prostate cancer; HRR, homologous recombination; NHEJ, non-homologous end joining.

weight between the groups (Fig. 6B). As compared with the control mice (DMSO+IR), significantly decreased PI of tumor volume was observed in mice treated with genistein, AG1024, and the combination ($P<0.05$; Fig. 6C). Moreover, at day 12 post the 1st administration of the agent, the PI of the tumor volume in mice treated with the combination of genistein and AG1024 was significantly lower than those in the mice treated with either genistein or AG1024 ($P<0.05$). The *in vivo* experiments showed that the treatment with either genistein or AG1024 radiosensitized PCa tumors, and the combination of them showed a synergistic effect on inhibiting tumor growth and sensitizing PCa to radiotherapy *in vivo*.

Discussion

The present study investigated whether the combination of genistein and AG1024 could enhance the radiosensitivity of PCa cells to X-irradiation. The data confirmed that the single and combination of genistein and AG1024 radiosensitized PCa cells to X-irradiation by arresting the cell cycle, promoting cell apoptosis, and inhibiting DNA repair via the inactivation of the NHEJ pathway and/or HRR pathway.

Radiotherapy to cancer cells induces cell damage by causing genomic DNA damage overloading DNA repair capacity, which finally results into mitotic catastrophe in tumor cells (5). Cell cycle arrest, apoptosis and autophagy are typical impairments induced by radiotherapy (6-8). Both genistein and AG1024 regulate DNA damage and enhance the radiosensitivity of tumor cells (5,17,28). Genistein has been proven to induce DNA damage, apoptosis, and cell cycle arrest at the G2/M phase via the ATM-p53-dependent pathway (Fig. 7), p38 MAPK activation, NF-κB and other pathways (12,27-29). In addition, genistein inhibited IGF-1 signaling pathway and inactivated Akt signaling pathways (30). In addition, genistein has been reported to arrest cell cycle at the G0/G1 phase in MCF-7, HB4a and BG-1 cells (31,32). Tyrphostin AG1024 is an inhibitor of IGF1R. The inhibition of IGF1R enhances tumor cell radiosensitivity (13). In this study, we confirmed that the pretreatment of genistein and AG1024 before X-irradiation promoted DNA damage and cell apoptosis in PC3 and DU145 cells, thus, enhancing the radiosensitivity of PCa cells to X-irradiation.

The cell cycle is regulated by check point proteins, including G0/G1 phase checkpoints, S phase checkpoints and G2/M phase checkpoints. These checkpoints are responsible for the genomic instability of tumor cells and are responsive to irradiation-induced DNA damage (14,33). These checkpoints control cell cycle arrest and the radiosensitivity of tumor cells (Fig. 7). Under irradiation-mediated genotoxic stress, increased DNA-DSB challenges the stability of genomic DNA and the DNA repair capacity determines the cell survival and the radiosensitivity of cancer cells (5). Under irradiation, DNA damage is sensed by DNA-PK/ATM and ATM-related kinase (ATR)-mediated signaling. The activation of this signaling promotes p53 phosphorylation and activates p53-mediated responses (34,35). The DNA damage checkpoints, including G2/M phase, S phase and G0-S transition checkpoints, are sensitive to and responsible for irradiation-mediated DNA damage (36). The p53-dependent p21 phosphorylation promoted cell cycle arrest at the G1 phase by inhibiting cyclin D1, cyclin E and CDK2 (32,37), while G2/M cell cycle arrest was induced by decreased cyclin B and Chk2/cell division cycle 25C (Cdc25C)/Cdc2, which was negatively regulated by p53-dependent GADD45 expression (8,9,27,38,39). Genistein and AG1024-induced G2/M cell cycle arrest has been reported in human colon cancer cells and breast cancer cells (28,29,40), whereas genistein and AG1024-induced G1 cell cycle arrest has also been confirmed in BG-1 ovarian cancer and DU145 PCa cells (18,32). These results demonstrated that genistein or AG1024-mediated cell arrest is dependent on cell types or other unknown conditions. Our current data demonstrated that genistein induced PCa cell cycle arrest at the S phase, and AG1024 induced PCa cell cycle arrest at the G2/M phase. In addition, the combination of genistein and AG1024 arrested the cell cycle at the S phase, showing a synergistic effect on cell cycle distribution with a genistein tendency.

In tumor cells, the NHEJ and HRR pathways enhance DNA repair capacity and modulate cell sensitivity and resistance to radiotherapy (5). The inhibition of IGF1R enhances radiosensitivity and delays DSB repair by inactivating NHEJ and HRR pathways (13). Previous reports have shown that the expression of the Ku70/80 gene and DNA-PKcs activity did not correlate with DSB repair capacity and cellular radiosensitivity in normal human fibroblasts (41). In tumor cells, the NHEJ pathway is mediated by DNA-PK-dependent phosphorylation of Ku70/80 and the HRR pathway is mediated by BRCA1/Rad51 expression (4,41). In comparison with normal tumor cells, increased NHEJ and HRR pathways were detected in radio-resistant PCa cells (4). On contrast, Chang *et al* indicated that the combination of P13K/mTOR inhibitor and 6-Gy radiation inhibited both NHEJ and HRR pathways, thus, radiosensitizing PCa cells (4). It was reported that genistein radiosensitized breast cancer cells via inactivating the HRR repair pathway by inhibiting Rad51 expression (28). IGF1R inhibitor AZ12253801 radiosensitized PCa cells through inactivating NHEJ repair pathway (13). Our data showed that genistein radiosensitized PCa cells via inhibiting both NHEJ and HRR pathways, and AG1024 enhanced the radiosensitivity of PCa cells to X-irradiation via the inhibition of the NHEJ pathway. Additionally, the combination of AG1024 and genistein showed a synergistic effect on the inhibition of NHEJ and HRR pathways and the radiosensitivity of PCa cells to X-irradiation, showing the therapeutic potential of the combination treatment with AG1024 and genistein for PCa.

In summary, we confirmed that both AG1024 and genistein radiosensitized PCa cells by arresting the cell cycle at the G2/M and S phase, respectively. In addition, the genistein-induced radiosensitivity of PCa cells was mediated by the inhibition of both HRR and NHEJ repair pathways, while AG1024 induced radiosensitivity of PCa cells only through the inhibition of the NHEJ pathway. Both *in vitro* and *in vivo* experiments indicated that the combination of AG1024 and genistein synergistically enhanced the radiosensitivity of PCa cells. Even so, we are still not entirely clear concerning the crosstalk between AG1024 and genistein. The mechanisms and differences in cell cycle arrest and DNA repair pathways between AG1024 and genistein were not explored in the present study. We hypothesize that the investigation of cyclin expression may be helpful for uncovering the differences

in genistein and AG1024-mediated cell cycle arrest and the radiosensitivity of PCa cells. More experiments should be performed before the clinical therapy of PCa by using the combination of AG1024 and genistein.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

QT and JM conceived and designed the study. QT, JM, JS, LY, FY, WZ, RL and LW performed the experiments. QT and JM analysed the data. QT and JM wrote the manuscript. YW and HW reviewed and checked the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The animal experiment protocols were approved by the Ethics Committee of the Fourth Military Medical University (Xi'an, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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