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Original Paper

Chloroquine Enhances the Radiosensitivity of Bladder Cancer Cells by Inhibiting Autophagy and Activating Apoptosis

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Key Words
Bladder cancer • Radiosensitivity • Chloroquine • Autophagy • Apoptosis

Abstract
Background/Aims: Chloroquine was formerly used as an anti-malarial agent drug but has now been proven to be useful for various diseases. This study aimed to investigate the radiosensitizing effect of chloroquine in bladder cancer, with an emphasis on autophagy inhibition and apoptosis induction. ***Methods:*** Bladder cancer cell lines were irradiated with or without chloroquine. Cell proliferation was determined by a Cell Counting Kit 8 assay. The radiosensitization effect of chloroquine was evaluated by clonogenic survival and progression of xenograft tumors. Cell apoptosis was detected by flow cytometry and western blot. Radiation-induced DNA double strand break was measured by the staining of γ-H2AX. In addition, autophagy was detected by western blot, immunofluorescence staining, and electron microscopy. ***Results:*** The treatment with chloroquine alone inhibited the proliferation of bladder cancer cells in a dose-dependent manner. Low cytotoxic concentrations of chloroquine enhanced the radiation sensitivity of bladder cancer cells with a sensitization enhancement ratio of 1.53 and 1.40. Chloroquine also obviously weakened the repair of radiation-induced DNA damage. A combination of radiation and chloroquine enhanced the apoptosis rate of EJ and T24 cells and down-regulated the expression of Bcl-2 while up-regulating the expression of caspase-3. Additionally, the relevant markers of autophagy were obviously increased in the combined group, meaning that chloroquine inhibited autophagy induced by irradiation. Furthermore, subcutaneous xenograft tumors displayed that the combination of radiation and chloroquine could impede tumorigenesis *in vivo*. ***Conclusion:*** In summary, these results

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provided support that by inhibiting autophagy and activating apoptosis, chloroquine might be a potentially promising radiosensitizer in the radiation therapy of bladder cancer.

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Introduction

Urinary bladder cancer is one of the most common malignancies and will account for approximately 79, 030 new cases and 16, 870 deaths in the United States in 2017 [1]. Bladder cancer can be divided into two clinically different groups, muscle invasive bladder cancer (MIBC) and non-muscle-invasive bladder cancer (NMIBC) [2]. MIBC is the leading cause of bladder cancer-related death. Today, radical cystectomy with lymph node dissection remains the gold standard for MIBC. However, cancer-specific survival after cystectomy is relatively low [3]. The low cure rates with radical cystectomy imply that MIBC is a micrometastatic disease. This supports the use of systemic treatment to achieve a better disease control and improve survival. Radical radiotherapy (RT) is a valid option in the radical treatment of MIBC, with similar survival rates to cystectomy in previous studies [4, 5]. Radiotherapy, as an alternative strategy, is a noninvasive technique that affects surrounding tissues less and provides a promising, high-quality life. Nevertheless, due to the resistance of bladder cancers to radiotherapy, the recurrence and metastasis of tumors remain major clinical problems [6, 7]. Therefore, it is urgent to further understand the potential mechanisms of radioresistance and improve radiosensitivity in patients with bladder cancer.

Autophagy is a newly identified response of cancer cells to various anticancer therapies, including chemotherapy and radiotherapy [8-10]. It is a genetically programmed, evolutionarily conserved catabolic pathway in which cytoplasmic components are targeted to lysosomes for degradation. Persistent activation of autophagy can cause depletion of organelles and critical proteins in cells, resulting in a specific form of programmed cell death [11]. Autophagic cell death is referred to as programmed cell death type II, which is different from the apoptotic type I death pathway [12]. Recent studies have indicated that autophagy contributed to radioresistance in various tumors, such as breast cancer, glioma, and pancreatic cancer [13-16]. Chaachouay et al. [13] reported that autophagy caused radioresistance in breast cancer cell lines. Yao et al. [14] found that irradiation of human glioma cells led to increased autophagy. Another study [15] showed that the induction of autophagy by radiotherapy resulted in radioresistance of glioblastoma. However, little research has concentrated on the relationship between autophagy and radioresistance of bladder cancer.

Chloroquine has been widely used as an anti-malarial agent drug in recent years. Currently, chloroquine is drawing more attention as an autophagy inhibitor [17]. Due to its chemical properties as a weak base, it can inhibit H+-ATPase to increase the lysosomal pH, thus inhibiting enzymatic activity of lysosomal hydrolases and preventing the fusion of autophagosome and lysosome for lysosomotropic properties [18, 19]. Now, several studies have reported that chloroquine could enhance the efficiency of present tumor therapies in many cancers [20-23]. Qin et al. [20] found that chloroquine enhanced the efficacy of cisplatin in human adrenocortical carcinoma treatment by blocking autophagy. In addition, Golden et al. [23] suggested that chloroquine blocked autophagy and triggered endoplasmic reticulum stress, thereby increasing the chemosensitivity of glioma cells to temozolomide. In addition, Ye et al. [21] showed that chloroquine enhanced the radiosensitivity of glioma initiating cells. It is therefore of interest to elucidate the association between chloroquine and radiosensitivity in bladder cancer.

Combining chloroquine with radiotherapy might be a novel and promising strategy to for curing bladder cancer. In this study, we investigated the underlying radiosensitization effect and prime mechanisms of chloroquine on bladder carcinoma through inhibiting autophagy and activating apoptosis.

Materials and Methods

Cell culture and irradiation

Bladder cancer cell lines EJ and T24 were purchased from the American Type Culture Collection (ATCC, USA). Both cells were cultured in DMEM media (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA) and 1% penicillin/streptomycin (Invitrogen, USA) in humidified air at 37 °C with 5% CO2. The irradiation group of cells was performed using a 2, 4, 6, and 8 Gy X-ray IR from a medical linear accelerator (Elekta Precise, Stockholm, Sweden) at room temperature.

Cell viability assay

A Cell Counting Kit 8 (CCK8) assay was used to evaluate the proliferation of bladder cancer cells. The 96-well plates were seeded with cells at a concentration of 3×103 cells/well. After 24 h, cells were incubated with fresh medium with or without chloroquine (Sigma, USA). After 24 h and 48 h, a CCK8 cell proliferation and cytotoxicity assay kit (Obio Technology, USA) was used. The absorbance was measured at a wavelength of 450 nm, and all experiments were repeated three times. IC50 values were calculated using the SPSS 22.0 software.

Clonogenic survival assay

EJ and T24 cells were seeded onto six-well plates overnight and then treated with or without chloroquine (10 μM) for 24 h. Next, the cells were exposed to X-rays of different doses (0, 2, 4, 6, or 8 Gy), and cultured in a 5% CO2 incubator at 37 °C for 10-14 days. Finally, colonies containing more than 50 cells were counted under a microscope.

Apoptosis assay

The percentage of apoptotic cells was determined by Annexin-V/FITC and propidium iodide (PI) dual staining (Annexin-V/PI). The cells were seeded into six-well plates and subjected to X-ray irradiation (8 Gy) and chloroquine (10 μM) alone or combined. After 24 h, the cells were collected and analyzed by flow cytometry on a BD FACS flow cytometer (San Diego, CA).

Immunofluorescence staining

Cells were grown on glass coverslip for 24 h. After irradiation for 4, 8, 12, and 24 h, the cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 for 30 min at room temperature, and stained with anti-phospho-histone γ-H2AX (Millipore, France, diluted 1: 250) and the rabbit anti–human light chain 3 antibody (LC3) (1:200, Abcam, UK) at 4 °C overnight. After washing in PBS, a secondary antibody (1:100) conjugated to FITC was added and incubated for 1 h at room temperature. Then, cells were incubated with 0.25 mg/mL DAPI for 3 mins. The images were examined under a laser scanning confocal microscope.

Protein extraction and western blot analysis

The cells were lysed in RIPA buffer supplemented with protease and phosphatase inhibitors (Kaygen, USA). The total protein in the lysates was determined by a BCA kit (Pierce, USA). Samples were separated by SDS-PAGE and transferred to PVDF membranes (Millipore, France). After blocking with 5% skim milk at room temperature for 60 mins, the blots were surveyed with the primary antibodies rabbit anti-p62 (1:10000; Abcam, UK), rabbit anti-LC3 (1:1000; Abcam, UK), rabbit anti-Bcl-2 (1:1000; Cell Signal, USA), rabbit anti-Caspase 3 (1:1000; Cell Signal, USA), and mouse anti-GAPDH (1:1000; Cell Signal, USA) at 4 °C overnight. The membranes were washed three times with TBST buffer (20 mmol/L Tris-buffered saline and 0.1% Tween 20) for 10 mins and incubated with HRP-conjugated secondary antibodies such as anti-mouse-IgG/anti rabbit-IgG for 1 h at 37 °C. Immunoblotted proteins were visualized by an imaging system (Bio-Rad, CA, USA), and the singles were detected using the ECL plus western blotting detection system (Millipore, France).

Tumor xenograft mouse models

The animal studies were approved by the Ethics Committee of Nanjing Medical University. Five- to six-week-old female BALB/C nude mice were provided by Nanjing University Animal Center. T24 cells

(1×107 cells/mL) were injected subcutaneously in each mouse at one site of the left leg. Tumors were allowed to grow for 7 days before treatment. Then, the nude mice with established tumors (all ~200 mm3) were randomly divided into four groups (n=5): (1) vehicle (PBS), (2) chloroquine, (3) irradiation and (4) combined (chloroquine plus irradiation). The mice in the control group were treated with vehicle control. Then, both the chloroquine group and the combined group were administered with 50 mg/kg chloroquine as 4-day continuous intraperitoneal injections. Tumors were irradiated by an RS-2000 biological irradiator at 6 Gy (2 Gy/min) for 2 h on day 4 after drug treatment. The tumor volume was calculated as follows: tumor volume = (length(L)×width(W)2)/2. The change in volume was measured to evaluate the efficacy of each treatment. Growth delay time (GD) was calculated as the time for treated tumors to double in volume minus the time for the control tumors to reach double in volume. The enhancement factor (EF) was then designated according to the following formula: EF=(GDcombined – GDchloroquine)/GDirradiation. The volume change was measured daily until day 25.

Immunohistochemical staining

The mice were killed at the end of the experiments. Tumor tissues were fixed in 10% formalin, paraffinized, and cut into 5 μm-thick sections. After microwave pretreatment in a citrate buffer (pH=6.0; for antigen retrieval), the slides were immersed in 3% hydrogen peroxide for 20 min to block endogenous peroxidase activity. After intensive washing with PBS, the slides were incubated with LC3, p62 and Bcl-2 antibodies and then incubated with HRP-conjugated antibodies. The slides were visualized with a DAB Horseradish Peroxidase Color Development Kit (Beyotime, China), and counterstained with hematoxylin. The images were analyzed by Image-Pro Plus 6.0 software.

Transmission electron microscopy (TEM)

Cells were fixed overnight in ice-cold glutaraldehyde (3% in 0.1 M cacodylate buffer, pH=7.4) before being post-fixed in 1% OsO4 for 45 mins. Then, the cells were dehydrated in a graded series of 70% to 100% acetone and embedded in Epon812. Subsequently, ultra-thin sections were stained by uranium tetraacetate and lead citrate trihydrate and observed under a PhilipsTECNAI10 transmission electron microscope.

Statistical analysis

All experiments were performed three times and the results are presented as the mean ± standard deviation (SD). Statistical analysis was performed using STATA 22.0 software (StataCorp) and Prism 5.0 software (GraphPad Prism). Student's t test and the $\chi 2$ test were used to determine the differences between treatment groups. A P value <0.05 was consider statistically significant.

Results

Chloroquine promotes radiosensitivity of bladder cancer cell lines

Chloroquine inhibited the proliferation of bladder cancer cell lines in a dose- and time-dependent manner (Fig. 1A and 1B). At 48 h, the IC50 of chloroquine on EJ and T24 cell lines were 83.18 and 73.05 μM, respectively. Therefore, cells were treated at 48 h with low concentration of chloroquine (10 μM) to investigate the radiosensitivity of these cell lines.

The clonogenic survival assay was also performed to analyze potential chloroquine radiosensitization activity. The results showed that chloroquine increased irradiation-induced clonogenic cell death in a dose dependent manner (Fig. 1C and 1D). The surviving fraction (SF) data were fit with the single-hit multi-target model formula: SF=1−(1−e-D/D0) n. The results showed that the surviving fractions (2 Gy) (SF2) were 0.74 and 0.73 in EJ cells and T24 cells, respectively. After treatment with chloroquine at 10 μM, SF2 were 0.58 in EJ cells and 0.56 in T24 cells, respectively. The sensitization enhancement ratio (SER) of chloroquine at 10 μM was 1.53 and 1.40 in EJ cells and T24 cells, respectively. These data indicated that chloroquine resulted in a significant radiosensitization effect in bladder cancer. Radiobiological variables were calculated and summarized in Table 1.

Table 1. Radiosensitization effects of chloroquine on bladder cancer cells in vitro. D0: mean inactivation dose; Dq: quasi domain dose; SF2: surviving fraction of 2 Gy; SER: sensitization enhancement ratio

	D0	Dq	SF2	SER
EJ				
Control	2.81	1.88	0.74	
Chloroquine (10 μM)	1.84	1.38	0.58	1.53
T24				
Control	2.77	2.13	0.73	
Chloroquine (10 μM)	1.98	1.17	0.56	1.40

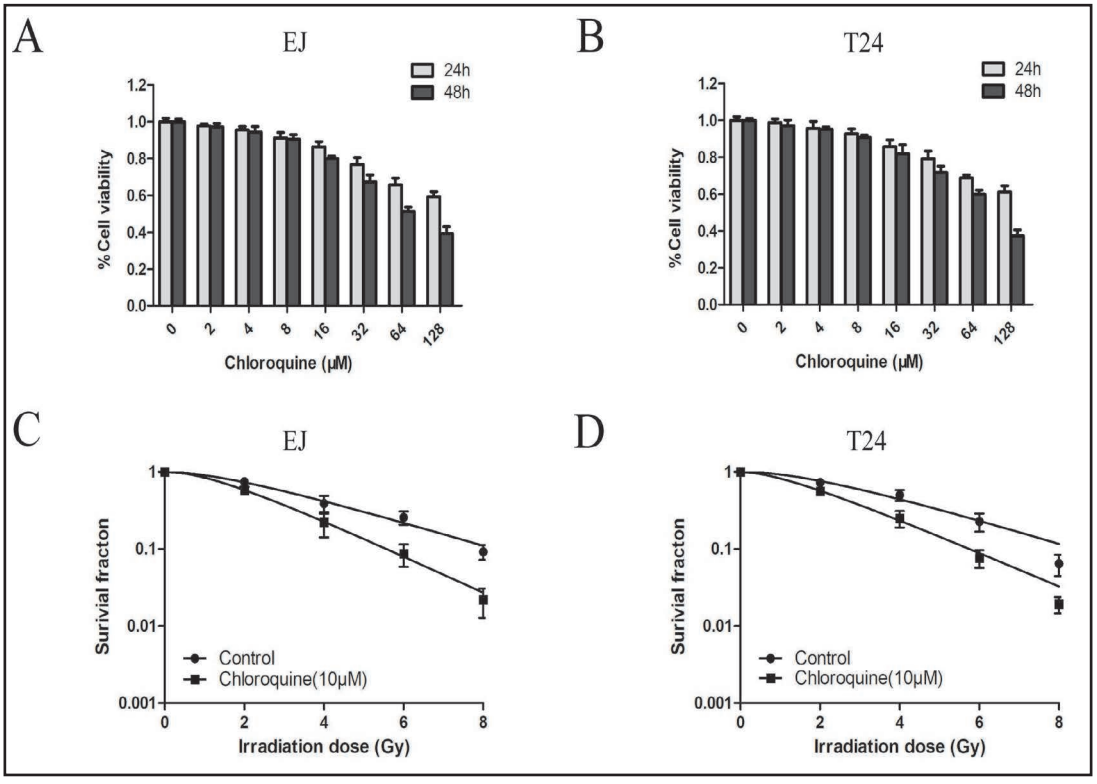

Fig. 1. Chloroquine promotes radiosensitivity of bladder cancer cell lines. (A, B) Bladder cancer cell lines EJ and T24 were treated with various concentrations of chloroquine for 24 or 48 h. Cell viability was determined by a CCK8 assay. (C, D) A cell survival curve was established by clonogenic survival assay. The cells were treated with 10 μM chloroquine and irradiation as illustrated and harvested after incubation for 10-14 d. Each bar represents the mean ± SD from 3 independent experiments.

Chloroquine induces apoptosis and DNA breaks in bladder cancer cells

Next, we investigated the effects of chloroquine on apoptosis of bladder cancer cells in response to irradiation. EJ cells and T24 cells were treated with X-ray (8 Gy) or chloroquine (10 μM) alone or with combination of both for 24 h. Annexin V-FITC/PI double staining was used to assess the induction of apoptosis. The results showed that the rate of apoptosis was higher than that of the control group in both EJ cells (Fig. 2A and 2C) and T24 cells (Fig. 2B and 2D) after treatment with chloroquine at 10 μM. The apoptosis rate was remarkably higher in the combination treatment group compared to the irradiation ($P<0.05$) or chloroquine group ($P<0.05$). In addition, western blot analysis showed that the combination of chloroquine and radiation down-regulated Bcl-2 and up-regulated caspase-3 (Fig. 2E and 2F).

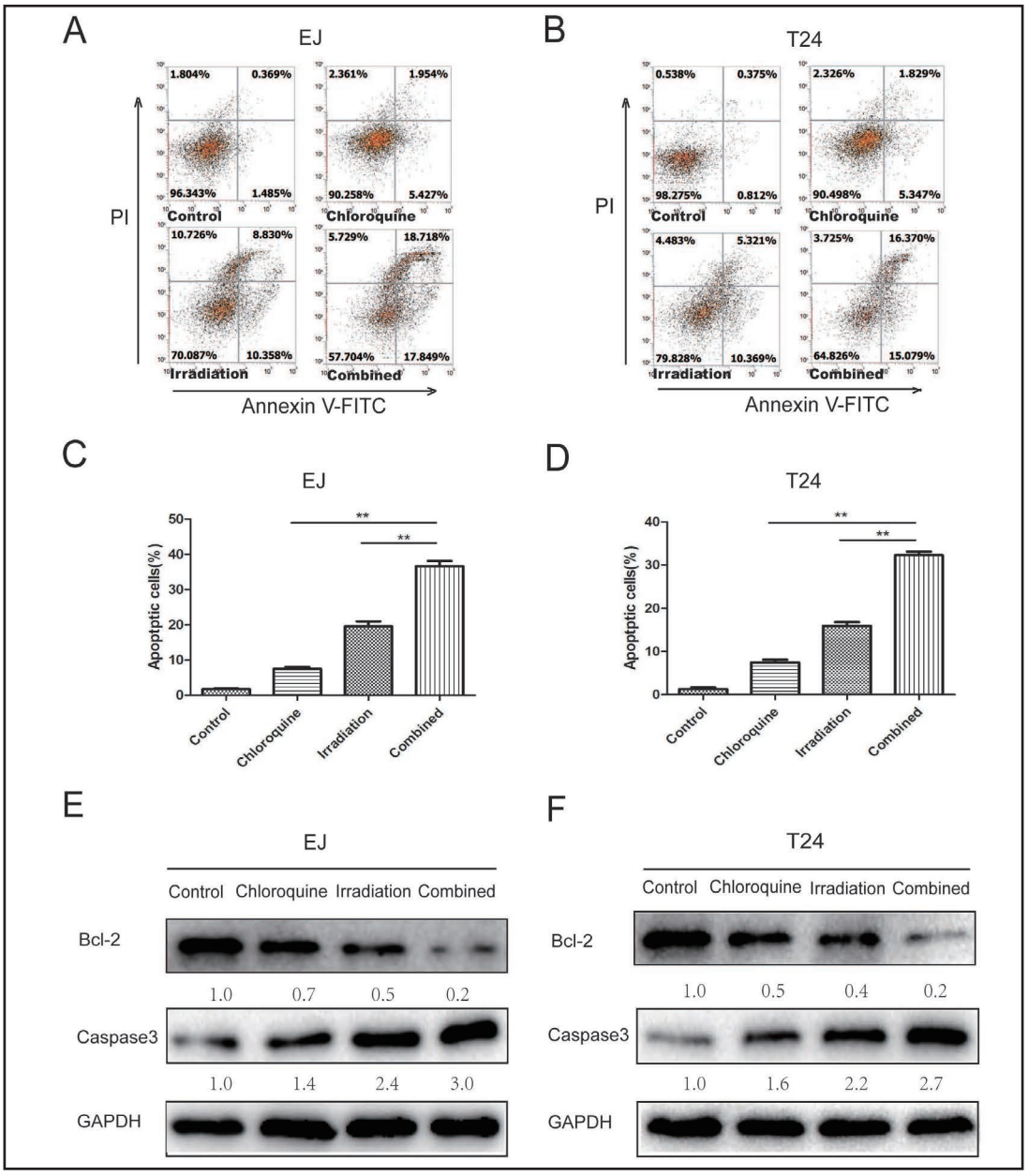

Fig. 2. Chloroquine induces apoptosis in bladder cancer cells. Cells were treated with IR (8 Gy) or chloroquine (10 μM) alone or in combination for 24 h. (A, B) The apoptosis rate was measured using Annexin V-FITC/PI double staining. (C, D) Quantitative analysis of apoptotic cells. The bars represent the means ± SD of three separate experiments. **P<0.01 compared with the response to IR or chloroquine alone. (E, F) Apoptosis-associated proteins were investigated by western blotting.

We detected the effects of chloroquine on irradiation-induced cellular genomic DNA damage by immunofluorescence staining of γ-H2AX foci in bladder cancer cells at different time points (0, 4, 8, 12 h) after irradiation (8 Gy). As anticipated, the peak foci value appeared in EJ and T24 cells at 4 h after irradiation, which was gradually declined to basal level at 24 h after irradiation. However, the combined treatment resulted in a significant increase in residual DNA damage compared with irradiation or chloroquine alone and excessive DNA damage existed at 24 h (Fig. 3A-D). These results suggested that chloroquine markedly inhibited the repair of DNA damage manifested as the persistence of irradiation-induced γ-H2AX foci from 4 h to 24 h after radiation.

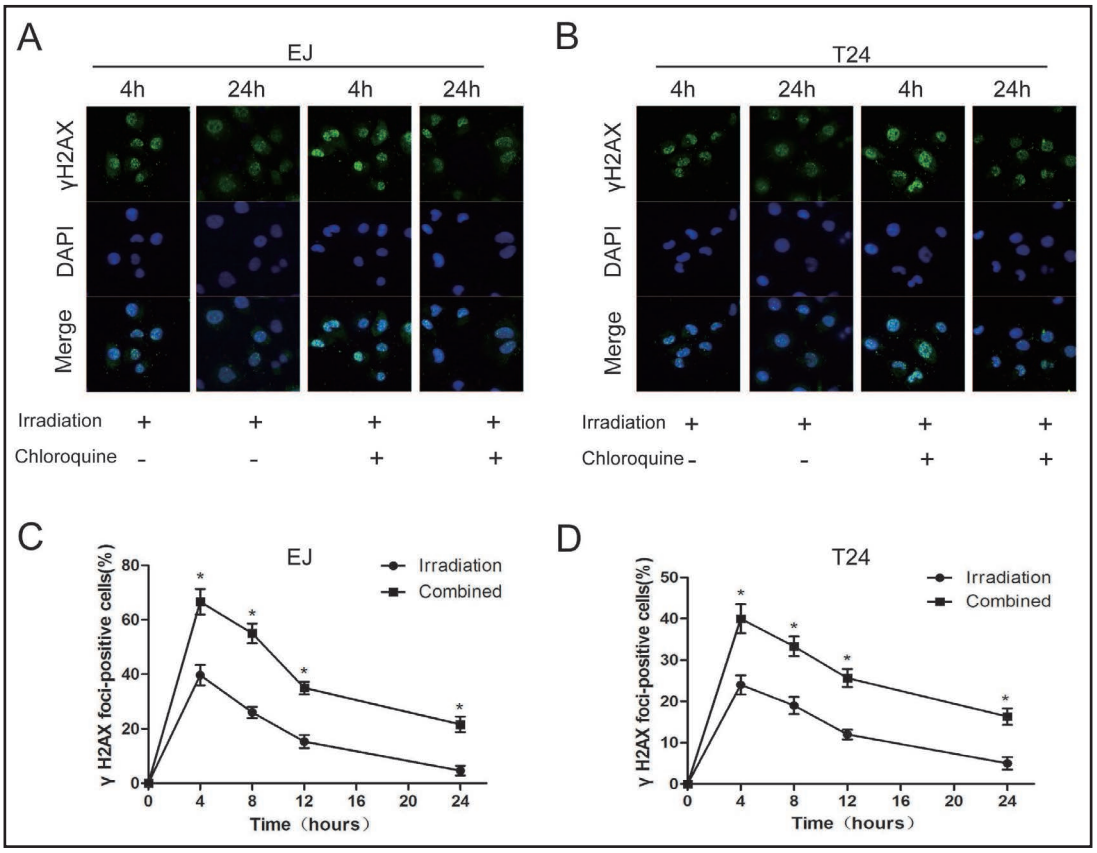

Fig. 3. Chloroquine induces DNA breaks in bladder cancer cells. (A, B) Cells were treated with IR (8 Gy) or chloroquine (10 μM) alone or in combination for 24 h, stained with phospho-γ-H2AX antibody, and observed under a confocal microscope. DNA damage was stained green, and the nuclei were stained blue. (C, D) DNA damage was quantified at each time point as the average number of foci in nuclei (n=3). *P<0.05 compared with the response to IR alone.

Chloroquine inhibits autophagy induced by irradiation

To evaluate the activity of autophagy, the LC3 II and p62 levels were detected with western blotting analysis (Fig. 4A and 4B). Quantitative analysis revealed that the accumulation of LC3 II and p62 protein levels were both increased by the treatment of chloroquine. The result indicated that chloroquine blocked autophagic flux, which promoted the accumulation of autophagosome-associated proteins by inhibiting lysosome fusion. After the treatment of 8 Gy in external radiation, the expression of LC3 II increased, and the p62 protein level decreased in both EJ and T24 cells, which suggested that irradiation activated autophagy. Moreover, the expression levels of LC3 II and p62 in the combined group were higher than irradiation alone, indicating that chloroquine inhibited autophagy induced by irradiation in bladder cancer. To further elucidate the autophagic activity, we measured the formation of LC3 II puncta in cells using immunofluorescence. Consistent with the western blotting, an obvious increase in the number of LC3 II puncta in EJ and T24 cells was detected under the treatment of the combination of chloroquine and irradiation (Fig. 4C-F).

Electron microscopy experiments detected the formation of acidic vesicular organelles (AVOs). The result showed that different morphology was observed with numerous double-walled membrane enclosed cytoplasmic vacuoles after treating T24 cells with irradiation. These vacuoles contained more intracellular organelles, primarily mitochondria, compared to the untreated cells. Treating cells with chloroquine prior to radiation led to a significant

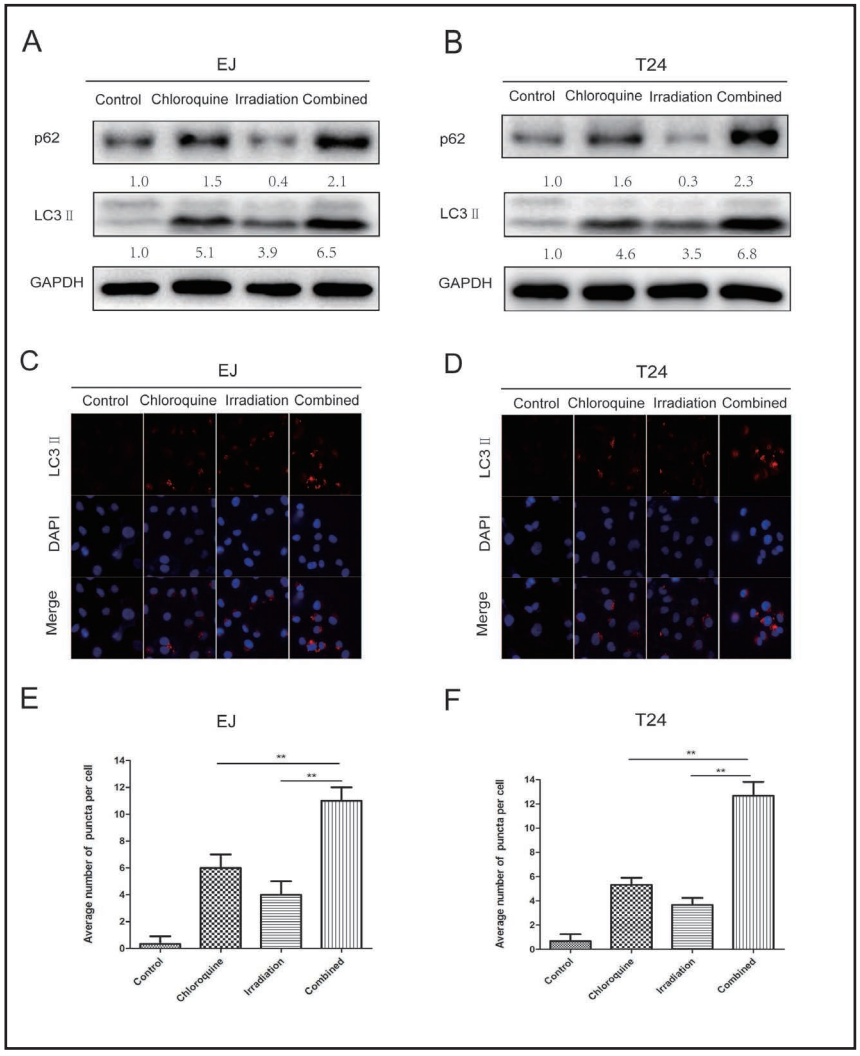

Fig. 4. Chloroquine inhibits autophagy induced by IR. Cells were treated with IR (8 Gy) or chloroquine (10 μM) alone or in combination for 24 h. (A, B) Western blotting analysis was used to determine the level of autophagy-associated protein LC3 II and p62. (C, D) Effects of chloroquine and irradiation alone or in combination on autophagy arrest assessed by using immunofluorescence staining of LC3 II. (E, F) Quantitative analysis of the accumulation of LC3 II punctum per cell. Data represent the mean ± SD from three independent experiments, **P<0.01 compared with the response to IR or chloroquine alone.

increase in the number of autophagic vacuole (Fig. 5). However, in untreated cells or cells treated with chloroquine alone, only a few autophagosomes were observed. Altogether, our findings showed that chloroquine inhibited irradiation-induced autophagy by blocking autophagosome -lysosome fusion in bladder cancer cells.

Chloroquine promotes the radiosensitivity of bladder cancer xenografts in nude mice

To confirm the potential radiosensitization effect of chloroquine on bladder tumor *in vivo*, T24 tumor-bearing mice were treated with a single fraction of 6 Gy irradiation, and mice received intraperitoneal injection of chloroquine (50 mg/kg) for four days before the irradiation. Compared to the control group, both irradiation and chloroquine was effective in delaying tumor growth, especially when the treatments were combined (Fig. 6). We also analyzed the doubling time required for the tumor to grow under different treatments. In the control group and the chloroquine group, the doubling times were 5.0 ± 0.3 and 6.2 ± 0.7 days, respectively. Compared to the irradiation treatment group (9.2 ± 1.2 days), the combination treatment extended the doubling time (16.2 ± 1.1 days) (Table 2). Intraperitoneal injection of chloroquine enhanced the response of bladder cancer xenografts to irradiation with an EF of 2.4. These results showed that chloroquine enhanced the radiosensitivity of bladder cancer xenografts.

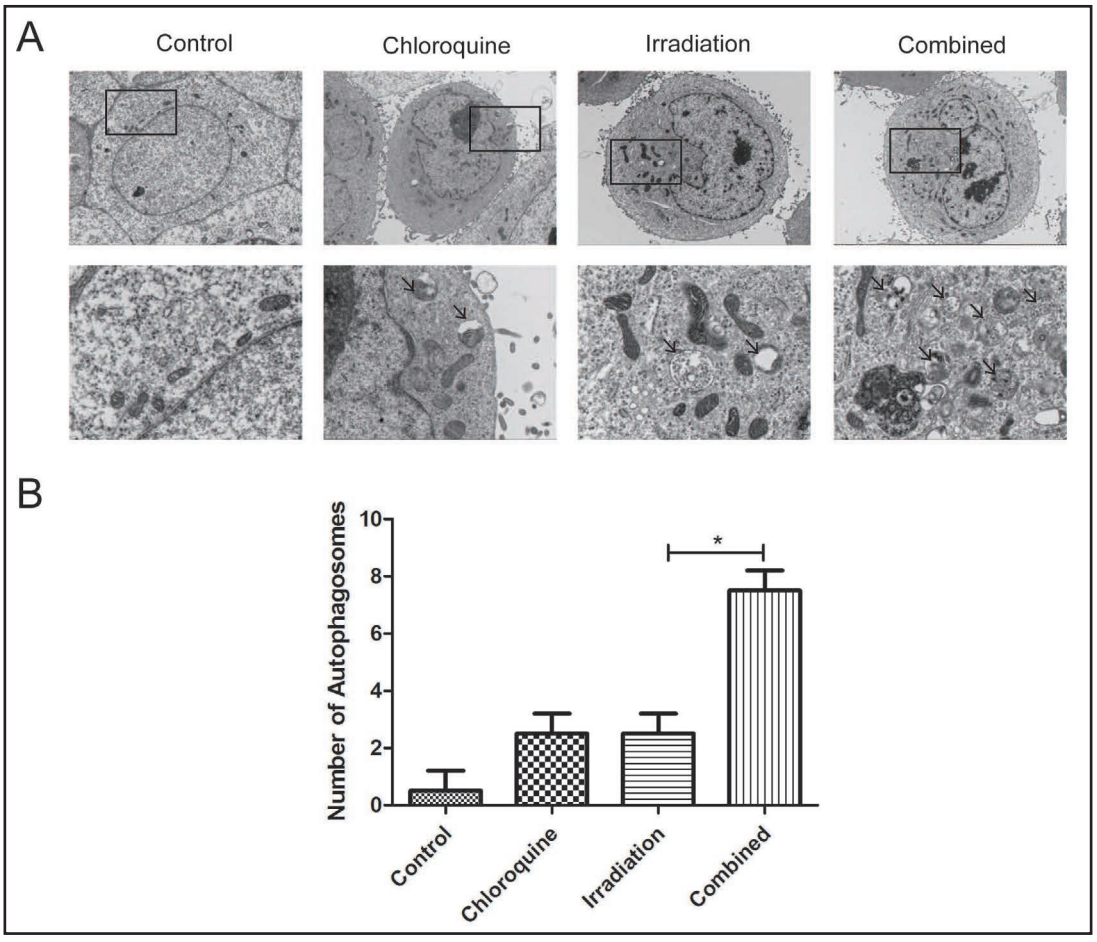

Fig. 5. Electron microscopy experiments detected the formation of acidic vesicular organelles (AVOs). (A, B) Electron microscopy micrographs of T24 cells treated with IR (8 Gy) or chloroquine (10 μM) alone or in combination for 24 h. The arrows indicate autophagic vacuoles and autolysosomes. Untreated cells were used as a control. Quantification of autophagosomes in 12 random fields in three independent experiments. *P<0.05 compared with the response to IR alone.

Table 2. Radiosensitization effects of chloroquine on T24 xenograft mouse model. aAbsolute growth delay, the doubling tumor time of treatment group minus that of control group; bNormalized growth delay, the time of absolute growth delay of tumor in irradiation combined with chloroquine group minus that of chloroquine group

Treatment	Doubling time (days)	Absolute growth delay (days)a	Normalized growth delay (days)b	Enhancement factor
Control	5.0 ± 0.3	0		
Chloroquine	6.2 ± 0.7	1.2 (6.2-5.0)		
Irradiation	9.2 ± 1.2	4.2 (9.2-5.0)		
Combined	16.2 ± 1.1	11.2 (16.2-5.0)	10 (11.2-1.2)	2.4 (10/4.2)

To investigate the relationship between the level of LC3 II, p62 and Bcl-2 in different treatments, tumor xenografts were sectioned for immunohistochemistry. The results demonstrated that the expression of LC3 II and p62 in the combined group were higher than irradiation treatment alone (Fig. 6). In addition, the combination of chloroquine and

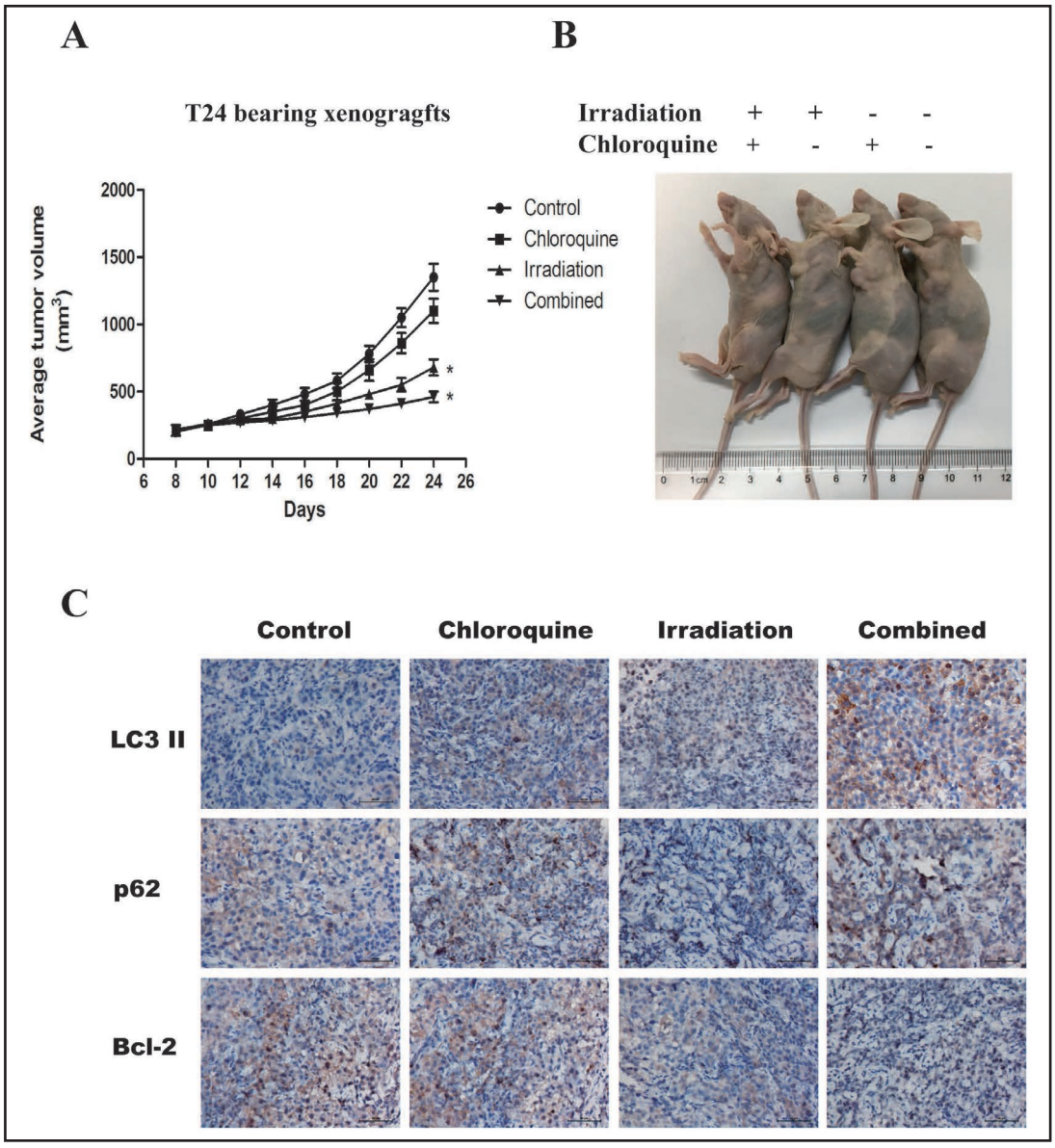

Fig. 6. Chloroquine promotes the radiosensitivity of bladder cancer xenografts. (A, B) The volume of bladder cancer xenografts treated by 50 mg/kg chloroquine as 4-day continuous intraperitoneal injections and/or 6 Gy on day 4 of drug treatment (mean ± SD, n=5), *P<0.05 vs. control. (C) Immunohistochemical staining for LC3 II, p62 and Bcl-2 in bladder cancer xenografts dissected from nude mice.

irradiation decreased the expression level of Bcl-2 compared to irradiation alone. The phenomenon showed that chloroquine potentiated the radiosensitivity of bladder cancer cells by inhibiting autophagy and activating apoptosis.

Discussion

Bladder cancer is the fourth most common malignancy and the second most frequent cause of mortality among cancers of the genitourinary system in men in the USA [1]. Radiotherapy was a valid option in the radical treatment of MIBC, with survival rates similar to cystectomy in the previous studies [4, 5]. However, the resistance of bladder cancer to radiotherapy has remained an important issue in recent years. Recently, studies have shown

that autophagy was strongly associated with radioresistance in various tumors [13, 16, 24]. Lomonaco et al. [24] have found that the induction of autophagy contributed to the radioresistance in glioma stem cells. Wang et al. [16] also reported that autophagy induced resistance to ionizing radiation in pancreatic cancer cells. A similar phenomenon was additionally reported in breast cancer cell lines by Chaachouay et al. [13]. Our study also showed that autophagy induced resistance to the radioresistance in bladder cancer.

Autophagy is a highly conserved cellular catabolic process that degrades and recycles cellular components through lysosomes [25]. The protein levels of LC3 and p62 have been the most widely accepted markers of autophagy [19, 26]. LC3 is a mammalian homolog of yeast ATG8 protein, with three isoforms. The transformation from LC3 I to LC3 II is deemed to be a good marker of autophagic activity. Thus, the level of LC3 II is related to the number of autophagosomes. However, in the conditions of up-regulated autophagy, the level of LC3 II can be increased or decreased because part of LC3 II is destroyed together with cargo in autophagolysosomes. Therefore, measuring the level of LC3 II alone does not determine whether autophagy is induced or inhibited. Wang et al. [27] showed that treatments of PC-12 cells with autophagy inhibitor chloroquine and autophagy inducer rapamycin can both increase LC3 II levels. The p62 protein, also called poly-ubiquitin binding protein sequestosome 1, is another marker of macroautophagy. P62 binds directly to LC3 and forms protein aggregates, which are degraded by autophagy [28]. When autophagy is inhibited, the level of p62 increases, whereas the p62 expression level decreases when autophagy is inducted. Thus, p62 protein is useful as a marker of autophagy flux. Our results showed that after the treatment of irradiation, the expression of LC3 II increased, whereas the p62 protein level decreased. In addition, we have also used other methods to explore the relationship between irradiation and autophagy. Consistent with the previous results, we found that the number of LC3 II punctain and autophagic vesicles increased in the irradiation group, using immunofluorescence and electron microscopy, respectively. Therefore, the results suggested that irradiation activated autophagy in bladder cancer with LC3 II increased and p62 decreased.

To further elucidate the relationship between autophagy and radioresistance in bladder cancer, we used chloroquine, known as an inhibitor of autophagy. It is reported that chloroquine can inhibit the fusion of autophagosomes to lysosomes and block the subsequent degradation step, thus inducing the accumulation of autophagic bodies [18, 19]. As shown in our results, the accumulation of LC3 II and p62 protein levels both increased under the treatment of chloroquine. After the chloroquine treatment, the number of LC3 II puncta also increased by immunofluorescence. Some studies have shown that chloroquine could be a potential radiation sensitizer by inhibiting autophagy [21, 29]. In the current study, we explored the radiosensitizing effects of chloroquine in bladder cancer *in vitro* and *in vivo*. The results showed that chloroquine in combination with irradiation delayed the repair of radiation-induced DNA damage, and chloroquine resulted in a significant radiosensitization effect in bladder cancer as shown by the clonogenic survival assay. Compared to irradiation alone, the expression level of LC3 II and p62, the number of LC3 II by immunofluorescence, and the number of autophagic vacuoles increased in the combined treatment. Therefore, the results showed that chloroquine increased the radiosensitivity in bladder cancer by inhibiting the autophagy induced by irradiation.

Chloroquine in combination with irradiation also increased the apoptosis rate. Apoptosis is involved many diseases, especially malignant tumors [30-32]. There are two major apoptotic pathways, namely, the extrinsic or death receptor pathway and intrinsic or mitochondrial pathways [33]. Both pathways are also involved in radiation-induced apoptosis [34]. Here, we found that the combination of chloroquine and irradiation down-regulated the expression of anti-apoptotic protein Bcl-2 and up-regulated the expression of caspase-3. Thus, these results demonstrated that chloroquine played a synergetic role in radiation-induced viability inhibition and apoptosis. Furthermore, we used a xenograft nude mouse model to provide *in vivo* evidence that chloroquine enhanced the radiosensitivity of T24 xenografts. The result showed that chloroquine combined with radiotherapy

significantly inhibited tumor growth. We also found that the combined treatment group had increased levels of LC3 II and p62 compared to the irradiation group, which confirmed that chloroquine inhibited autophagy and activated apoptosis in bladder cancer *in vivo*.

Conclusion

Our results demonstrated that the radiosensitization efficiency of chloroquine in combination with irradiation was enhanced by the inhibition of autophagy and the activation of apoptosis. Thus, by inhibiting autophagy and activating apoptosis, chloroquine might be a potentially promising radiosensitizer in the radiation therapy of bladder cancer.

Acknowledgements

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Disclosure Statement

No conflict of interest.

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