Original Article

Mechanism Study on Radiosensitization Effect of Curcumin in Bladder Cancer Cells Regulated by Filamin A

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Abstract

Objective: To study the radiosensitization effect of curcumin, a natural product with anti-inflammatory and anti-cancer properties, in bladder cancer cells and identify the specific role of FLNA gene in that process.
Methods: CCK-8 method was initially adopted to identify the proper interventional concentration of curcumin. T24 bladder cancer cells were subjected to CCK-8, flow cytometry, and colony formation assay to study the cell biological behaviors under different interventions. γ-H2AX test was performed to test the level of damage in T24 cells. RT-qPCR and Western blot were conducted to measure FLNA mRNA and protein levels.
Results: Low-dose curcumin (10, 20 μM) following X-ray exposure resulted in increased DNA damage, augmented apoptosis, and reduced proliferation of T24 cells. Certain radiosensitization was demonstrated when curcumin was applied at 10 μM. Additionally, elevation of FLNA gene and protein levels was also indicated upon combination treatment.
Conclusion: Low-dose curcumin has certain radiosensitization effect in bladder cancer, where FLNA plays a certain regulatory role.

Keywords

curcumin, bladder cancer, filamin A

Introduction

Bladder cancer is one of the top 10 prevalent cancers in the world. Over the past three decades, there remains no effective treatment strategy, and the muscle-invasive and metastatic cancer subtypes are still associated with a high mortality.[1] Currently, treatments against bladder cancer mainly are surgery, immunotherapy, and radiotherapy. Of note, radiotherapy is significant for bladder cancer and in palliative treatment, and proper radiotherapy could improve patient prognosis with tolerable adverse reactions.[2] Radiation therapy generally requires dose increase for radio-resistant tumors, which is always accompanied by increased damage to surrounding normal cells. In this context, radiosensitizers can compensate for the deficiency as they can reduce damage to normal cells while killing more tumor cells under the same radiation dose. Curcumin is a component of *Curcuma longa* (turmeric), a traditional Chinese medicine (TCM). It is a kind of phenolics of multiple biological effects and has been widely studied in a variety of tumors for its anti-tumor effect. Studies in different cancers revealed that curcumin could significantly improve the therapeutic efficacy of radiotherapy, demonstrating radiosensitization effect. Similarly, the radiosensitization effect of curcumin was also demonstrated in

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head and neck squamous cell carcinoma,[3] non-small cell lung cancer (NSCLC)[4,5], pancreatic cancer[6], colon cancer,[7] rectal cancer,[8] and human urethral scar fibroblasts[9]. Furthermore, it was also reported that curcumin exhibited certain radiosensitization effects in bladder cancer cell lines UM-UC5 and UM-UC6[10]. We thus speculated that curcumin can also play certain radiosensitization effects in other bladder cancer cell lines.

Filamin A (FLNA), also known as actin-binding protein 280 (ABP 280) and non-muscle actin filament crosslinking protein,[11] is a member of the Filamin family with the largest number and the widest distribution. By now, FLNA serves as a scaffold for more than 90 DNA binding-proteins in multiple cellular functions, such as signal transduction, transcriptional regulation, transmembrane receptor, DNA damage repair, phosphorylation, ion channel regulation, cellular proliferation, migration, and adhesion.[12] In our previous study,[13] we found that the FLNA gene could be an important therapeutic target in treatment of bladder cancer. In addition, both in vivo and ex vivo experiments demonstrated that overexpression of FLNA gene could regulate autophagy of tumor cells, in turn to suppress proliferation and advance apoptosis. A concern is raised that whether the FLNA gene is involved in the cell-killing process by radiation, especially that whether the FLNA gene plays a regulatory role in the radiosensitization of curcumin to the bladder cancer cells. In the present study, bladder cancer cells were exposed to curcumin or X-ray irradiation or both to study the potential effects on cell biological behaviors, and the role of FLNA in that process was also investigated.

Materials and Methods

Materials

Bladder cancer cell line T24 (Shanghai Zhongqiaoxinzhou Biotech); RPMI-1640, Fetal bovine serum (FBS), Cell Counting Kit-8 (CCK-8), Annex V/FITC Cell Apoptosis Kit, Trypsin (Meilun Cell); Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) Kit (EpiZyme, Shanghai); 5× Tris-glycine buffer, 10× transfer buffer, 10× Tris Buffered Saline with Tween 20 (TBST), Dimethyl sulfoxide (DMSO) (Solarbio); RC112-01 RNA Extraction Kit, R333-01 Reverse Transcription Kit, Q711-02 sybr green PCR Kit (Vazyme Biotech Co,Ltd); Penicillin-Streptomycin Solution (Biosharp); Primary anti-FLNA antibody (Abcam); Primary anti-β-Tubulin antibody (CST); Secondary rabbit antibody (Abclonal); Primary anti-γ-H2AX antibody (GeneTex); Immunofluorescence Staining Kit (anti-mouse cy3), Antifade Mounting Medium containing 4′,6-diamidino-2-phenylindole (DAPI) (Beyotime).

Methods

Cell Culture and Grouping

T24 bladder cancer cell line was cultured in RPMI-1640 medium, supplemented with 10% FBS, and maintained in surroundings of 37°C and 5% CO2. Cell count and grouping were determined according to the purpose of each experiment.

To identify the optimal drug interventional concentration, CCK-8 was performed in cells from three groups: Blank control group, DMSO control group, and Curcumin group (10, 20, 30, and 40 μM). In the cell proliferation, apoptosis, RT-qPCR, and Western blot assays, cells were exposed to either curcumin group (0, 10, 20 μM) or X-ray (0, 5 Gy) or both (X-ray addition 48 h after curcumin application). The same treatment strategy was applied in the colony formation assay, except that the X-ray radiation was provided at a dosage of 0, 2, 4, 6, and 8 Gy.

CCK-8 Cell Survival Assay

T24 cells at logarithmic growth phase were firstly digested with trypsin, and seeded into a 96-well plate containing 100 μL of culture medium per well (3-5 ×10^3 cells/well). Culture environment was an incubator with 5% CO2 and a temperature of 37°C. After 24 h of culture, 10 μL of curcumin at different concentrations (0, 10, 20, 30, 40 μM) was added for co-culture for another 48 h. DMSO corresponding to 40 μM curcumin was added as control. Subsequently, the medium in each well was replaced by 10 μL of CCK-8 solution. After 45 min of incubation in the dark, a microplate reader was used to read the optical density (OD) at 450 nm. Cell survival rate = (experimental − blank)/(DMSO − blank) × 100%.

Cell Apoptosis Assay

T24 cells at logarithmic growth phase were inoculated into a 6-well plate at 2 mL per well (1.5×10^4 cells/mL). AnnexinV-FITC kit was used for cell apoptosis assay. Single staining was provided as control with either PI or AnnexinV-FITC, in an attempt to adjust fluorescence compensation, remove spectral overlaps, and mark the crosses. Cell apoptosis was assessed in a total of 1×10^4 cells.

Plate Colony Formation Assay

T24 cells were intervened by curcumin after 24 h of culture and by X-ray irradiation (0, 2, 4, 6, and 8 Gy) after 48 h. The cell count per well was according to the dosage of X-ray irradiation, from 200 to 400, 600, 800, and 1000 cells/well. The cells were cultured for further 10–14 days after irradiation and the medium was replaced at 2–3 days intervals. The culture was terminated until visible colonies occurred, and crystal violet staining was provided to count the colonies. Clonogenic survival rate = colony count/inoculated cell count × 100%. Colony formation rate = clonogenic survival rate at each dosage/clonogenic survival rate at 0 Gy irradiation × 100%. Radiotherapy sensitization ratio was calculated by using Origin software according to the single-hit multi-target model. Radiation sensitization ratio (SER)

=Dq (irradiation group)/Dq (curcumin combined irradiation group).

Immunofluorescence Assay

Prepared T24 cells were washed with PBS for three times, 5 min per wash. Fixation at room temperature was completed with 4% paraformaldehyde for 15 min. PBS washing was performed. Punch-solution was added and the cells were incubated at 4°C for 15 min. Following another PBS washing, blocking solution was added at room temperature for 1 h. Primary (4°C overnight) and secondary antibodies (37°C, 1 h) were successively added. PBST washing was provided three times (5 min per) before and after hybridization. Finally, DAPI-contained Antifade Mounting Medium was used to prepare slides. Scanning laser confocal microscope (FV1200) was used to take pictures under ×600 oil objective, and the IMaris x64.7.4.2 was run to count the foci.

RT-qPCR

Cells of each group were washed with PBS twice, digested by trypsin, suspended by PBS, and collected by centrifugation. RNA was extracted from cells using the RC112-01 RNA extraction kit and then treated by R333-01 kit to obtain cDNA. RT-qPCR was operated with the Q711-02 sybr green kit according to predevised reaction system.

Western Blot

Cells were collected following the same procedures as described before. Subsequently, the cells were lysed on ice with RIPA lysis buffer for 30 min. The lysates were centrifuged at 1200 r/min at 4°C for 5 min to collect the supernatant and BCA method was adopted to quantitate the proteins. After that, the protein samples were denatured at 100°C with loading buffer for 10 min, and then subjected to SDS-PAGE at 10 μg per lane. Separated proteins were transferred to a PVDF membrane under a constant voltage of 300 mA within 120 min. 5% skim milk was used to block unspecific binding at room temperature. After 1.5 h, the membrane was exposed to primary (4°C, overnight) and secondary antibodies (37°C, 1.5 h) successively. ECL solution was prepared to develop protein bands and chemiluminescence imaging system was operated to catch images.

Statistical Analysis

Measurement data in mean ± standard deviation were analyzed on SPSS 20.0 statistical software. Between-group comparisons were completed by one-way analysis of variance. Difference on P<.05 was considered to have statistical significance.

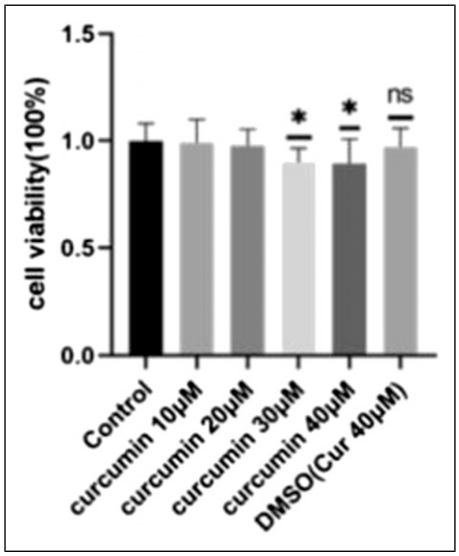

Figure 1. T24 cell viability with increasing curcumin concentration (10, 20, 30, 40 μM) was observed by CCK-8 kit (A). Values represent mean ±SD (n=4) and asterisks signs represent significant differences from controls:*$P < .05$.

Results

Proper Interventional Concentration and Time of Curcumin

Curcumin at different concentrations was used, and a decreasing trend was observed in T24 cell viability with increasing curcumin concentration. Under low concentrations (10, 20 μM), there was no significant difference in cell viability (P>.05). When the concentration increased to 30 and 40 μM, the proliferation of T24 cells was remarkably suppressed as demonstrated by reducing cell survival viability (P<.05) (Figure 1A). Hence, concentrations of 10 and 20 μM were selected for further experiments.

The Radiosensitization Effect of Curcumin in X-Ray Irradiation

The additional X-ray irradiation in the presence of curcumin at 10 and 20 μM showed radiosensitization effect. As compared to the irradiation by X-ray, it was found that the additional X-ray irradiation in the presence of curcumin further weakened the cell viability (P<.05) (Figure 2A).

Similar changing trends were observed in the Annexin V-FITC/PI cell apoptosis assay. The cell apoptosis reversely increased in the presence of both curcumin and X-ray irradiation (P<.05) (Figures 2B-2E).

In the colony formation assay, the presence of both curcumin and X-ray irradiation resulted in low abilities in colony formation (P<.05).(Figures 3A and 3B). And it seem like that there is no cell colony in higher dosages (8 Gy). The calculated results of SER were as follows: D0 and Dq of irradiation

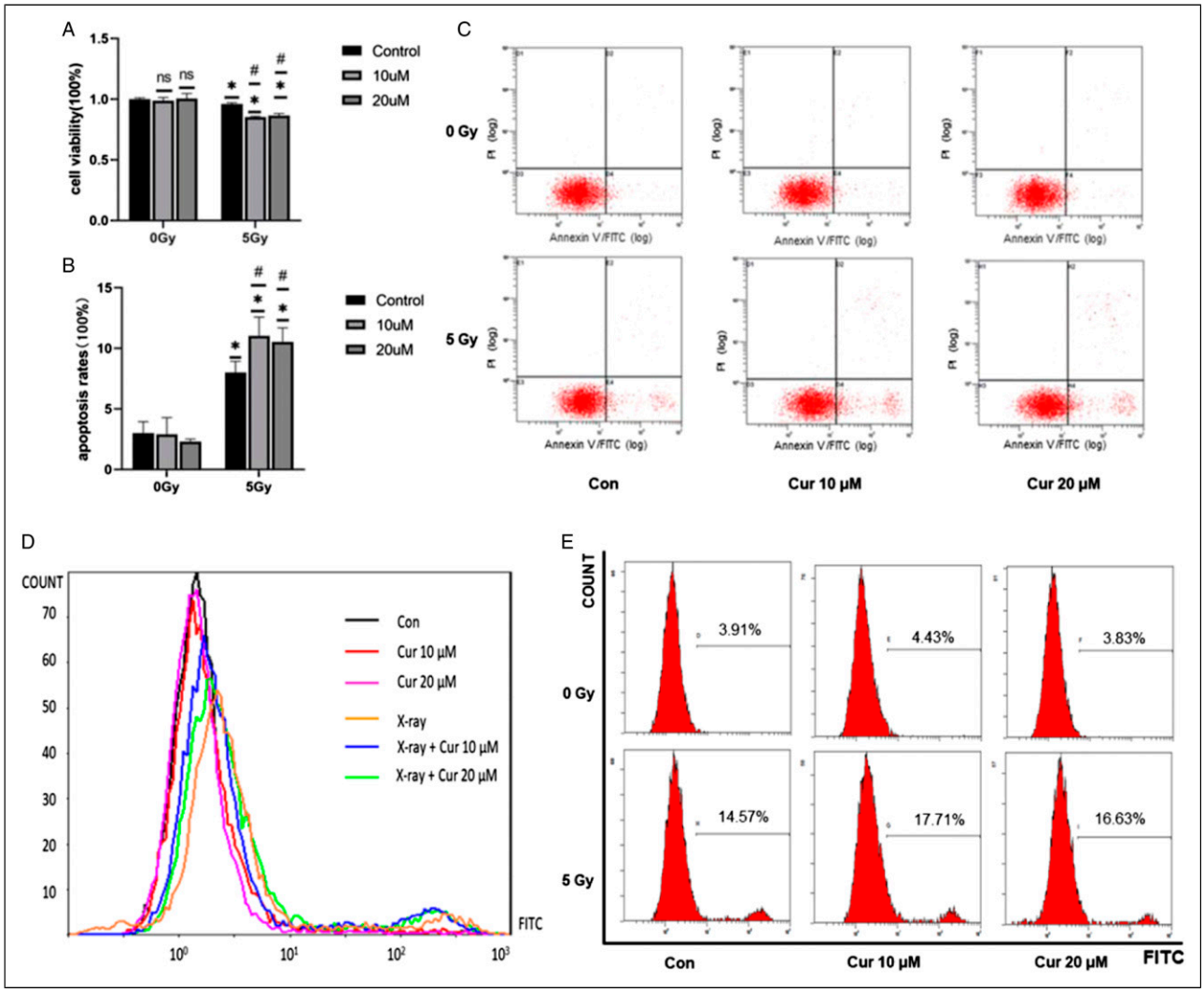

Figure 2. (A) T24 cell viability with both increasing curcumin concentration (10, 20 μM) and X-ray irradiation (5 Gy) was observed by CCK-8 kit. (B, C, D, E) Apoptosis of T24 cells analyzed by flow cytometry exposed to both curcumin (10, 20 μM) and X-ray irradiation (5 Gy). Figure 2. C, D, and E are the scatter diagram and histogram of T24 cell apoptosis, respectively, in which Figure D is the superposition of Figure E. Values represent mean ± SD (n=4), asterisks and pound signs represent significant differences from controls and Single IR:*$P < .05$, #$P < .05$.

group, 10 μM and 20 μM combined curcumin irradiation group were .86, 1.85, 2.16, and 3.95, 2.83, 2.12, respectively. The SER for curcumin at 10 and 20 μM was 1.40 and 1.86, respectively.

In the immunofluorescence assay, γ-H2AX proteins, specific to DNA double-strand break (DSB), the number of foci was much higher upon exposures to both curcumin and X-ray, indicative of aggravated DNA injury (P<.05) (Figures 3C and 3D).

FLNA mRNA and Protein Levels

RT-qPCR was performed to measure FLNA mRNA gene expression in each group. Results indicated that exposure to both curcumin (10, 20 μM) and X-ray (5 Gy) contributed to higher FLNA mRNA expression, as compared to the single exposure to X-ray (P<.05), which was much remarkable at a lower curcumin concentration (P<.05) (Figure 4A). Further Western blot was conducted to find the same changing trend in FLNA protein expression (Figure 4B). X-ray exposure increased the level of FLNA proteins, and the increase was significantly higher after curcumin was administrated. At different durations of radiation, FLNA was mainly distributed in the cytoplasm as 280 kda FLNA at 24 h. After 48 h, nuclear translocation was induced and FLNA mainly expressed as 90 kda FLNA in the nuclei. (Figure 4B).

Discussion

As population aging goes increasingly significant, the incidence of cancer raises accordingly. Radiotherapy is one of the effective methods for treatment of cancer, while radioresistance is inevitable and becomes a great challenge in clinic. To address this issue, radiosensitizers have emerged and attracted people's attention.

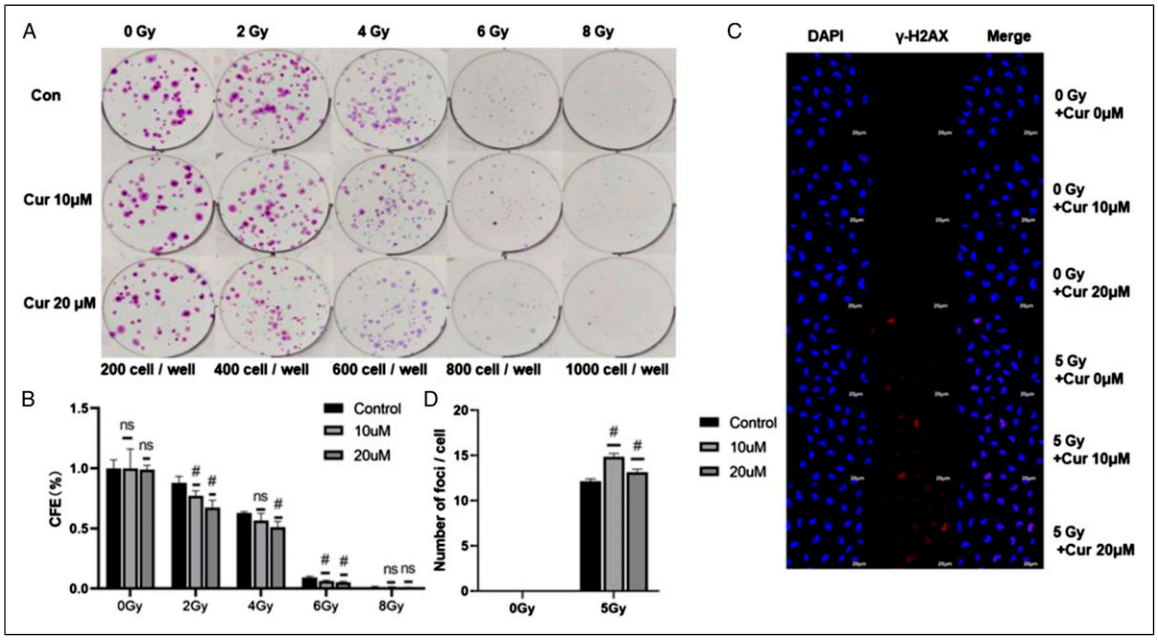

Figure 3. (A,B) Colony formation efficiency of T24 cell intervened by curcumin concentration (0, 10, 20 μM) and X-ray irradiation (0, 2, 4, 6, and 8 Gy). (C,D) Immunofluorescence analysis of T24 in 2h after IR. The nucleus are labeled with DAPI (blue). Quantitative analysis of foci points were measured by IMaris x64.7.4.2. Values represent mean ± SD (n=3, n=50), asterisks and pound signs represent significant differences from controls and Single IR:*$P < .05$, # $P < .05$.

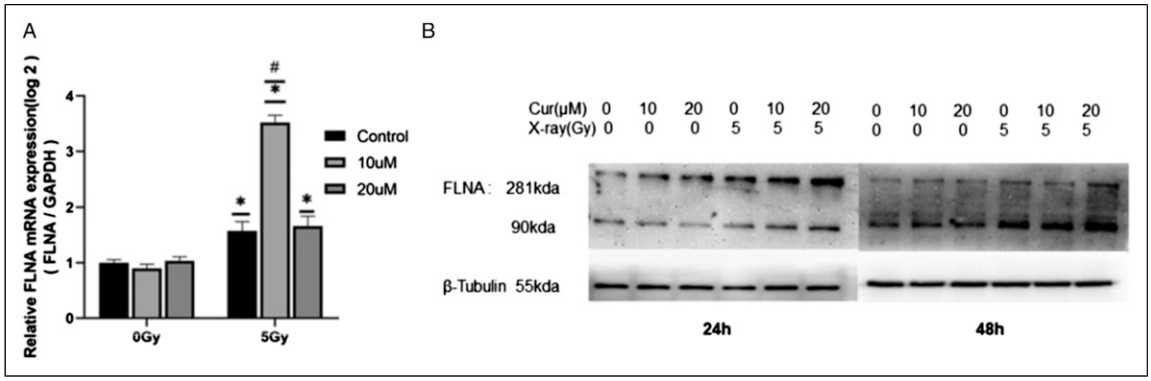

Figure 4. (A)Relative expression levels of FLNA compared to GAPDH. Values represent mean ± SD (n=3), asterisks and pound signs represent significant differences from controls and Single IR:*$P < .05$, # $P < .05$. (B) Western blot analysis of FLNA protein expression in T24 cells exposed both curcumin (10, 20 μM) and X-ray (5 Gy). The expression of FLNA protein compared to β-tubulin after X-ray irradiation (24h, 48h).

For the past few years, increasing studies have proved that curcumin has certain radiosensitization effects in cancer cells via affecting production of reactive oxygen species (ROS), cell cycle process, apoptosis, related signaling pathways such as NF-κB, and the DNA damage repair. For example, curcumin could induce apoptosis of HCT-115 cells by increasing the production of ROS[7]; through G2/M arrest, curcumin could augment the radiosensitivity of human glioma cells U87[14]; in neuroblastomas, curcumin could suppress the activation of NF-κB signaling pathway to enhance the radiosensitivity of tumor cells[15]; in addition, curcumin (analogues) was recently reported to be synergetic with radiation in neuroblastomas and pancreatic cancers by regulating the radiation-induced NF-κB-DNA binding activity and inhibiting NF-κB.[16,17] The role of curcumin in radiosensitization via DNA damage repair captures our interest. It is well known that DNA is the major target for studies of biological effects of radiation. DNA damage is a result of a synergistic combination of multiple protein complexes, including DNA damage response proteins at DNA breaks, signal transduction proteins, and repair proteins. Research found that radiation-sensitive mutant strains might be directly associated with the DNA damage repair process in

Yeasts or mammalian cells. For instance, mutations in X-ray repair cross-complementing gene 1 (XRCC1) could result in 1.7-fold increase in radiation sensitivity, which might be involved in the repair of DNA single strand breaks.[18-20] In addition, curcumin could induce DNA damage in cancer cells and affect the self-repair by decreasing the expression of DNA damage repair genes and proteins, such as BRCA1, MDC1, MGMT, DNA-PKcs.[21-24] Wang et al [8] revealed that curcumin had radiosensitization effects on rectal cancer cells HT-29 by regulating the expression of DNA ligase-4 (LIG4), polynucleotide kinase/phosphatase (PNKP), x-ray repair cross-complementing protein 5 (XRCC5), and Cyclin H (CcnH). Katharina Schwarz et al[6] found that curcumin could increase the radiation-induced DNA double stranded breaks thereby to enhance the radiosensitivity of human pancreatic cancer cells (Panc-1 and MiaPaCa-2). All the studies above indicated that the radiosensitization effect of curcumin is closely associated with DNA damage responses. We reasoned that it might be also a mechanism of action of curcumin in bladder cancer.

In our previous study, we noted that the FLNA gene served as a tumor-suppressor gene in bladder cancer cells T24[25]. Compelling evidence is also revealed in some other cancers, such as gastric cancer, colon adenocarcinoma, and renal cell carcinoma.[13,26-32] Further research unraveled that the FLNA gene plays its tumor-suppressive role via participating in cell signal transduction, transcriptional regulation, cell proliferation, migration, and adhesion.[12,33] To the contrary, the FLNA gene was also reported to act as an oncogene in pancreatic cancer and lung cancer.[34-37] It has been established that the FLNA gene plays different roles with cancer types and its nucleoplasmic localization. It was reported that cytoplasm-localized 280 kda FLNA proteins can be phosphorylated to 90 kda FLNA proteins, which triggers nuclear translocation and then induces interaction with transcription factors.[25,33,38] As a consequence, the tumor growth and metastasis will be suppressed, showing the anti-tumor role of the FLNA gene.[12] Additionally, Roble G. Bedolla et al[38] also reported the anti-tumor role of the FLNA gene in prostate cancer. They found that regulation of the nuclear androgen receptor (AR), a member of the steroid hormone receptor superfamily, by the cleavage and nuclear translocation of FLNA proteins could be suppressive for tumor progression. The findings suggest that the FLNA gene could be used as a potential therapeutic target in treatment of cancer and agents capable of inducing nuclear translocation of FLNA proteins are viable options.

It is noteworthy that the FLNA proteins also have implications in DNA damage repair.[39] FLNA can act as DNA-binding proteins to participate in DNA damage repair with the recruitment of DNA repair proteins, such as BRCA1, BRCA2, and RAD51[40-43] In addition, FLNA can positively regulate BRCA1 to exhibit anti-tumor effects.[44] p53 binding protein 1 (53BP1) is a key regulator of DNA double-strand break repair[45-47] and is considered as a tumor-suppressor protein through the activation of p53[48]. A study found that increase in nuclear FLNA and 53BP1 could advance DNA damage repair to some extent.[49] Furthermore, FLNA could interact with SQSTM1 thereby to participate in the autophagy after DNA damage.[50] It can be seen that the FLNA as a scaffold protein in DNA damage repair is closely associated with multiple DNA repair proteins. It was thus speculated that the FLNA is involved in the cell-killing process by radiation and might play a part in the radiosensitization effect of curcumin in bladder cancer cells (T24).

In the current study, curcumin at safe doses (10, 20 μM) was applied to study its radiosensitization effect in bladder cancer cells T24. It was found that curcumin followed by X-ray radiation further inhibited the proliferation and colony formation, advanced apoptosis, and interfered with DNA damage repair of T24 cells, as compared to X-ray radiation alone. This result demonstrated that curcumin had certain radiosensitization effects in bladder cancer cells. In addition, we noted that there was no significant difference between curcumin at 10 and 20 μM, which might be associated with the typical S-shape of drug dose-response curves and that the curcumin at 10 μM and 20 μM are within the safe range. The SER for curcumin at 10 and 20 μM was 1.40 and 1.86, respectively. As analyzed by the SH-MT model, curcumin at 20 μM was associated with a more narrow shoulder region of the survival curve of T24 cells and a smaller Dq value, indicating less sublethal damage repair. This can be used to interpret the radiosensitization effect of curcumin. Furthermore, the γ-H2AX test also identified the effect of curcumin on repair of the radiation-induced DNA damage in T24 cells by measuring the DNA damage level. Considering involvement of the FLNA in DNA damage repair, we reasoned that the FLNA gene is involved in the radiosensitization effect of curcumin on T24 cells. To validate this speculation, RT-qPCR and Western blot were performed and showed increased mRNA and protein levels of FLNA under curcumin administration and radiation exposure. In the meantime, FLNA was localized in T24 cells by Western blot. It was noted that FLNA proteins transferred from the cytoplasm to the nucleus, which suggested increased expression of nuclear FLNA (90 kda) proteins and enhanced tumor-suppressive effect after curcumin administration followed by radiation exposure. These results imply that curcumin exhibited its radiosensitization effect by increasing FLNA expression and inducing FLNA nuclear translocation to affect the DNA damage repair in the nucleus, which validates our speculation. In all, curcumin exhibits its radiosensitization effect probably through up-regulating intracellular FLNA expression, inducing its phosphorylation and subsequent nuclear translocation, and then affecting the DNA damage repair via regulation of related transcription factors.

Conclusion

To conclude, the current study identified that low-dose curcumin (10, 20 μM) has certain radiosensitization effects in bladder cancer cells, during which the FLNA gene might play a regulatory role.

Author Contributions

Na Chen and Zhengfan Wang conceived and designed the experiment. Shuqing He performed the biological experiments. Minjun Jiang and Xueli Li collected all the data. Shuqing He analyzed the data. Zhengfan Wang and Shuqing He wrote the article. All authors contributed to the final approval of this manuscript.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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