Traditional Chinese Medicine Curcumin Sensitizes Human Colon Cancer to Radiation by Altering the Expression of DNA Repair-related Genes

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Abstract. *Background/Aim: The aim of the present study was to investigate the radio-sensitizing efficacy of curcumin, a traditional Chinese medicine (TCM) on colon cancer cells in vitro and in vivo. Materials and Methods: Human colon cancer HT-29 cells were treated with curcumin (2.5 μM), irradiation (10 Gy) and the combination of irradiation and curcumin. Cell proliferation was assessed using the MTT assay. Apoptotic cells were detected by Annexin V-PE/7-AAD analysis. PCR was performed to determine differential-expression profiling of 95 DNA-repair genes in irradiated cells and cells treated with both irradiation and curcumin. Differentially-expressed genes were confirmed by Western blotting. In vivo radio-sensitizing efficacy of curcumin was assessed in a xenograft mouse model of HT-29 colon cancer. Curcumin was administrated daily by intraperitoneal injection at 20 mg/kg/dose. Mice received irradiation (10 Gy) twice weekly. Apoptosis of the cancer cells following treatment was determined by TUNEL staining. Results: Irradiation induced proliferation inhibition and apoptosis of HT-29 cells in vitro. Concurrent curcumin treatment sensitized the HT-29 tumor to irradiation (p<0.01). DNA repair-related genes CCNH and XRCC5 were upregulated and LIG4 and PNKP downregulated by the combination of curcumin and irradiation compared with irradiation alone* (p<0.05). *Combined treatment of curcumin and irradiation resulted in a significantly greater tumor-growth inhibition and apoptosis compared to irradiation treatment alone* (p<0.01). *Conclusion: Curcumin sensitizes human colon cancer in vitro and in vivo to radiation. Downregulation of LIG4 and PNKP and upregulation of XRCC5 and CCNH DNA-repair-related genes were involved in the radio-sensitizing efficacy of curcumin in colon cancer.*

Curcumin [1,7-bis (4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5dione] is a traditional Chinese medicine (TCM) derived from the rhizome of *Curcuma longa* (1). Curcumin has been shown to inhibit cancer-cell proliferation and induce cell apoptosis in many types of cancer cells (1-7) and modulate multiple cell-signaling pathways (8). Additionally, curcumin induces radio-sensitivity of cancer cells *in vitro* and *in vivo* (9-11). Curcumin also alters the expression of radio-sensitivity-related signaling pathways (12, 13). The detailed mechanism of the radio-sensitizing effects of curcumin on colon cancer is poorly understood.

In the present study, we investigated the radio-sensitizing efficacy of curcumin on the proliferation and apoptosis of colon cancer cells *in vitro* and tumor growth in a xenograft model of human colon cancer. To determine the mechanism of radio-sensitization, we performed a real-time polymerase chain reaction (PCR) assay in order to identify DNA-repair genes involved in the radio-sensitizing mechanism of curcumin.

Materials and Methods

Colon cancer cells and culture. Human colon-cancer cell line (HT-29) was purchased from the Shanghai Cell Bank of the Chinese

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Key Words: Curcumin, radiosensitivity, DNA repair gene, colon cancer.

Academy of Sciences. Cells were cultured in RPMI 1640 medium (Thermo Fisher Scientific, CA, USA) containing 10% fetal bovine serum (Thermo Fisher Scientific, CA, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Thermo Fisher Scientific, CA, USA) at 37°C with 5% CO_2.

Cell proliferation assay. Cell proliferation was determined using the MTT assay. HT-29 cells were seeded in 96-well plates with $5×10^3/200$ μl and cultured overnight. Cells were divided into four groups: control group; curcumin group; irradiation group (IR); and irradiation + curcumin (IR+curcumin) group. Cells treated with dimethyl sulfoxide (0.1% DMSO) were used as a vehicle control. Each treatment included six duplicate wells. The curcumin and IR+curcumin groups were treated with 2.5 μM curcumin (94% purity, Sigma, St. Louis, MO, USA). The IR and IR+curcumin groups were irradiated with X ray at 6 MV using a 600C/D linear accelerator (RS2000Pro, RADSOURCE, Buford, GA, USA) to deliver the indicated doses (10 Gy). All cells were further cultured for 24, 48, or 72 h. To measure cell viability, 20 μl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5 μg/μl) (ATCC, Manassas, VA, USA) were added to each well and incubated for 2 h at 37°C. An extraction buffer (20% SDS and 50% dimethylformamide) was added, and the cells were incubated overnight at 37°C. The absorbance (OD) at 490 nm was measured with a micro-plate reader to determine the cell viability. The above experiment was repeated three times. The resulting MTT absorbance in negative control cells was used as 0% cell inhibition.

Annexin V detection of apoptotic cells. Apoptotic cells were detected by Annexin V-PE/7-AAD analysis. Cells were divided into four groups (Control; curcumin; IR; and IR+curcumin group) $(5×10^5$ cells/well) and seeded in 6-well plates. After attachment to the dish, the cells in the curcumin and radiation+curcumin were treated with 2.5 μM curcumin. The IR and IR+curcumin groups were treated with X ray at 6 MV using a 600C/D linear accelerator to deliver the indicated doses (10Gy). All cells were further cultured for 48h. Cells were digested by 0.25% trypsin without EDTA (Thermo Fisher Scientific, CA, USA) and then were stained using an Annexin V-PE/7-AAD apoptosis kit (Nanjing KeyGen BioTech, Nanjing, P.R.China) according to the manufacturer's instructions. Stained cells were detected and analyzed using flow cytometry (Becton Dickinson, NJ, USA). Each experiment was performed in triplicate.

Real-time PCR array analysis of gene expression. Samples from each group were submerged in 2 ml Trizol (Invitrogen, Carlsbad, CA, USA) for RNA extraction and stored at −80°C until further processed. cDNA synthesis was performed on 4 μg RNA in a 10 μl sample volume using SuperScript II reverse transcriptase (Invitrogen) as recommended by the manufacturer. Real-time PCR array analysis was performed in a total volume of 20 μl including 1 μl cDNA, 10 μl qPCR Master Mix 2x (Roche, Mannheim, Germany) and 9 μl ddH_2O. Reactions were run on an Lightcycler 480 (Roche) using universal thermal cycling parameters (95°C for 5 min, 40 cycles of 15 sec at 95°C, 15 sec at 60°C and 20 sec at 72°C; followed by a melting curve: 5 sec at 95°C, 60 sec at 60°C and continued melting). Data were obtained using the Lightcycler 480 sequence detection software and analyzed using Microsoft Excel. For quality control purposes, melting curves were acquired for all samples. The comparative Ct method was used to quantify gene expression. The target gene expression level was normalized to expression of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) within the same sample (−ΔCt). The relative expression of each gene was calculated using Log2 (−ΔCt).

Western blotting. The differential gene expression of CCNH, LIG4, XRCC5 and PNKP in the various groups was determined by Western blotting. Cells were lysed in 100 μl RIPA lysis buffer (50 mmol/l Tris-HCl, pH 7.5, 1% NP-40, 150 mmol/l NaCl, 1 mg/ml aprotinin, 1 mg/ml leupeptin, 1 mmol/l Na_3VO_4, 1 mmol/l NaF) at 4°C for 30 min. Cell debris was removed by centrifugation at $12,000 × g$ for 20 min at 4°C. Protein concentrations were determined with the Bradford assay (Bio-Red, Hercules, CA, USA). An equal amount of lysate (40 μg) was resolved by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). The membranes were blocked with 5% non-fat milk at room temperature for 1 h and then incubated for 2 h with primary antibodies to CCNH, LIG4, XRCC or PNKP (Cell Signaling Technology, Boston, MA, USA). The membranes were then incubated for 1 h with an appropriate horseradish peroxidase-linked secondary antibody (Santa Cruz Biotechnology, Dallas, TX, USA). Electro-chemi-luminescence was performed according to the manufacturer's instructions using a ChemiDoc™ Touch Imaging System (Bio-Rad, Hercules, California, USA). Quantity One software (Bio-Rad, Hercules, CA, USA) was used to quantify band density.

Animal care. Twenty-eight [28] BALB/C female nude mice aged 4-6 weeks weighing 20-25g, were purchased from Shanghai SLAC Laboratory Animal Co., Ltd., Shanghai, P.R.China (SCXK, Hu. 2007-0005). All mice were maintained in a HEPA-filtered environment at 24-25°C and humidity was maintained at 50-60%. All animals were fed with autoclaved laboratory rodent diet. Animal experiments were approved by the Animal Committee of Nanjing Origin Biosciences, P.R. China (OB1431).

In vivo anti-tumor efficacy. A mouse model of human colon cancer [14] was established with the HT-29 cancer cell line. Stocks of HT-29 tumors were established by subcutaneously injecting $5×10^6$ HT29 cells in the flank of nude mice. The resulting tumors were harvested at the exponential growth phase and resected under aseptic conditions. Necrotic tissues were removed and viable tissues were cut with scissors and minced into $1-mm^3$ pieces. Animals were anesthetized by injection of 0.02 ml of solution of 50% ketamine, 38% xylazine, and 12% acepromazine maleate. Two tumor fragments were transplanted to the flank of nude mice. All surgical procedures and animal manipulations were conducted under HEPA-filtered laminar-flow hoods.

Treatments were initiated when the average tumor size reached $100 mm^3$. The mice with tumors were randomly divided into four groups of seven. Group 1 (Control) received intraperitoneal saline injection only; Group 2 (curcumin); Group 3 received irradiation; Group 4 (irradiation and curcumin) received intraperitoneal curcumin treatment at 20 mg/kg/dose. Six hours after saline or curcumin treatment, Groups 3 and 4 received irradiation (10 Gy). For irradiation, animals were anesthetized and immobilized in the treatment position with their right legs extended. Radiation was delivered at a dose rate of 2.0 Gy/min through a single posterior to anterior collimated 3-cm cobalt beam with a 5-mm bolus placed over the tumor. Tumor growth was measured twice a week with calipers. Tumor volume was calculated using the formula $(L × W^2) × ½$, where W and L represent the perpendicular minor dimension and
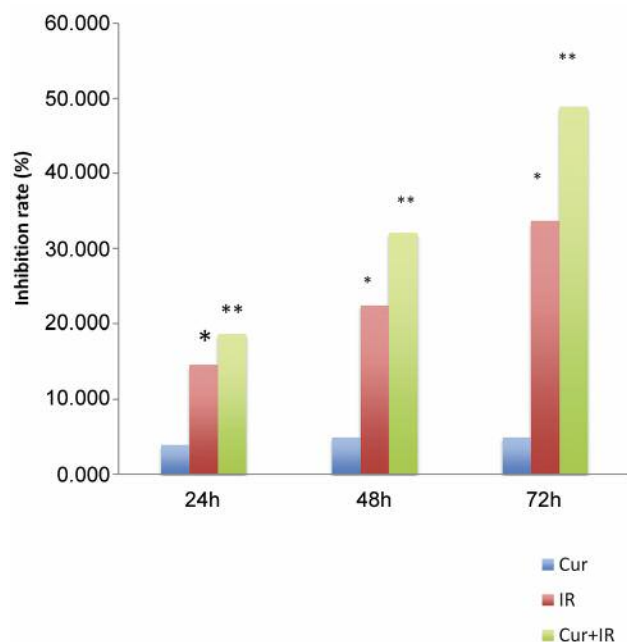
Figure 1. *Curcumin sensitizes colon cancer cells to radiation (IR). Cell proliferation was measured using the MTT assay as described in the Materials and Methods. IR had significantly greater proliferation inhibition on HT-29 cells than curcumin at each time point. IR-induced proliferation inhibition was significantly increased by combination curcumin treatment compared with IR-alone at each time point. *p<0.01, when compared with curcumin. **p<0.01, when compared with IR. Cur: Curcumin; IR: irradiation.*
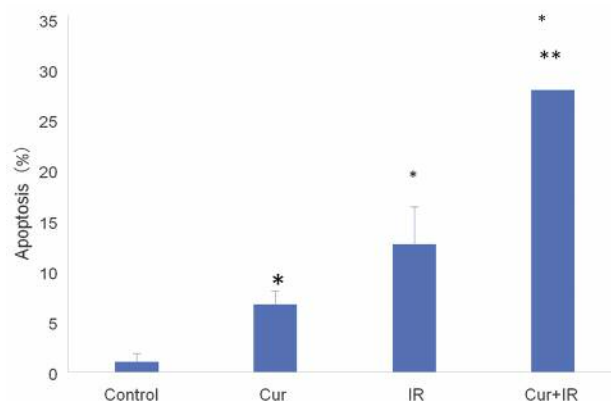

Figure 2. *Curcumin potentiates IR-induced cancer-cell apoptosis. Apoptotic cells were detected with Annexin V-PE/7-AAD as described in the Materials and Methods. All treatments increased cancer-cell apoptosis compared with the control. IR-induced cancer-cell apoptosis was significantly increased by curcumin as compared with IR-alone. *p<0.01, when compared with IR-alone. **p<0.01, when compared with IR-alone. Cur: Curcumin; IR: irradiation.*

major dimension, respectively. Animal body weights and clinical signs were recorded over the course of the experiments. All animals were sacrificed 15 days after treatment initiation. At autopsy the tumor was removed and weighed. The tumor tissue was formalin-fixed and paraffin-embedded for further analysis.

TUNEL detection of apoptosis. Apoptosis of the tumor cells following *in vivo* treatment was determined by TUNEL staining, using a commercially-available kit (In Situ Cell Death Detection Kit, POD; Roche). Tumor sections were deparaffinized and dehydrated according to standard protocols. Tissue sections were incubated with Proteinase K working solution for 30 min at 21-37˚C. The slides were then washed with PBS (pH 7.2-7.6) twice. The positive control was incubated with DNase I for 10 min at 15-25˚C. The negative control was incubated with label solution (without terminal transferase) instead of the TUNEL reaction mixture. The slides were then washed with PBS (pH 7.2-7.6) three times. Converter-POD was added on the slides which were then incubated in a humidified chamber for 30 min at 37˚C. The slides were then washed with PBS (pH 7.2-7.6) three times. DAB substrate was added on the slides which were then incubated for 10 min at 15-25˚C. The slides were then washed with PBS (pH 7.2-7.6) three times. The slides were mounted and analyzed by microscopy (Olympus, Melville, NY, USA). The apoptotic index (AI) was calculated by counting the number of TUNEL+ nuclei visible with a high-power-field 40x

objective in at least 3 fields/sample. The results were expressed as the percentage of apoptotic cells with respect to the total number of cells in each field. Apoptotic cells were identified by the appearance of brown- or tan-stained nuclei.

Statistical analysis. Data are expressed as means ± SD, and were analyzed by one-way analysis of variance (ANOVA), using SPSS software version 19.0, where *p*<0.05 was considered to be statistically significant.

Results and Discussion

Curcumin sensitizes HT-29 cells to radiation. As shown in Figure 1, irradiation (IR) induced significantly greater proliferation inhibition of HT-29 cells than curcumin treatment at each time point (*p*<0.01). However, IR-induced proliferation inhibition was significantly increased by combination curcumin treatment (*p*<0.01).

Curcumin potentiates IR-induced cancer-cell apoptosis. As shown in Figure 2, IR induced significant cancer-cell apoptosis (13.11%) compared with curcumin (6.91%) (*p*<0.01). IR-induced cell apoptosis was significantly increased by curcumin (27.92%) compared with IR-alone (*p*<0.01).

Curcumin sensitizes cancer cells to IR by altering the expression of DNA-repair-related genes. PCR was performed to compare differential-expression profiling of 95 DNA repair-related genes between the cells treated with IR-alone and the cells treated with both IR and curcumin. Four DNA repair-related genes had significant differential expression, including *CCNH, LIG4, XRCC5* and *PNKP* (Figure 3A). CCNH and
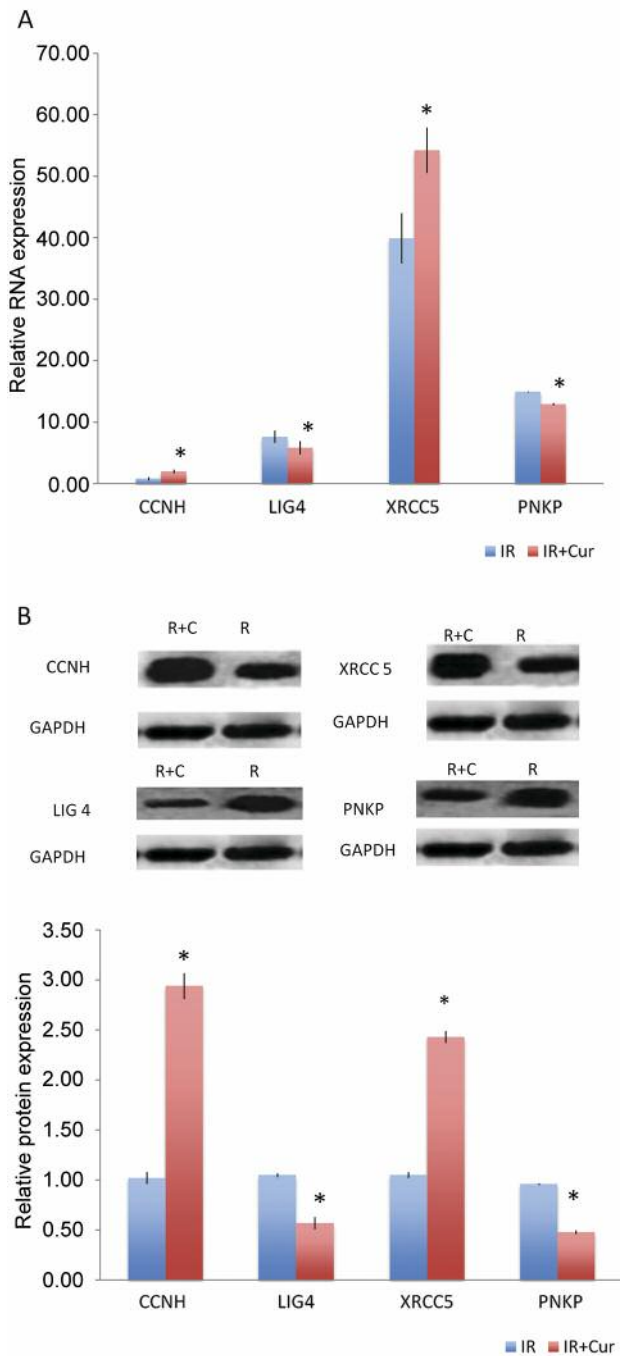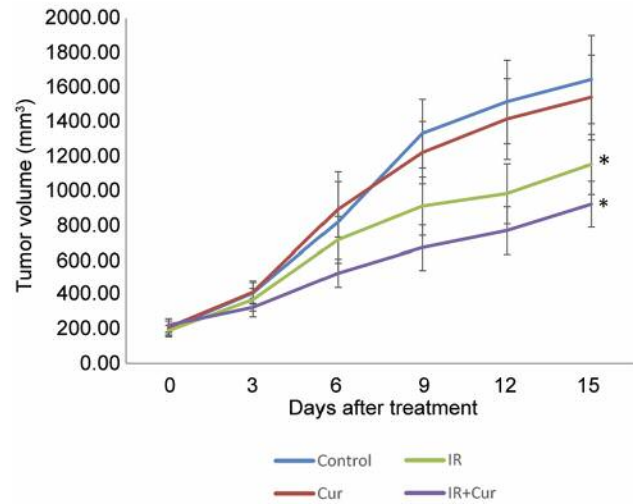
A

B

Figure 4. *Curcumin sensitizes colorectal cancer to IR in vivo. The effect of curcumin on tumor radio-sensitivity was assessed on a human colon cancer HT-29 xenograft tumor as described in the Materials and Methods. Tumor growth was significantly reduced by IR and by IR combined with curcumin compared to the control group. IR plus curcumin resulted in significantly greater tumor-growth inhibition compared to IR alone. *p<0.01, when compared with the control. **p<0.01, when compared with IR. Cur: Curcurmin; IR: irradiation.*

XRCC5 mRNA expression was up-regulated 2.49- and 2.15-fold, respectively, in the cells treated with both IR and curcumin compared to the cells treated only with IR (*p*<0.05). LIG4 and PNKP mRNA expression was down-regulated 2.63- and 1.70-fold, respectively in the cells treated with both IR and curcumin compared to cells treated with IR-only (*p*<0.05).

The differential expression of four DNA repair-related genes was confirmed by Western blot analysis of protein expression. As shown in Figure 3B, the IR and curcumin-treated group had higher protein expression of CCNH and XRCC5 and lower protein expression of LIG4 and PNKP, compared to the IR-alone treated group.

Curcumin sensitized the HT29 colorectal tumor to IR in vivo. To determine whether curcumin can sensitize colorectal cancer to IR *in vivo*, we examined the effect of curcumin alone, IR alone or the combination of curcumin and IR on the growth of subcutaneous HT-29 tumors in nude mice. As shown in Figure 4, the curcumin group did not show significant tumor growth inhibition compared to the control group (p>0.05). However, tumor growth was significantly reduced by IR and by the combination of IR and curcumin compared to the control group starting from day 9 after treatment initiation (both *p*<0.01). Combined treatment with IR and curcumin resulted in significantly greater tumor growth inhibition compared to IR alone (*p*<0.01).

Figure 3. *Curcumin sensitizes cancer cells to IR by altering the expression of DNA repair-related genes. (A) PCR was performed to determine differential-expression profiling of 95 DNA repair genes between the IR and IR plus curcumin groups as described in the Materials and Methods. CCNH and XRCC5 mRNA expression was upregulated by IR + curcumin compared to IR-alone. LIG4 and PNKP mRNA expression was down-regulated by the combination of IR and curcumin compared to IR-alone. *p<0.05, when compared with IR. (B) The differential protein expression of four DNA-repair-related genes was confirmed by Western blotting as described in the Materials and Methods. *p<0.05, when compared with IR. Cur: Curcumin; IR: irradiation.*
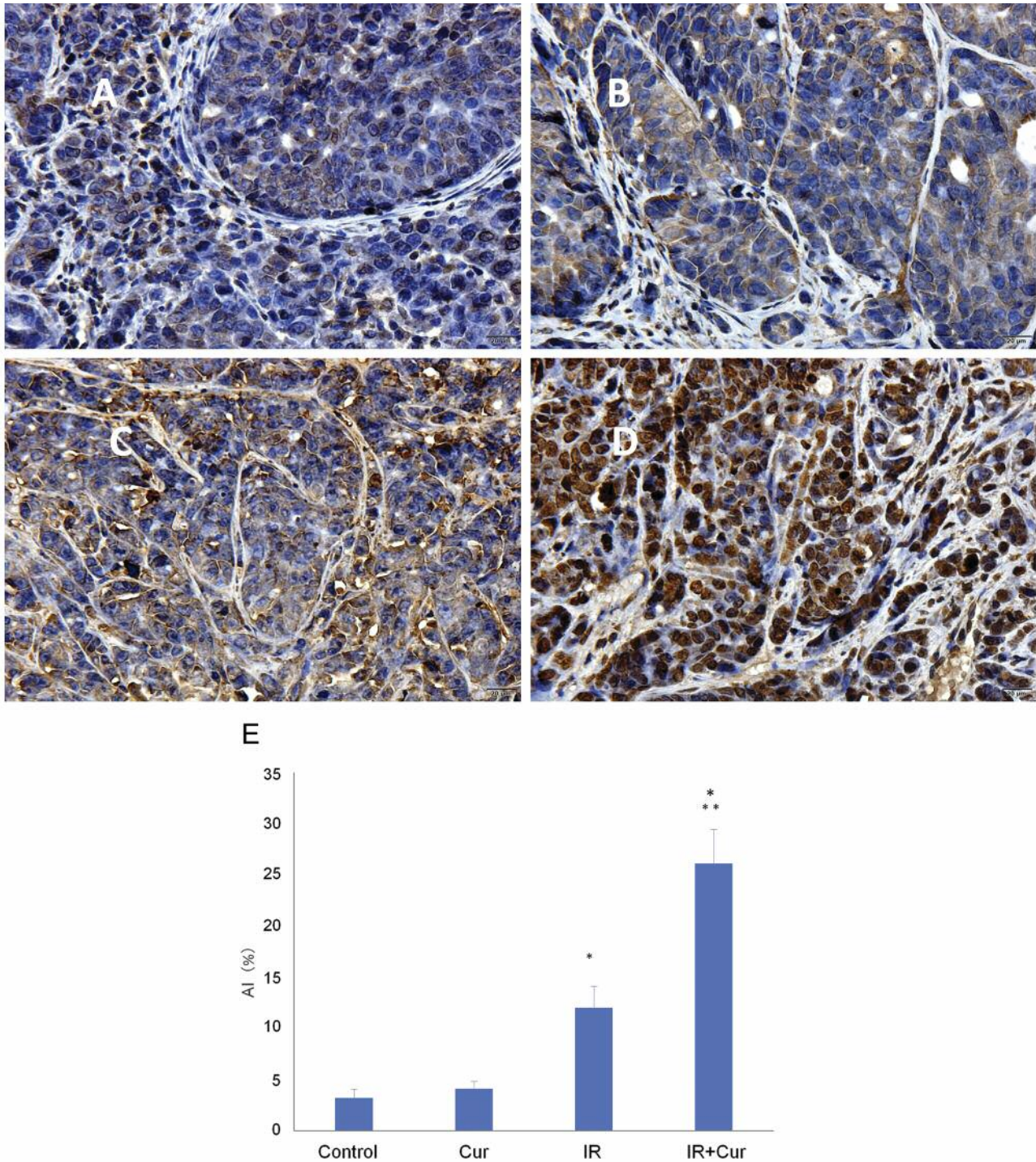
Figure 5. *Curcumin increased IR-induced tumor apoptosis in vivo. Apoptosis was detected by TUNEL staining as described in the Materials and Methods. (A-D) Representative TUNEL images for cell apoptosis in each group (100× magnification). A: Control; B: Curcumin; C: IR; D: IR plus curcumin. (E) Quantitation of cell apoptosis with the apoptosis index (AI). *p<0.01, compared with the control. **p<0.01, compared with IR-alone.*

Curcumin increased IR-induced tumor apoptosis in vivo. To investigate the effect of curcumin on IR-induced tumor apoptosis *in vivo*, we analyzed the apoptosis of the tumor tissues by TUNEL staining. Representative fields of view from each group are shown in Figure 5A-D. The curcumin group did not show significant tumor apoptosis compared to

the control group (p>0.05). However, combination treatment of IR and curcumin induced greater tumor apoptosis than IR treatment alone ($p<0.01$) (Figure 5E).

The *LIG4* gene encodes ATP-dependent DNA ligase IV which is involved in the repair of DNA double-strand breaks (DSBs). LIG4 somatic-knockout cell lines display extreme radiation sensitivity (15, 16). Polynucleotide-kinase phosphatase (PNKP) is a DNA repair enzyme which plays a critical role in radiation-induced DNA-end repair (17). X-ray repair cross-complementing 5 (XRCC5), encoding the Ku80 protein, is an important functional factor in the *NHEJ* repair pathway (18). If the *NHEJ* repair gene is defective, the cells have increased their radiation sensitivity (19). CCNH is mainly involved in the regulation of cyclin-dependent protein kinases and is also involved with RNA polymerase II (20). Our results indicated that curcumin enhanced the radio-sensitivity of human colon cancer cells *via* down-regulation of LIG4 and PNKP expression and up-regulation of XRCC5 and CCNH expression (Figure 3).

The present study demonstrates the potential of a traditional Chinese medicine (curcumin) as an effective radio-sensitizer for human colon cancer.

Conflicts of Interest

None of the Authors have a conflict of interest in regard to this study.

Acknowledgements

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