Isoegomaketone improves radiotherapy efficacy and intestinal injury by regulating apoptosis, autophagy and PI3K/AKT/mTOR signaling in a colon cancer model

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Abstract. The current study aimed to investigate the effect of isoegomaketone (IK) as a radiosensitizer for colon cancer and its effect on intestinal injury, and to verify its potential mechanism. A total of 40 BALB/c nude mice were selected to construct a HT-29 tumor-bearing mice model with T lymphocyte deficiency. Tumor size was measured every other day, and the survival of mice was counted. Intestinal and tumor tissues of mice were harvested when the experiment ended. The levels of inflammatory factors and markers of oxidative stress in intestinal tissues of different groups of mice were analyzed by ELISA. Western blotting was used to examine the expression of apoptosis- and autophagy-related proteins, and the phosphorylation levels of the PI3K/AKT/mTOR signaling pathway in HT-29 cells and tumor tissues. Radiotherapy (RT) combined with IK significantly reduced the viability of HT-29 cells. The optimal dose proportion of RT combined with IK was 8 Gy and 100 μg/ml, and the combination index was <1, suggesting a strong combination effect. In addition, IK could further promote radiation DNA damage in HT-29 cells by inhibiting the PI3K/AKT/hypoxia inducible factor 1α (HIF-1α) signaling pathway, while upregulating the expression of proapoptotic and autophagy-related proteins in HT-29 cells. In HT-29 tumor-bearing mice, RT in combination with IK significantly inhibited the growth of xenografts and improved mouse survival. In addition, the combination of RT and IK significantly upregulated BAX and Beclin-1 expression, downregulated BCL-2 expression, and promoted the conversion of LC3 I to LC3 II. Radiation induced an increase in inflammatory cytokine levels as well as oxidative stress marker levels in intestinal tissue. Western blot analysis showed that the combination of RT and IK significantly inhibited the phosphorylation level of the PI3K/AKT/mTOR signaling pathway compared with the control and monotherapy groups. IK could significantly enhance the efficacy of RT by regulating the apoptosis and autophagy of colon cancer tumors, and alleviate inflammation and oxidative stress by regulating the PI3K/AKT/mTOR signaling pathway to alleviate intestinal injury. The present findings suggest that IK can be used as a promising sensitizer and has the potential to enhance the efficacy and safety of RT for colon cancer.

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Introduction

In recent years, the incidence and mortality of colon cancer have risen (1). Colon cancer accounts for 10.0 and 9.4% of all cancer types, ranking third and second, respectively, as the leading cause of cancer-related mortality worldwide (2). By 2030, the burden of colon cancer is expected to increase by 60% to >2.2 million new cases and 1.1 million mortalities (3). The occurrence and development of colon cancer are closely related to the tumor microenvironment, including inflammatory response and heredity, and are associated with risk factors such as population aging, dietary habits, obesity, lack of physical exercise and smoking (4). Among colon cancer cases, 20-30% are familial inherited due to genetic factors, and ~70% are affected by environmental factors (5). With the intervention of early screening methods such as colonoscopy, the overall incidence of colon cancer has shown a slow decreasing trend, but the age at diagnosis tends to be younger (5). In addition, the early symptoms of colon cancer are more insidious, and the majority of patients are diagnosed in middle and advanced stages (6).

At present, the combined treatment of surgery and chemoradiotherapy is the standard modality for patients with advanced rectal cancer (7). Surgical resection of the lesion is the preferred treatment modality (8). However, there is a high incidence of local recurrence and distant metastasis after surgical treatment alone (8). Systematic radiotherapy (RT) and chemotherapy are essential to relieve the symptoms and prolong

the life of most patients, particularly those with metastatic symptoms of cancer cells (9). Current first-line chemotherapy regimens for colon cancer include combination of fluorouracil, leucovorin and oxaliplatin or fluorouracil, irinotecan and bevacizumab (10). However, the unavoidable side effects of chemotherapeutic drugs (for example, diarrhea, nausea, vomiting, acute myocardial infarction and cerebrovascular injury) greatly reduce the quality of life of patients, and are serious and even potentially life-threatening (11). Preoperative RT can effectively eliminate subclinical lesions around the tumor, reduce tumor volume, reduce tumor stage, and increase the possibility of anus preservation for patients (12). In addition, preoperative RT can reduce local recurrence and increase patient survival opportunities (13). However, tolerance to radiation therapy for colon cancer as well as the side effects of RT remain the biggest problems affecting clinicians (13). Therefore, it is important to screen and search for natural products with low toxicity and high antitumor activity.

In recent years, the status of traditional Chinese medicine has gradually increased in the study of radiosensitizers (14). The antitumor effect of isoegomaketone (IK) (PubChem Compound ID: 5318556) in the perilla extract is particularly prominent (15). It has been shown that IK can promote apoptosis and inhibit cell proliferation, and exert radio-sensitizing effects on lung cancer cells by regulating the expression of endoplasmic reticulum stress proteins (15). In addition, IK has biological functions such as anti-inflammatory, antifungal, healing-promoting activities and resistance to toxicity induced by immunotherapy (16). However, the RT sensitizing effect of IK on colon cancer and whether IK can alleviate the side effects of RT on intestinal injury have not been reported to date. Therefore, the present study intended to investigate the RT sensitizing effect of IK in colon cancer and its effect on reducing intestinal side effects and related potential mechanisms by using the combination of RT and IK in *in vitro* and *in vivo* experiments.

Materials and methods

Materials and reagents. HT-29 cells (cat. no. HTB-38) and CCD-18Co cells (cat. no. CRL-1459) were purchased from the American Type Culture Collection. IK was isolated from *Perilla frutescens* Britt by the supercritical carbon dioxide (SC-CO_2) method (17). DMEM (cat. no. SH30285.02), fetal bovine serum (FBS; cat. no. SH30088.03IR25-40), trypsin (cat. no. SH30042.02) and penicillin-streptomycin (cat. no. SV30010) were purchased from HyClone (Cytiva). Cell Counting Kit-8 (CCK-8) assay kit (cat. no. PA137267) was purchased from Pierce (Thermo Fisher Scientific, Inc.). Specific antibodies against HIF-1α (1:1,000; cat. no. 36169), PI3K (1:1,000; cat. no. 4292), phosphorylated (p)-PI3K (1:1,000; cat. no. 4228), AKT (cat. no. 9272), p-AKT (1:500; cat. no. 9271), mTOR (1:500; cat. no. 2972), p-mTOR (1:1,000; cat. no. 2971), BCL-2 (1:500; cat. no. 15071), BAX (1:1,000; cat. no. 2772), LC3 I/II (1:1,000; cat. no. 4108S), Beclin-1 (1:1,000; cat. no. 3738) and β-actin (1:1,000; cat. no. 4967) were purchased from Cell Signaling Technology, Inc. Specific antibodies against γH2AX (1:1,000; cat. no. ab81299) were purchased from Abcam. A total of 40 male BALB/c nude mice (age, 4 weeks-old; weight, 18~22 g) were purchased from Beijing HFK Bioscience Co. Ltd. X-ray irradiator (X-ray RAD 320; Precision X-ray Inc.). All mice were housed in an SPF environment with free access to food or water at a temperature of 21-25˚C and humidity of 60-70%, ensuring 12 h of alternating light.

Cell culture and assays. HT-29 and CCD-18Co cells were cultured in DMEM containing 10% FBS, 100 U/ml penicillin and 100 U/ml streptomycin, and placed in an incubator at 37˚C with 5% CO_2 and saturated humidity. After 48 h of incubation, cells were rinsed twice with PBS, and 1 ml trypsin was added for digestion at 37˚C for 5-6 min. After complete digestion, 2 ml fresh DMEM was added to terminate the reaction, and a cell suspension was prepared, which was pipetted into a 10-ml Eppendorf tube and centrifuged at 1,000 x g for 3 min at room temperature. Next, fresh DMEM was added in a 1:3 ratio, and the mixture was transferred to a flask and incubated in a 37˚C in a 5% CO_2 incubator. HT-29 and CCD-18Co cells were incubated with different concentrations of IK (0, 50, 100 or 200 μg/ml) for 48 h at ≤65% cell confluence. At the same time, all grouped cells were irradiated with different doses (0, 1, 2, 4, 6, 8, 12 or 16 Gy) of X-rays (X-ray RAD 320 irradiator; Precision X-ray, Inc.). Cells from each group were seeded in 96-well plates at a concentration of $6x10^3$ cells/well. A total of 10 μl CCK-8 solution was added to each well and incubated at 37˚C for 4 h. Finally, the optical density (OD) 450 nm values were determined by using a plate reader. Cell viability was calculated by average cell viability of control wells. DNA damage was detected by single cell gel electrophoresis at 0, 15, 30, 60 and 120 min after X-ray irradiation. Olive tail moment (OTM) was analyzed with Comet assay software project (http://casplab.com/).

Animal experiments. BALB/c nude mice were fed normally for 7 days after acquisition. A mouse tumor model was established by injecting cancer cells into mice. Briefly, log-proliferating human colon cancer HT-29 cells were washed with PBS and digested with trypsin for 4-6 min, and the digestion was terminated by adding fresh DMEM. After centrifugation, cell suspensions were obtained after two resuspensions in serum-free DMEM. The cell concentration in the mixture was adjusted to $2.5x10^7$ cells/ml. A total of 40 healthy BALB/c-nu nude mice were inoculated subcutaneously with 150 μl ($3.75x10^6$ cells) cell suspension on the dorsal side of the right axilla to obtain a HT-29 tumor-bearing nude mouse model. All HT-29 tumor-bearing nude mice were randomly divided into i) control group, ii) RT, iii) IK and iv) RT + IK groups (n=10). Mice in both the RT and combination groups were treated with 8 Gy doses of X-ray radiation once a day for 14 consecutive days, while mice in the IK and combination groups were intraperitoneally injected with 100 mg/kg IK daily for 14 consecutive days. Placebo was administered during RT to the control group. Tumor size was measured every other day and the survival of mice was counted. Mice were euthanized when the tumor size exceeded 3,000 mm^3. All surviving mice were anesthetized 24 h after the last administration. The anesthetic agent used was sodium pentobarbital, prepared as a 3% sterile saline solution at a dose of 30 mg/kg body weight. Sodium pentobarbital was administered via intraperitoneal injection, and the animals' responses were continuously observed. Once

the animals were fully anesthetized, they were euthanized by cervical dislocation.

The procedures for sampling tumor and intestinal tissue were as follows: Using surgical instruments, the skin over the tumor site of the mouse was carefully cut and exposed. Next, the tumor was carefully separated from the surrounding tissues and the intact tumor tissue was placed in a sterile container for subsequent experimental analysis or preservation. For intestinal tissues, upon disinfecting the skin with alcohol, the abdominal skin and muscle layers were cut open to expose the abdominal cavity. After locating the intestine, the desired intestinal segment was excised. All operations were performed under sterile conditions to ensure that the samples were not contaminated. The obtained tumor and intestinal tissues were washed with normal saline to remove residual blood, dried with absorbent paper and stored at -80˚C for later use. Intestinal tissues were fixed in a 4% paraformaldehyde solution, then embedded in paraffin and cut into 5-μm thick sections. The sections were sequentially stained with hematoxylin for 5 min and eosin for 2 min at room temperature. Intestinal images were obtained using a light microscope. Peripheral blood was collected from mice and analyzed for peripheral blood leukocyte, neutrophil and monocyte numbers using a LH-750 hematology analyzer (Beckman Coulter, Inc.).

The present animal studies complied with all relevant national regulations and institutional policies, and were approved [approval no. 2023 (30)] by the Animal Experimental Ethics Committee of the First Affiliated Hospital, Zhejiang University School of Medicine (Hangzhou, China).

ELISA. The levels of malondialdehyde (MDA) (cat. no. ab238537; Abcam), TNF-α (cat. no. ab181421; Abcam), NF-κB (cat. no. ab279874; Abcam) and IL-1β (cat. no. BMS6002-2TEN; Invitrogen; Thermo Fisher Scientific, Inc.), and the activities of glutathione (GSH) (cat. no. EEL155; Invitrogen; Thermo Fisher Scientific, Inc.) and catalase (CAT) (cat. no. KTB9040; Abbkine Scientific Co., Ltd.) were measured by ELISA. Briefly, intestinal tissue frozen at -80˚C was mixed with 1 ml tissue lysis buffer, ground to homogenate in a glass mill in an ice bath, lysed at 4˚C for 30 min and centrifuged at 3,000 x g for 20 min at 4˚C. The levels of MDA, TNF-α, NF-κB and IL-1β in tissue homogenate, and the activities of GSH and CAT were measured using commercial ELISA kits.

Western blotting. Western blotting was used to analyze protein expression levels in different cells and tissues. Briefly, tumor and intestinal tissues frozen at -80˚C were mixed with 1 ml tissue NP-40 lysis buffer (cat. no. P0013F; Beyotime Institute of Biotechnology), ground to homogenate in a glass mill in an ice bath, lysed at 4˚C for 30 min and centrifuged at 3,000 x g for 20 min at 4˚C. A total of 10 μl HT-29 cell homogenates, tumor tissue homogenates or intestinal tissue homogenates were isolated by 10% SDS-PAGE and transferred to nitrocellulose membranes, which were then blocked with 5% bovine serum albumin (cat. no. ST2249-5g; Beyotime Institute of Biotechnology) for 1 h at room temperature and washed with TBS-Tween 20 (TBST; 20 mM Tris buffer, 0.1% Tween 20, pH 7.4) for 5 min. Subsequently, the membranes were incubated at 4˚C with a primary antibody (1:500) overnight and then with a horseradish peroxidase-labeled secondary antibody (1:1,000; cat. no. 58802; Cell Signaling Technology, Inc.) for 1 h at room temperature. After washing with TBST, the membrane was immersed in an enhanced chemiluminescence solution (SuperSignal™ West Atto; cat. no. A38556; Thermo Fisher Scientific, Inc.). The gray value of the bands was analyzed by ImageJ software (version 1.8.0; National Institutes of Health). The target protein expression level was calculated as the target band gray value divided by the reference material strip gray value.

Statistical analysis. Data were analyzed using SPSS 25.0 statistical software (IBM Corp.), and the data were expressed as the mean ± SD. Inter-group comparisons were performed using one-way ANOVA followed by Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

IK enhances the sensitivity of HT29 cells to X-rays. To investigate whether IK can synergistically promote the inhibitory effect of X-rays on the proliferation of human colorectal cancer cells, the viability of HT-29 cells treated with RT combined with IK was examined by CCK-8 assay. As shown in Fig. 1A, RT at different irradiation doses inhibited the viability of HT-29 cells. In addition, RT in combination with different concentrations of IK further reduced HT-29 cell viability. Notably, for normal human colon fibroblasts (CCD-18Co cells), IK effectively reversed the RT-induced decrease in cell viability (Fig. 1B). The optimal dose ratio of RT in combination with IK was further analyzed (Fig. 1C and D). At different doses of RT and IK, the proliferation inhibition rate of HT-29 cells in the combination group was significantly greater than that in the RT alone or IK alone groups (both $P<0.05$). In addition, the proliferation inhibition of CCD-18Co cells in the combination group was significantly lower than that in the RT alone group (all $P<0.01$). CCD-18Co cell viability was significantly decreased ($P<0.05$) in the 12 Gy/200 μg/ml group compared with the RT/IK dose of 8 Gy/100 μg/ml, but had no significant effect on HT-29 cell viability ($P>0.05$). Therefore, 8 Gy/100 μg/ml was selected as the optimal RT/IK dose ratio for subsequent studies. The combination index (CI) values were further calculated (data not shown), and they were observed to be <1 at each concentration, suggesting that RT combined with IK had a synergistic inhibitory effect on the proliferation of colon cancer cells.

IK enhances DNA damage in HT-29 cells by inhibiting HIF-1α and the PI3K/AKT signaling pathway. After X-ray irradiation, the repair time of DNA damage is usually 4-6 h. Therefore, single cell gel electrophoresis was used in the present study to analyze the initial DNA damage in HT-29 cells at 0, 15, 30, 60 and 120 min after irradiation. As demonstrated in Fig. 2A, OTM in control HT-29 cells remained low from 0 to 120 min. RT alone significantly increased the OTM levels of HT-29 cells at 0, 15, 30, 60 and 120 min compared with the controls ($P<0.01$). Notably, IK alone also slightly increased OTM levels in HT-29 cells at 0 and 15 min (both $P<0.05$). In addition, RT in combination with IK increased the OTM levels

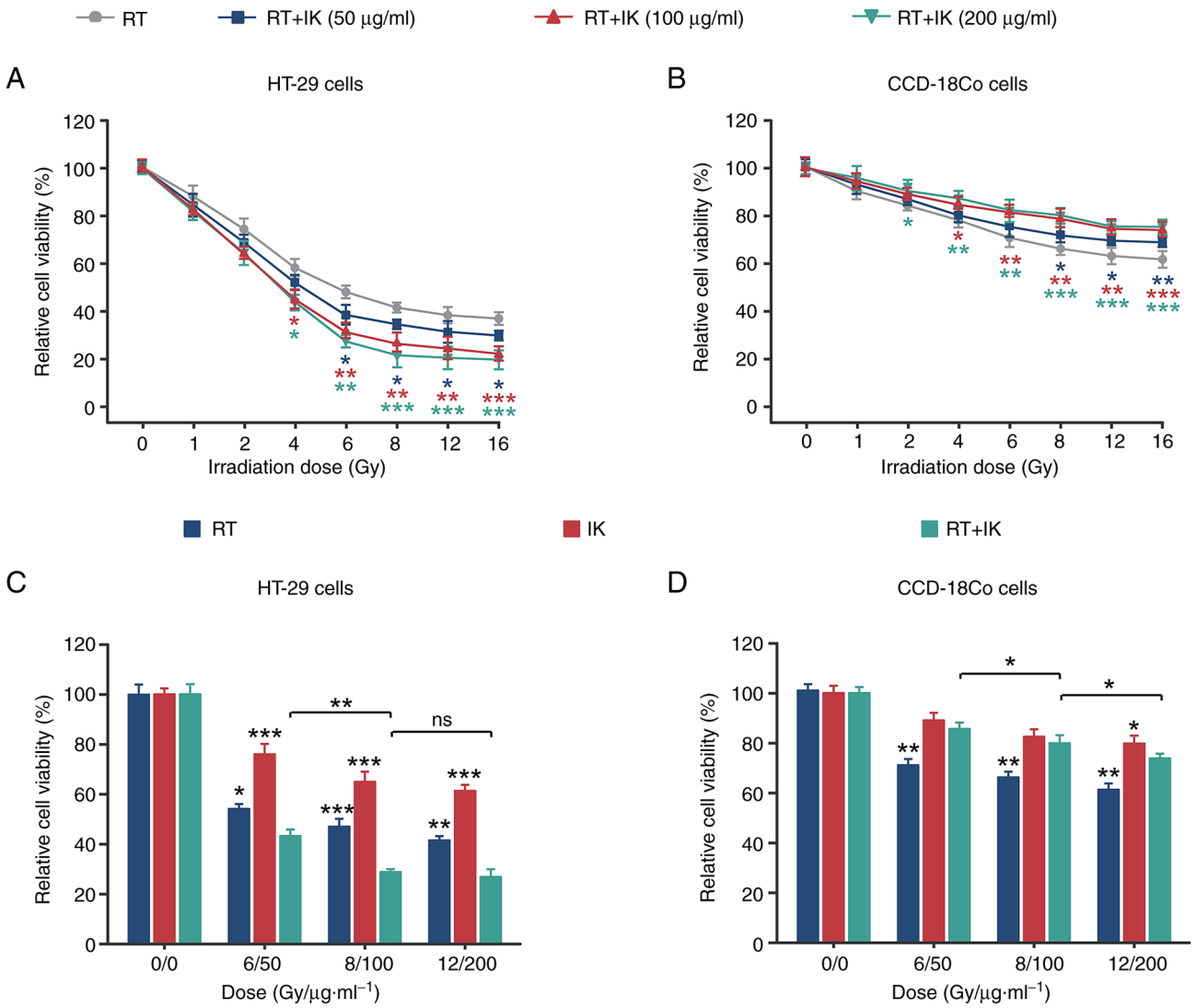

Figure 1. Effect of RT combined with IK on relative cell viability of HT-29 and CCD-18Co cells. (A and B) Cell viability of (A) HT-29 cells and (B) CCD-18Co cells treated with different dose of RT and IK. (C and D) Cell viability of (C) HT-29 cells and (D) CCD-18Co cells treated with different dose ratio of RT combined with IK. Data are presented as the mean ± SD (n=6). *P<0.05 **P<0.01 and ***P<0.001 vs. the RT group in (A) and (B); and vs. the RT + IK group in (C) and (D). RT, radiotherapy; IK, isoegomaketone; ns, not significant.

of HT-29 cells to a greater extent and significantly higher than either treatment alone (all P<0.001).

The expression levels of HIF-1α and the P13K/AKT signaling pathways in HT-29 cells were analyzed by western blotting. As revealed in Fig. 2B-E, RT or IK alone significantly decreased the expression levels of HIF-1α, p-PI3K and p-AKT compared with controls (all P<0.01). In addition, RT in combination with IK further reduced the expression level of HIF-1α and the phosphorylation level of the PI3K/AKT signaling pathway.

IK combined with RT promotes the apoptosis and autophagy of HT-29 cells. The expression of apoptosis and autophagy-related proteins in HT-29 cells was further analyzed by western blotting. As demonstrated in Fig. 3A-C, both RT or IK alone significantly increased the expression of the proapoptotic factor BAX and decreased the expression of the anti-apoptotic factor BCL-2 compared with the control group (all P<0.05). As expected, RT in combination with IK increased BAX but decreased BCL-2 expression, compared with controls (both P<0.05), and the extent of the change was significantly higher than either treatment alone. In addition, RT or IK alone promoted the conversion of LC3 I to LC3 II and increased Beclin-1 expression levels compared with controls (both P<0.05). Notably, the combination treatment group further increased the changes in these levels. These results suggest that RT combined with IK can significantly promote apoptosis and autophagy in HT-29 cells.

Combination treatment with RT effectively inhibits colon cancer growth in a xenograft model. Based on the efficacy of RT in combination with IK in inhibiting HT-29 cell proliferation, the *in vivo* antitumor potential of this combination therapy was further evaluated in HT-29-bearing mice. As shown in Fig. 4A and B, the tumor volume of control mice continued to increase over the 14-day treatment period, but there was no significant change in body weight. The maximum tumor diameter and volume was 16.12 mm and 1,425 mm^3, respectively. Both RT and IK treatments significantly reduced

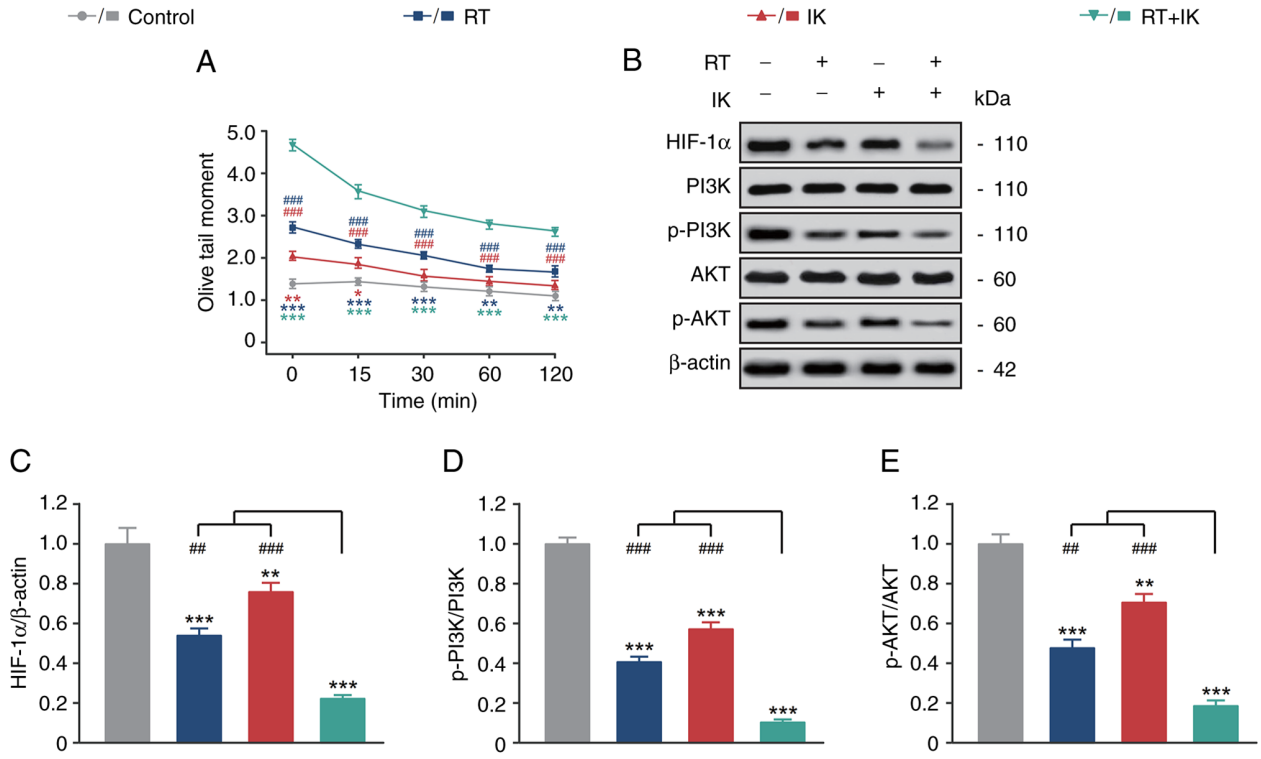

Figure 2. Effect of RT combined with IK on DNA damage of HT-29. (A) Olive tail moment of HT-29 cells treated with RT combined with IK. (B) Western blot images and quantitative analysis of (C) HIF-1α, (D) p-PI3K/PI3K, and (E) p-AKT/AKT of HT-29 cells treated with RT (8 Gy) combined with IK (100 μg/ml). Data are presented as the mean ± SD (n=6). *P<0.05 **P<0.01 and ***P<0.001 vs. the control group; $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. the RT + IK group. RT, radiotherapy; IK, isoegomaketone; p-, phosphorylated.

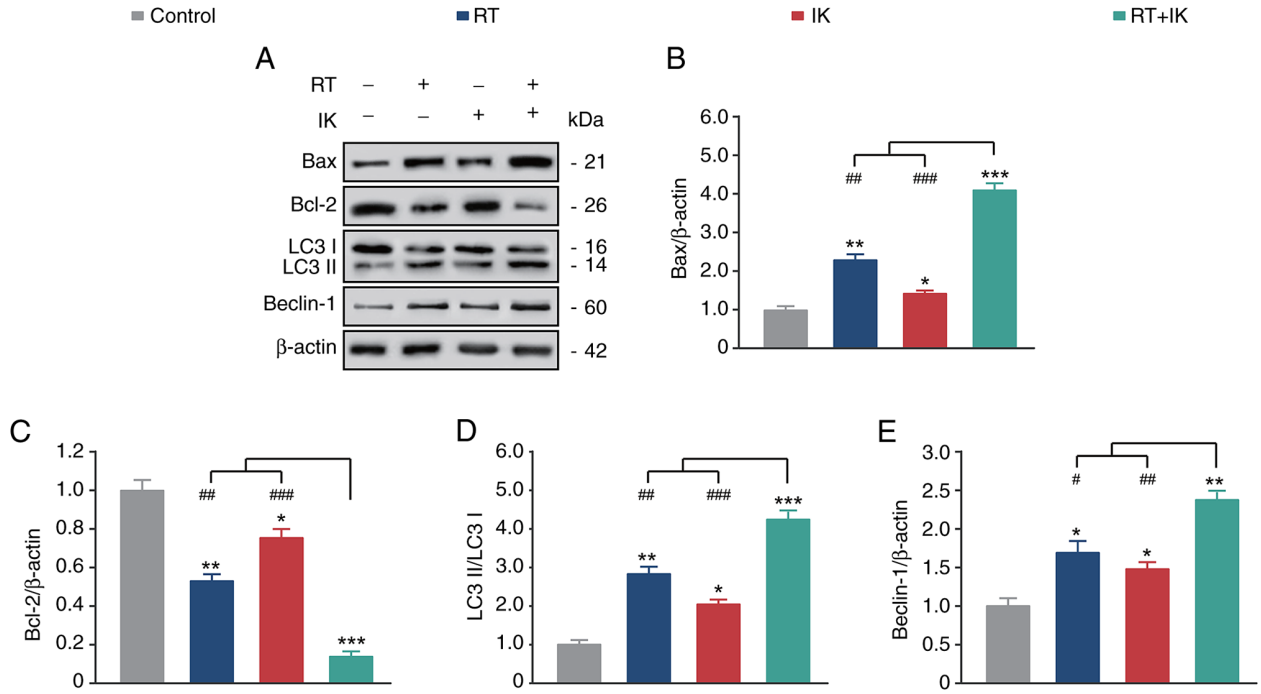

Figure 3. Effect of RT combined with IK on apoptosis and autophagy in HT-29 cells. (A) Western blot images and quantitative analysis of (B) BAX (C) BCL-2, (D) LC3 II/LC3 I and (E) Beclin-1 of HT-29 cells treated with RT (8 Gy) combined with IK (100 μg/ml). Data are shown as the mean ± SD (n=6). *P<0.05, **P<0.01 and ***P<0.001 vs. the control group. $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. the RT + IK group. RT, radiotherapy; IK, isoegomaketone.

tumor size in mice compared with controls (both P<0.001). In addition, the combination of RT and IK significantly reduced tumor size in HT-29-bearing mice compared with controls (P<0.001), and to a significantly greater extent than RT or IK alone. Notably, after 14 consecutive days of RT in combination with IK, the transplanted tumors in HT-29-bearing mice were

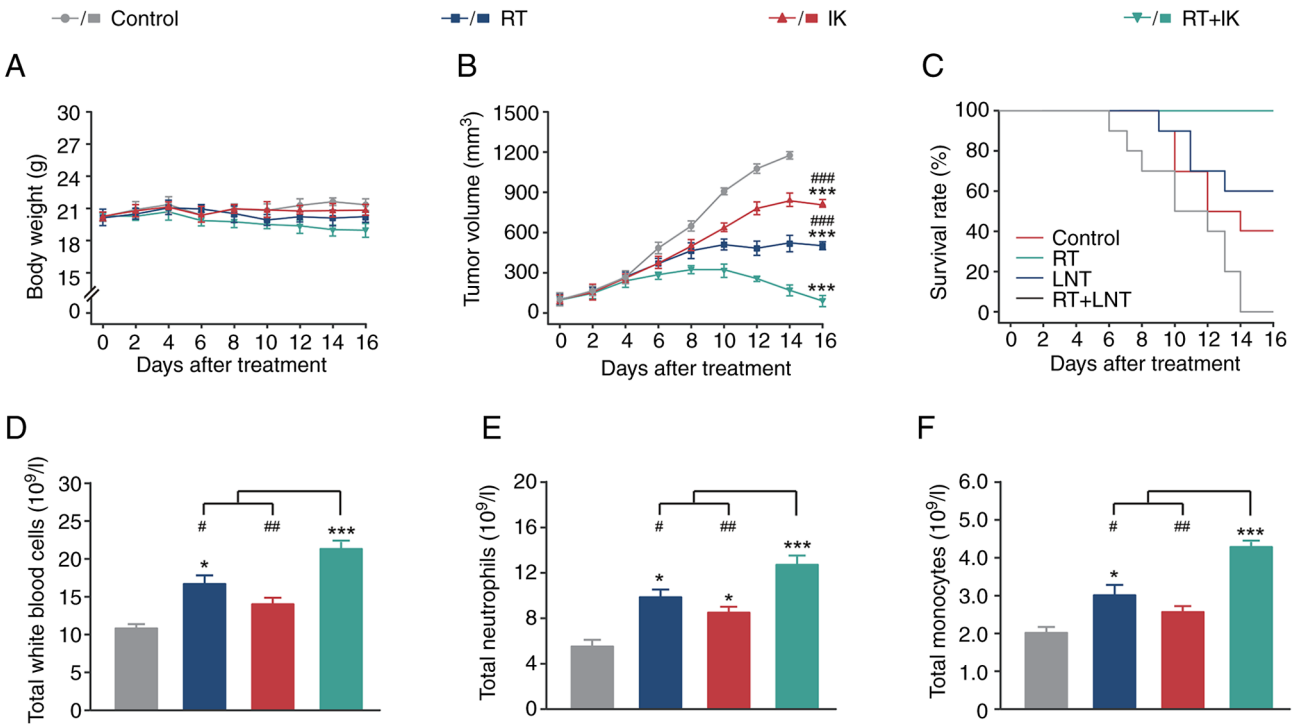

Figure 4. Effect of RT combined with IK on xenograft tumors in HT-29-bearing mice. (A) Body weight, (B) Tumor volume, (C) Survival rate, (D) Total white blood cells, (E) Total neutrophils and (F) Total monocytes of HT-29-bearing mice treated with RT (8 Gy) combined with IK (100 mg/kg). Data are presented as the mean ± SD (n=10). *P<0.05 and ***P<0.001 vs. the control group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. the RT + IK group. RT, radiotherapy; IK, isoegomaketone.

almost completely eliminated, followed by no tumor recurrence. The survival rates of different groups of mice during 14 days of continuous treatment were statistically analyzed (Fig. 4C). Xenograft mice in the control group commenced to succumb to disease 6 days after modeling and all succumbed within 14 days. Treatment with RT or IK alone significantly improved mouse survival compared with controls, but survival was only 40-60%. Of note, xenograft mice in the combination treatment group did not die during 14 consecutive days of treatment (survival rate of 100%), indicating that RT combined with IK treatment is effective in prolonging the survival of HT-29-bearing mice.

Non-specific immunity plays an important role in the treatment of solid tumors. Therefore, the levels of leukocytes, neutrophils and monocytes in the peripheral blood of different groups of HT-29-bearing mice were further analyzed. As revealed in Fig. 4D-F, RT alone significantly increased the levels of white blood cells, neutrophils and monocytes in the peripheral blood of mice compared with controls (all P<0.05). In addition, the levels of neutrophils in mice treated with IK alone were significantly higher than those in the control group (P<0.05), whereas the levels of white blood cells and monocytes were not significantly different (both P>0.05). As expected, the levels of white blood cells, neutrophils and monocytes in peripheral blood were significantly enhanced in the combination group, and were significantly higher than in the monotherapy or control groups.

IK combined with RT enhances the autophagy and apoptosis of tumor cells in a xenograft model. Western blotting was used to study the expression of apoptosis and autophagy-related proteins in tumor tissues of HT-29-bearing mice. As shown in Fig. 5A-C, RT or IK alone significantly upregulated the expression of BAX and downregulated the expression of BCL-2 compared with the control group (all P<0.01). Furthermore, RT in combination with IK further significantly upregulated BAX expression and downregulated BCL-2 expression compared with both treatment groups alone (both P<0.01). On the other hand, both RT and IK alone significantly upregulated LC3 II expression and downregulated LC3 I expression compared with controls, indicating an increased conversion of LC3 I to LC3 II (both P<0.01) (Fig. 5D). In addition, treatment with RT or IK alone also significantly upregulated Beclin-1 expression (both P<0.01) (Fig. 5E). The combination of RT and IK further significantly enhanced the conversion of LC3 I to LC3 II (both P<0.001), and significantly upregulated Beclin-1 expression (both P<0.001) compared with the two treatment groups alone. These results suggest that RT combined with IK can promote tumor cell apoptosis by regulating the expression of apoptosis-related proteins, and ultimately inhibit the xenograft tumor growth.

Effect of IK on intestinal injury induced by RT in mice. Radiation therapy damages normal tissue around the lesion. Oxidative stress and inflammation are the main manifestations of radiation side effects. Therefore, the present study further investigated the protective effect of IK on normal intestinal tissue after RT in mice. As demonstrated in Fig. 6A-C, after RT, MDA levels in normal intestinal tissues of xenograft mice were significantly increased (P<0.001), whereas the activities of GSH and CAT were significantly decreased (both P<0.001), suggesting development of oxidative stress. Notably, IK

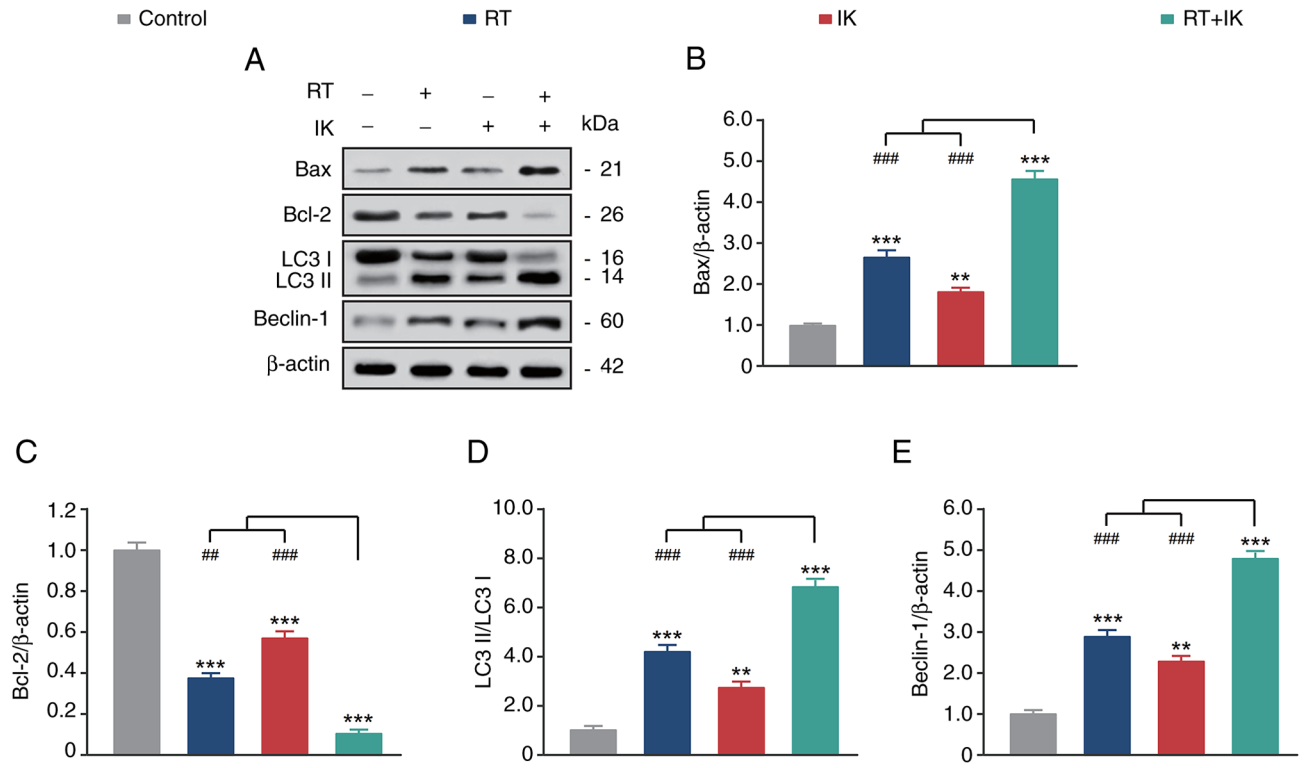

Figure 5. Effect of RT combined with IK on apoptosis and autophagy in tumor tissues of HT-29-bearing mice. (A) Western blot images and quantitative analysis of (B) BAX, (C) BCL-2, (D) LC3 II/LC3 I and (E) Beclin-1 of tumor tissues in HT-29-bearing mice treated with RT (8 Gy) combined with IK (100 mg/kg). Data are shown as the mean ± SD (n=6). **P<0.01 and ***P<0.001 vs. the control group; $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. the RT + IK group. RT, radiotherapy; IK, isoegomaketone.

treatment significantly decreased MDA levels in the intestinal tissue of irradiated mice (P<0.01), and significantly increased the activities of GSH and CAT (both P<0.01). On the other hand, RT treatment significantly increased the levels of inflammatory factors, including TNF-α, NF-κB and IL-1β, compared with controls (all P<0.001) (Fig. 6D-F). In addition, these changes in inflammatory cytokine levels were significantly reversed by IK treatment (all P<0.01). These results suggest that IK significantly ameliorates radiation-induced oxidative stress and inflammation.

IK attenuates radiation-induced intestinal injury in rats by inhibiting the phosphorylation of proteins in the PI3K/AKT/mTOR signaling pathway. The results of H&E staining in normal intestinal tissues of xenograft tumor models are shown in Fig. 7A. Intestinal microvilli were structurally intact in the control and IK groups. By contrast, in the RT group, the tip of some villi in intestinal mucosa was necrotic, the height and width of villi were disorganized, and some villi exhibited shortening and atrophy. In the IK group, only partial villus shortening and structural integrity were observed in intestinal microvilli. In the RT+IK group, histopathological changes were relieved, and the submucosal, muscular and serosal tissue structures were significantly improved.

The PI3K/AKT/mTOR signaling pathway plays an important role in inflammation and oxidative stress. Activation of the PI3K/AKT/mTOR signaling pathway promotes the expression of numerous proinflammatory cytokines as well as the development of oxidative stress. Thus, the expression of proteins of the PI3K/AKT/mTOR signaling pathway in normal intestinal tissues of tumor-bearing mice was further analyzed by western blotting. As revealed in Fig. 7, RT treatment significantly upregulated the expression level of γH2AX and the phosphorylation levels of PI3K, AKT and mTOR compared with controls (all P<0.001). In addition, the expression level of γH2AX and the phosphorylation levels of PI3K, AKT and mTOR in the intestinal tissue of mice treated with IK alone did not change significantly compared with the control group (all P>0.05). As expected, IK treatment significantly reversed the RT-induced phosphorylation of PI3K/AKT/mTOR (all P<0.01). These results suggest that IK can ameliorate radiation injury in the intestinal tissue of xenografted mice by inhibiting the phosphorylation of the PI3K/AKT/mTOR pathway.

Discussion

Numerous previous studies have focused on various radiosensitive markers and sensitizers (18-20). Radiosensitive markers include mutations in the tumor suppressor gene P53, cell proliferation marker Ki-67 and high expression of proliferating cell nuclear antigen (19). Radiosensitizers include chemotherapeutic agents such as 5-fluorouracil and oxaliplatin, or targeted therapeutic agents such as cetuximab (19). However, the reason of RT tolerance of colorectal cancer cells remains unclear, and effective radiosensitive markers and sensitizers need to be further explored. In addition, radiation enteritis is one of the most common potential complications of RT for abdominal tumors and a major issue affecting patient quality of life and treatment outcome (20). Therefore, it is necessary to prevent or reduce radiation damage to the intestine. *Perilla frutescens* is

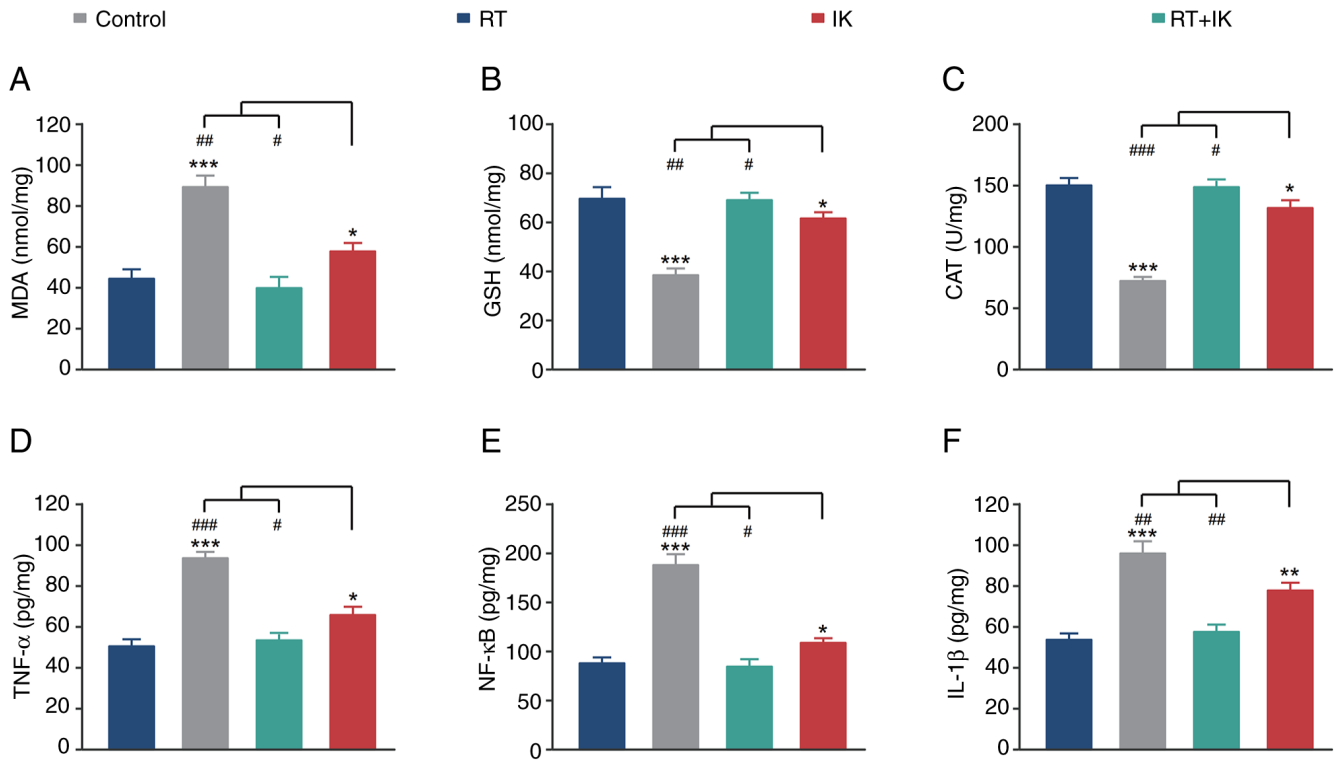

Figure 6. Effect of RT combined with IK on oxidative stress and inflammation in normal intestinal tissues of HT-29-bearing mice. Quantitative analysis of (A) MDA, (B) GSH, (C) CAT, (D) TNF-α, (E) NF-κB and (F) IL-1β of normal intestinal tissues in HT-29-bearing mice treated with RT (8 Gy) combined with IK (100 mg/kg). Data are presented as the mean ± SD (n=10). *P<0.05, **P<0.01 and ***P<0.001 vs. the control group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. the RT + IK group. RT, radiotherapy; IK, isoegomaketone; MDA, malondialdehyde; GSH, glutathione; CAT, catalase.

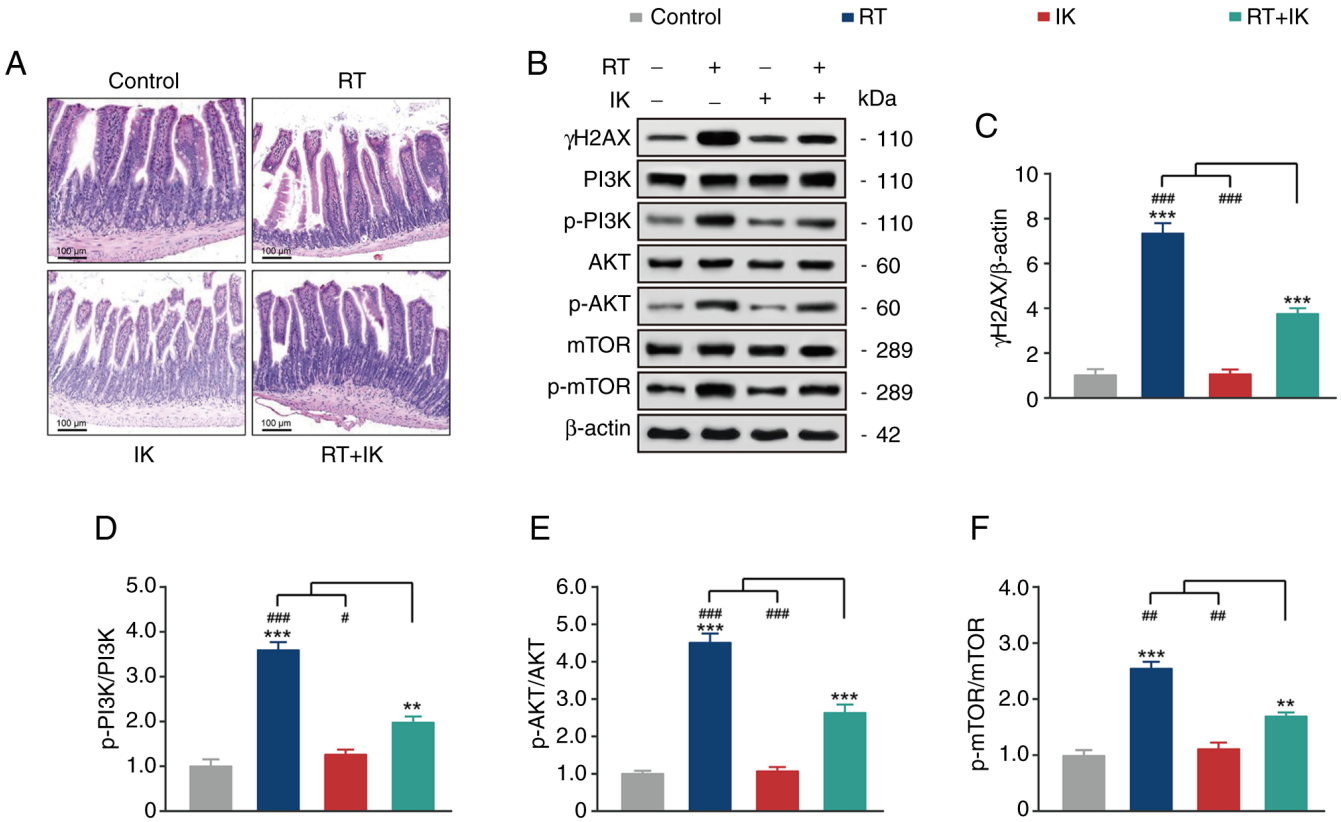

Figure 7. Effect of RT combined with IK on expression of PI3K/AKT/mTOR in normal intestinal tissues of HT-29-bearing mice. (A) Representative images of H&E staining. (B) Western blot images and quantitative analysis of (C) γH2AX, (D) p-PI3K/PI3K, (E) p-AKT/AKT and (F) p-mTOR/mTOR of normal intestinal tissues in HT-29-bearing mice treated with RT (8 Gy) combined with IK (100 mg/kg). Data are shown as the mean ± SD (n=6). **P<0.01 and ***P<0.001 vs. the control group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. the RT + IK group. RT, radiotherapy; IK, isoegomaketone; p-, phosphorylated.

favored by researchers for its mild drug properties and favorable antitumor effects. IK is the main component of *Perilla* extract, which can promote enhanced cleavage of BID, BAX translocation, release of cytochrome *c*, translocation of apoptosis-inducing factors into the nucleus, and enhancement of the activities of apoptosis-related enzymes such as caspases-3, -8 and -9 (21). In addition, IK can inhibit the phosphorylation of signal transducer and activator of transcription 1 and the activation of NF-κB, thereby blocking apoptosis escape and inhibiting cell proliferation (21). In addition, it has been identified that IK also attenuates the development of inflammation and oxidative stress (22). Therefore, the aim of the present study was to investigate whether IK could act as a RT radiosensitizer for colon cancer and to mitigate the side effects of RT on normal intestinal tissue.

Firstly, CCK-8 assay was used to investigate whether IK could synergistically promote the inhibitory effect of RT on the proliferation of human colorectal cancer cells. The results showed that different concentrations of IK significantly enhanced the viability of HT-29 cells, which was inhibited by RT. Remarkably, IK significantly reduced the inhibitory effect of RT on the viability of normal colonic histiocytes (CCD-18Co cells). Inhibition of HT-29 cell viability was maximal at RT and IK doses of 8 Gy and 100 μg/ml, respectively, whereas inhibition of CCD-18Co cell viability was not evident. Therefore, 8 Gy/100 μg/ml was selected as the optimal dose ratio for RT/IK in subsequent experiments.

Hypoxia is a ubiquitous phenomenon in the microenvironment of solid tumors (23). The ability of tumor cells to resist ionizing radiation exceeds that of cells with normal oxygen content by 2-3-fold, mainly due to reduced damage to DNA proliferation by oxygen free radicals (24). Hypoxia in solid tumors is the main cause of RT tolerance, and overcoming radiation resistance due to hypoxia is an important approach to improve the therapeutic effect of tumors (25). HIF-1 is a transcription factor for hypoxic cells and consists of two subunits, α and β, in which HIF-1α is oxygen-regulated (26). High expression of HIF-1α predicts adverse outcomes such as poor RT effect, local recurrence and reduced overall survival time (26). In addition, the PI3K/AKT pathway is one of the major pathways regulating HIF-1α protein expression and is also closely associated with RT resistance (27). Freudlsperger *et al* (27) found that PI3K/AKT-mediated DNA damage repair may be an important mechanism of radiation resistance, and inhibition of the PI3K/AKT pathway could increase radiation-induced apoptosis and attenuate intrinsic radiation resistance of cells to exert a role in radiosensitization. Miyasaka *et al* (28) found that regulation of the protein expression of HIF-1α downstream of the PI3K/AKT pathway similarly enhanced the radiosensitivity of endometrial cancer cells. Blockade of the PI3K/AKT pathway could inhibit the expression of HIF-1α and improve the hypoxic status of tumor cells, thereby increasing the sensitivity of endometrial cancer cells to radiation, which is consistent with the results of the present study (28). The current findings revealed that IK could enhance the DNA damage induced by RT in HT-29 cells by reducing the expression level of HIF-1α and the phosphorylation level of proteins in the PI3K/AKT signaling pathway. In addition, apoptosis, also known as programmed cell death, is an important process of cell life-sustaining activities (29), and is considered key to effective anticancer treatment options (29).

The BCL-2 family plays an important role in apoptosis, with the pro-apoptotic gene BAX and the anti-apoptotic gene BCL-2 leading to programmed cell death by activating downstream caspase proteins (30). Autophagy is a critical cellular process that typically protects cells and organisms from stressors such as nutritional deficiencies (29). In addition to their roles in normal physiological processes, they also play important roles in pathological processes such as cancer (31). Autophagy has been found to inhibit tumor growth, and deletion of autophagy genes leads to tumorigenesis (32). Beclin-1 was originally identified as a tumor suppressor gene, and is one of the key molecules between apoptosis and autophagy, and can suppress tumors by promoting autophagy (33). When the autophagic process begins, LC3 I is converted to LC3 II; thus, the levels of autophagy can be reflected by detecting LC3 I and LC3 II levels (34). The present results demonstrated that RT combined with IK significantly upregulated the expression of BAX and downregulated the expression of BCL-2. In addition, the combination of RT and IK further enhanced the conversion of LC3 I to LC3 II, and upregulated Beclin-1 expression to a greater extent than that observed in the treatment alone and control groups. These results confirmed that the combination of RT and IK significantly promoted apoptosis and autophagy in HT-29 cells.

Based on the antitumor effect of RT in combination with IK *in vitro*, HT-29-bearing mice were further generated, and the antitumor potential of RT in combination with IK *in vivo* was evaluated in the present study. Combination therapy for 14 consecutive days significantly reduced tumor size in xenograft mice to an extent of almost complete elimination. In addition, combination therapy significantly improved the survival of mice with xenografts, with no mice dying during the 14-day treatment period, and the survival rate being 100%. Mice in the combination treatment group had significantly reduced tumor size and significantly improved survival compared with controls and either monotherapy groups. Furthermore, the levels of non-specific immune factors in the peripheral blood of tumor-bearing mice were analyzed. As expected, peripheral blood leukocyte, neutrophil and monocyte levels were significantly enhanced in the combination group compared with the monotherapies and control groups. In addition, the levels of apoptosis- and autophagy-related proteins in tumor tissues were evaluated by western blotting. The results showed that the combination of RT and IK significantly upregulated and downregulated the expression of pro- and anti-apoptotic factors, respectively, and promoted the upregulation of autophagy-related protein levels. These results suggest that the combination of RT and IK can significantly increase the non-specific immunity of HT-29 bearing mice, promote the apoptosis and autophagy of tumor tissue cells, and further inhibit the growth of tumor xenografts.

Radiation therapy is widely used for the treatment of various abdominal malignancies (35). However, radiation damages surrounding normal tissues, leading to epithelial barrier dysfunction and mucosal inflammation, delayed mucosal atrophy and intestinal wall fibrosis, thus reducing the quality of life of patients with cancer and

causing enterotoxicity or chronic intestinal dysfunction in long-term cancer survivors, leading to acute gastrointestinal syndromes such as abdominal pain, anorexia and bleeding (35). The pathogenesis of radiation-induced intestinal injury is multifaceted, with inflammatory cytokines potentially involved in ionizing radiation-induced cell injury (36). It has been observed that TNF-α, IL-1β and NF-κB are markedly elevated in patients after RT (36). At the same time, the PI3K/Akt/mTOR pathway is involved in various cellular processes, including cell proliferation, differentiation, survival, metabolism, cellular immunity, inflammation and intestinal epithelial apoptosis (37). There is considerable evidence that the PI3K pathway modulates inflammatory responses and is strongly associated with chemokine-mediated recruitment of immune cells to sites of inflammation (38). Inhibition of the PI3K/Akt/mTOR signaling pathway has been reported to be a potential target for inflammation-related diseases (39). Furthermore, the PI3K/Akt/mTOR signaling pathway is equally closely associated with the development of oxidative stress (40). Consistent with the aforementioned studies, RT combined with IK treatment reduced the development of intestinal tissue inflammation and oxidative stress in the present study. The underlying mechanism may be through downregulation of phosphorylation levels in the PI3K/Akt/mTOR signaling pathway, thereby reducing radiation-induced intestinal injury. In addition, paradoxical effects were observed in tumor and normal intestinal tissues. Notably, the underlying molecular mechanisms are not contradictory. Specifically, the combination of RT and IK significantly decreased PI3K/AKT phosphorylation compared with the RT alone group in both HT-29 cells and normal intestinal tissues. In HT-29 cells, decreased phosphorylation of PI3K/AKT further improved DNA damage in HT-29 cells. On the other hand, decreased PI3K/AKT phosphorylation is a potential molecular mechanism underlying decreased inflammation and oxidative stress in normal intestinal tissues. Moreover, the specific mechanism by which IK enhances the efficacy of RT remains elusive and needs to be elucidated in subsequent in-depth studies. In addition, the toxicological effects of RT combined with IK therapy are future research topics. In the future study, the IK neutralizers, inhibitors, or even similar competitors, will be applied in an attempt to reverse the radiosensitivities of IK to colorectal cancer and to explore how does the IK sensitize the tumor cells to radiation.

In summary, IK synergistically increased the antitumor effect of RT *in vitro* and attenuated radiation damage to normal cells. In addition, IK could inhibit RT-induced DNA damage repair through the HIF-1α/PI3K/AKT signaling pathway, and further promote apoptosis and autophagy in HT-29 cells. Furthermore, the combination of RT and IK could effectively exert antitumor effects in HT-29 bearing mice, inhibit the growth of xenografts and improve the survival rate of mice. RT and IK combination therapy has potential therapeutic value in improving radiation-induced inflammation and oxidative stress, and reducing intestinal inflammation and oxidative stress in irradiated mice. IK may be a promising RT adjuvant to improve therapeutic effect and safety by enhancing radiosensitivity to tumor tissue and reducing intestinal complications after RT.

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Availability of data and materials

The data generated in the present study are included in the figures and/or tables of this article.

Authors' contributions

SFX and HYW analyzed the data and drafted the manuscript. XWH and LLY contributed to the study design and critical revision. All authors read and approved the final version of the manuscript. SFX and XWH confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Research related to animal use complies with all relevant national regulations, institutional policies, and was approved [approval no. 2023 (30)] by the animal Experimental Ethics Committee of the First Affiliated Hospital, Zhejiang University School of Medicine (Hangzhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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