IRF1 regulates the progression of colorectal cancer via interferon-induced proteins

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Abstract. Radiation is one of the main methods for the treatment of colorectal cancer (CRC) before or after surgery. However, radiotherapy tolerance of patients with CRC is often a major concern. Interferon regulatory factor 1 (IRF1) is a member of the IRF family and is involved in the development of multiple diseases, including tumors. The present study investigated the role of IRF1 in the development and radiation sensitivity of CRC. Immunohistochemistry was performed to examine the expression levels of IRF1 in tissue samples from patients with CRC, as well as in nude mice. MTT, 5-ethynyl-20-deoxyuridine, colony formation, cell cycle alteration and apoptosis assays were performed in CRC cell lines. Western blotting and immunofluorescence were used to detect the expression levels of a series of proteins. RNA sequencing was applied to identify genes whose expression was upregulated by IRF1 overexpression. Xenograft nude mouse models and hematoxylin and eosin staining were used to validate the present findings in vivo. It was revealed that the expression levels of IRF1 were significantly lower in CRC tissues than in adjacent tissues. IRF1 upregulation inhibited cell proliferation and colony formation, caused G1 cell arrest, promoted cell apoptosis, and enhanced the sensitivity of CRC cells to X-ray irradiation. The role of IRF1 in promoting the radiosensitivity of CRC was further demonstrated in nude mice with CRC xenografts. In addition, RNA sequencing revealed that overexpression of IRF1 in CRC cells significantly increased the expression levels of interferon-induced protein family members interferon α inducible protein 6, interferon induced transmembrane protein 1 and interferon induced protein 35 (fold change >2.0). In summary, the present study demonstrated that the upregulation of IRF1 inhibited the progression and promoted the radiosensitivity of CRC, likely by regulating interferon-induced proteins.

Introduction

Colorectal cancer (CRC) is the fourth leading cause of cancer-associated mortality worldwide (1). According to the American Cancer Society, there were 145,600 new cases of CRC and 51,020 associated deaths in the United States in 2019, and it ranked third in terms of incidence among all types of cancer (2). Additionally, the incidence and mortality rate of CRC have increased during the past decade in China (3). At present, radiotherapy is recognized as one of the main adjuvant treatment methods for CRC surgery (4). However, postoperative recurrence and metastasis of the tumor still occur due to radiation tolerance (5). Therefore, identifying related biological targets by which the radiosensitivity of CRC can be improved is a topic of great interest.

Interferon regulatory factor 1 (IRF1) is a member of the IRF family and can be activated by cytokines, such as IFN-γ, TNF-α and interleukins-1-6, which are involved in the regulation of various cytokines (IL-4, IL-5, IL-12 and IL-13) and immune cells (Th1, Th2 and Th9) (6,7). Furthermore, studies have demonstrated that differences in the sensitivity of IRF1 to IFN-γ responses can lead to different clinical outcomes (8,9). IRF1 has been demonstrated to be involved in the pathogenesis of different types of cancer, including breast cancer (10), cervical cancer (11,12), hepatocellular carcinoma (13), pancreatic ductal adenocarcinoma (14), prostate cancer (15,16) and CRC (17,18). It has been revealed that there is positive feedback regulation between IRF1 and microRNA-29b, which could promote the sensitivity of CRC cells to IFN-γ (17). Blocking this feedback regulation inhibits the growth and metastasis of CRC (17). Hong et al (18) reported that IRF1 suppresses CRC metastasis and proliferation by promoting Ras association domain-containing protein 5 expression and inhibiting the RAS-RAC1 signaling pathway.

In addition, previous studies have documented that IRF1 affects tumor radiation therapy (11,19,20): One study revealed...
that nuclear IRF1 expression is higher in patients with cervical cancer with a complete response to radio/chemotherapy than in patients with only a partial response to therapy (11). Chen et al. (19) found that IRF1 expression is lower in radiation-tolerant cells than in radiation-sensitive head and neck squamous tumor cells. Furthermore, IRF1 is an important transcription factor for cytosolic DNA sensing-mediated type III IFN production induced by gamma rays in CRC cells (20). Therefore, it is clear that IRF1 is involved in the radiotherapy of tumors. However, to the best of our knowledge, it is unclear how IFN-γ-mediated IRF1 influences the development and radiotherapy of CRC. The present study investigated the role of IRF1 in the progression and radiosensitivity of CRC. The present study demonstrated that the upregulation of IRF1 inhibited CRC progression and promoted the sensitivity of CRC to X-rays in both in vitro and in vivo experiments.

Materials and methods

Tissue samples. For immunohistochemistry analysis, 200 samples from patients with CRC and 31 matched adjacent tissues were collected at The Second Hospital of Wuxi (Wuxi, China) between January 2010 and December 2012. Among the 200 patients, there were 142 male patients and 58 female patients. Their age ranged between 40 and 83 years, the mean age was 66.21 years and the median age was 66 years. All specimens were obtained during resection. Patients who had undergone radiotherapy or chemotherapy were excluded. Matched adjacent normal tissues were obtained >2 cm away from the tumor. All patients provided signed, informed consent for their tissues to be used for scientific research. Ethical approval for the study was obtained from Ethics Committee of The Second Hospital of Wuxi (approval no. KY-2020-001; Wuxi, China). All diagnoses were based on pathological and/or cytological evidence. The histological features of the specimens were evaluated by a senior pathologist according to the classification criteria from the World Health Organization (21).

Immunohistochemistry. Immunohistochemistry staining and the stained section scoring methods were described in a previous study (22). IRF1 (dilution, 1:500; cat. no. ab232861; Abcam) and Bcl-2 (dilution, 1:100; cat. no. ab182858; Abcam) antibodies were used. The secondary antibody was horseradish peroxidase-labeled goat anti-rabbit IgG (H+L) (dilution, 1:500; cat. no. A0208; Beyotime Institute of Biotechnology).

Human Protein Atlas database. The results of IRF1 in CRC were validated using the Human Protein Atlas database, which is a database for cancer and normal protein expression in tissues and the analysis of patient survival (23). The specific steps were as follows: The www.proteinatlas.org website was used to enter ‘IRF1’ in the search field. Subsequently, ‘Pathology’ was selected, and ‘Colorectal cancer’ was selected in ‘RNA EXPRESSION OVERVIEW’ to obtain the survival analysis result and figure.

Cell culture, treatment, transfection, infection and irradiation. Human CRC-derived HT29 and LoVo cell lines were resuscitated and resuspended in RPMI 1640 medium (HyClone; Cytiva), while HCT116, CCL244 and SW480 were resuspended in high-glucose DMEM (HyClone; Cytiva) supplemented with 10% (v/v) FBS (Biological Industries). Our previous study revealed that CCL244 and SW480 cells were relatively radiation-resistant cell lines among seven types of CRC cells: HT29, LoVo, Hce8693, CaCo2, HCT116, CCL244 and SW480 (24). The present study mainly explored how to enhance the radiation sensitivity of CRC. Therefore, these two types of cells were used for in vitro experiments. All cells were obtained from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences and maintained in medium supplemented with 10% FBS and 100 U/ml penicillin-streptomycin at 37°C with 5% CO2. IFN-γ (ProteinTech Group, Inc.) was added to the culture medium at 10 ng/ml (17) at 0, 0.25, 0.50, 1.50 and 2.00 h.

For plasmid transfection, the cells were grown in a 6-well culture plate to 70-80% confluence and then transfected with 2.5 µg plasmids for 20 min [either short hairpin RNA (shRNA)-negative control (NC), 5'-TTCTCCGAACGTGTA CGTGTTCTTAAGAGAAGGCTCGTTCAGTTCAAGGAGAACTTCTCACTGTGTTTA$agt$AGAATTTTTT-3' or shRNA-IRF1, 5'-GGCTCATTGATTAATAAGTTCAAGAGACTTTAATCATCACAGGACTGCCTTTTTT-3'; Vigene Biosciences] using Lipofectamine 2000 at room temperature (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. After 48 h, the cells were collected for assays.

For viral infection, the adenovirus expression vectors IRF1 (Ad-IRF1) and negative control (Ad-NC) were obtained from Hanbio Biotechnology Co., Ltd. The cells were infected with Ad-IRF1 and Ad-NC (multiplicity of infection, 3) for 4 h and then the medium was changed according to the manufacturer's protocol. The cells were used for relevant experiments at 48 h after infection. Furthermore, the present study mainly focused on the inhibitory effect of IRF1 on CRC and the enhanced radiation sensitivity of CRC. Therefore, the cells were divided into two groups in in vitro experiments: Ad-NC and Ad-IRF1.

SW480 and CCL244 cells were irradiated with an X-ray linear accelerator (Rad Source Technologies) at a dose rate of 1.15 Gy/min.

Cell proliferation assay. After cells infected with Ad-NC and Ad-IRF1, SW480 or CCL244 cells were seeded into 96-well plates at a density of 5x10³ cells per well with eight parallel wells per group. At 24, 48 and 72 h after 6 Gy X-ray treatment, cell proliferation was measured using the MTT assay. Briefly, 20 µl MTT solution (5 mg/ml) and 100 µl complete medium were added to each well, and the cells were incubated for 4 h at 37°C. The medium was then aspirated, and 150 µl dimethyl sulfoxide was added to dissolve the crystals. The optical density (OD) was measured at 490 nm using a microplate reader (Bio-Rad Laboratories, Inc.). The viability index was calculated as the experimental OD value/the control OD value. Three independent experiments were performed in quadruplicate.

Colony formation assay. The cells were seeded into 6-well plates at different densities of 4x10² (for both cell lines with 0 Gy) and 8x10² (for CCL244 cells with 6 Gy and SW480 cells with 4 Gy) cells per well with three parallel wells per group. After 24 h, CCL244 cells were exposed to X-rays with different irradiation doses of 0 and 6 Gy, and SW480 cells were exposed to X-rays with different irradiation doses of 0 and 4 Gy. After 14 days of incubation at 37°C, the colonies were fixed with anhydrous methanol (100%) at room temperature for 15 min and stained.
with Giemsa at room temperature for 30 min, and those with a minimum of 50 viable cells were counted under a light microscope (Leica Microsystems GmbH). This process was repeated three times. The cloning efficiency was calculated as the ratio of the number of colonies formed divided by the total number of cells plated. The GraphPad software (version 9.0.0; GraphPad Software, Inc.) was used to analyze the cloning efficiency.

5-Ethynyl-2-deoxyuridine (EdU) incorporation assay. The Cell-Light EdU DNA Cell Proliferation kit (Guangzhou RiboBio Co., Ltd.) was used to determine the proliferation rate of CCL244 or SW480 cells after treatment according to the manufacturer's protocols. Briefly, the cells were incubated at 37°C with 50 µM EdU for 2 h prior to fixation with 4% paraformaldehyde for 30 min at room temperature, 0.5% Triton-X-100 permeabilization for 10 min at room temperature and EdU staining for 30 min at room temperature. The cell nuclei were stained with Hoechst 33342 at a concentration of 5 µg/ml for 30 min at room temperature, and the cells were examined under a fluorescence microscope (Leica Microsystems GmbH). The GraphPad software (version 9.0.0; GraphPad Software, Inc.) was used to analyze the proportion of red to blue.

Flow cytometric analysis of cell apoptosis. Following conventional digestion, cells in the logarithmic growth phase were used to prepare a single cell suspension, 2x10^5 cells/ml were seeded into 6-well plates with three parallel wells per group. At 48 h after irradiation (0 and 6 Gy), all cells were collected and centrifuged at 300 x g for 5 min at 4°C. The supernatant was discarded after washing with PBS, and centrifugation at 300 x g for 5 min at 4°C was repeated twice. The cells were then stained with fluorescein FITC-conjugated Annexin V (Shanghai Yeasen Biotechnology Co., Ltd.) and PI (Shanghai Yeasen Biotechnology Co., Ltd.), and directly analyzed using the flow cytometry software equipped in the machine (BD FACScanVerse; Becton-Dickinson and Company). The ratio of both early and late apoptotic cells was counted. The GraphPad software (version 9.0.0; GraphPad Software, Inc.) was used to statistically analyze the data (24).

Flow cytometric analysis of the cell cycle. At 48 h after irradiation with 0 and 8 Gy, cells infected with Ad-NC and Ad-IRF1 in logarithmic growth phase were collected and fixed with 70% precooled ethanol at 4°C overnight. After staining with propidium iodide (10 µg/ml; Shanghai Yeasen Biotechnology Co., Ltd.) in the dark for 30 min at 37°C, flow cytometry was performed on the BD FACScanCalibur Flow Cytometer system (Becton-Dickinson and Company), and the cell cycle distribution was analyzed using FlowJo software (version 7.6; Becton-Dickinson and Company) and ModFit LT software (version 3.2; Becton-Dickinson and Company) (22).

RNA sequencing analysis. Total RNA from three paired Ad-IRF1 and Ad-NC CCL244 cell lines was isolated with an RNA Isolation kit (cat. no. 12183018A, Ambion; Thermo Fisher Scientific, Inc.). RNA purity and quantification were evaluated using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc.). RNA integrity was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.). Subsequently, using RNA with an initial quality of 4 µg as raw material for sequencing, the libraries were constructed using TruSeq Stranded mRNA LT Sample Prep kit (Illumina, Inc.) according to the manufacturer's protocols. The transcriptome sequencing and analysis were conducted by OE Biotech Co., Ltd. The libraries were sequenced on an Illumina HiSeq X Ten platform and 150 bp paired-end reads were generated. The clean reads were mapped to the human genome using HISAT2 (25). FPKM (26) value of each gene was calculated using Cufflinks (27), and the read counts of each gene were obtained by HTSeq-count (28). Differential expression analysis was performed using the DESeq (2012) R package (29). P<0.05 and fold change >2 or fold change <0.5 was set as the threshold for significantly differential expression. Hierarchical cluster analysis of differentially expressed genes (DEGs) was performed to explore gene expression patterns. GO enrichment (30) and KEGG (31) pathway enrichment analysis of DEGs were performed respectively using R based (R 3.6.2; https://cran.r-project.org/bin/windows/base/old/3.6.2/) on the hypergeometric distribution. All sequencing procedures and analyses were performed at OE Biotech Co., Ltd.

Immunofluorescence. The treated cells were plated in 24-well plates with circular slides, up to 50-60% confluence fixed with 4% paraformaldehyde at room temperature for 10 min after X-ray radiation for 0, 3 and 6 h, and then treated with 1% Triton X-100 for 10 min at room temperature. After blocking with 1% BSA (Beyotime Institute of Biotechnology) for 1 h at room temperature, the samples were incubated with the IRF1 antibody (dilution, 1:1,000; cat. no. ab26109; Abcam) overnight at 4°C, and then incubated with FITC-conjugated goat anti-rabbit secondary antibody (dilution, 1:1,000; cat. no. A0562; Beyotime Institute of Biotechnology) for 2 h at room temperature. The nuclei were stained with DAPI (Sigma-Aldrich; Merck KGaA). The cells were observed under a confocal microscope (Olympus Corporation).

Western blotting. Cells were lysed in 1X RIPA (cat. no. P0013B; Beyotime Institute of Biotechnology) lysis buffer. The total protein concentration was detected using a BCA kit (cat. no. P0010S; Beyotime Institute of Biotechnology). The samples were centrifuged at 12,000 x g for 15 min at 4°C and then boiled, and ~20 µg protein/lane was loaded onto 12 or 15% SDS-PAGE (cat. no. P0012A; Beyotime Institute of Biotechnology) gels. The electrophoresed samples were then transferred onto PVDF membranes (cat. no. IPVH0010; EMD Millipore), which were then blocked with PBS (HyClone; Cytiva)/Tween 20 (0.1%; WVR International, LLC) containing 5% BSA (Beyotime Institute of Biotechnology) for 1 h at room temperature. The membranes were incubated with antibodies against IRF1 (dilution, 1:1,000; cat. no. ab26109; Abcam), interferon induced protein 35 (IFIT3; dilution, 1:1,000; cat. no. ab233415; Abcam), interferon α inducible protein 6 (IFI6; dilution, 1:1,000; cat. no. ab192314; Abcam), Bel-2 (dilution, 1:1,000; cat. no. ab182858; Abcam), cleaved caspase-3 (dilution, 1:1,000; cat. no. ab2302; Abcam), cleaved caspase-9 (dilution, 1:1,000; cat. no. ab2324; Abcam), interferon induced transmembrane protein 1 (IFITM1; dilution, 1:1,000; cat. no. 60074-1-Ig; ProteinTech Group, Inc.), β-actin (dilution, 1:1,000; cat. no. AF0003; Beyotime Institute of Biotechnology), GAPDH (dilution, 1:1,000; cat. no. AF0006; Beyotime Institute of Biotechnology) and β-actin (dilution, 1:1,000; cat. no. AF0003; Beyotime Institute of Biotechnology) antibodies separately.

Cluster analysis of differentially expressed genes (dEGs) was performed respectively on the R platform using the hypergeometric distribution. All sequencing procedures and analyses were performed at OE Biotech Co., Ltd.
Tubulin (dilution, 1:1,000; cat. no. AT819; Beyotime Institute of Biotechnology) overnight at 4°C. Goat anti-mouse secondary antibodies (dilution, 1:1,000; cat. no. A0286) and goat anti-rabbit secondary antibodies (dilution, 1:1,000; cat. no. A0277) were purchased from Beyotime Institute of Biotechnology. The membranes were incubated with the appropriate secondary antibody for 1 h at room temperature. After being washed, the membranes were incubated with enhanced chemiluminescence (ECL) stable peroxide solution (Beyotime Institute of Biotechnology). All blots were visualized using a G: BOX Chemi XRQ gel doc system (Syngene Europe) at room temperature and ImageJ (version 1.8.0; National Institutes of Health) was used for densitometry.

**Animal experiments and irradiation.** A total of 25 male BALB/c nude mice (4 weeks old and weighing 18-22 g) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. The mice were maintained under standard laboratory conditions with a 12-h light-dark cycle at a controlled temperature (21-23°C) and humidity (40-60%), and given access to sterilized food and water in a specific pathogen-free environment throughout all experiments. All experimental procedures were performed in accordance with the Guide for the Use and Care of Laboratory Animals from the National Institutes of Health (32). For the subcutaneous injection, CCL244 cells (5x10^6) were suspended in 100 µl PBS and then inoculated subcutaneously into the right posterior flank region of BALB/c nude mice. The mice were divided into five groups: i) Control group without treatment; ii) Ad-IRF1 group; iii) Ad-NC group; iv) shRNA-IRF1 group; and v) shRNA-NC group. Each group consisted of five nude mice. Two-dimensional measurements were taken with an electronic caliper every 3 days, and the tumor volume (in mm³) was calculated using the following formula: Volume=a x b²/2, where a is the longest diameter, and b is the shortest diameter of the tumor. When the tumor volume reached 80 mm³ on the fifth day, the local tumor was irradiated with 8 Gy X-ray. The histopathological scores indicated that IRF1 expression is downregulated in CRC tissues and positively associated with the survival rate. Immunohistochemical staining was performed for 200 CRC tissues and 31 matched tumor-adjacent tissues. The results revealed that IRF1 expression was weaker in CRC tissues compared with in adjacent tumor tissues. In addition, IRF1 was expressed in both the cytoplasm (green arrow) and nucleus (red arrow) (Fig. 1A). The histopathological scores indicated that IRF1 expression in CRC tissues was significantly lower than that in adjacent tissues (Fig. 1B). The present study further searched the Human Protein Atlas database and identified that the 5-year survival rate of patients with high IRF1 expression was significantly higher than that of patients with low IRF1 expression (Fig. 1C). Therefore, IRF1 expression was lower in the tumor tissues and its high expression was associated with improved survival of the patients.

Both IFN-γ and X-rays induce IRF1 expression. Western blotting was used to detect the expression levels of IRF1 in different CRC cells, and it was found that there was no significant difference in the expression levels of IRF1 in different cell lines (Fig. S1). Based on the results of a previous study (24), two cell lines (CCL244 and SW480) were used in *in vitro* experiments. Consistent with a previous study (17), the present study identified that IFN-γ induced IRF1 expression in colon cancer cells. IRF1 expression gradually increased after IFN-γ treatment and started to rise within 1 h, as demonstrated by...
Figure 1. IRF1 expression in CRC tissues is significantly lower than that in adjacent tissues. (A) Representative images of the different staining patterns. Scale bar, 300, 200 60 µm. Red arrows indicate the nucleus and green arrows indicate the cytoplasm. (B) IRF1 staining scores as assessed by IHC in CRC tumors (n=200) and adjacent tissues (n=31). *P<0.05. (C) Association between IRF1 expression and the 5-year survival rate. Data were acquired from the Human Protein Atlas database. CRC, colorectal cancer; IHC, immunohistochemistry; IRF1, interferon regulatory factor 1.

Figure 2. IRF1 expression in colorectal cancer cell lines is activated by IFN-γ or X-rays. (A) IFN-γ (10 ng/ml) was added to a 6-well plate containing CCL244 and SW480 cells. Cells were harvested at 0.25, 0.50, 1.00, 1.50 and 2.00 h after IFN-γ was added, proteins were extracted and the expression levels of IRF1 were detected by western blotting. (B) CCL244 and SW480 cells were treated with 0, 6 and 8 Gy X-rays. After 48 h, the cells were collected for protein extraction, and the expression levels of IRF1 were detected by western blotting. (C) X-ray irradiation of 0 and 6 Gy was administered to the two cell lines. The cells were fixed at 0, 3 and 6 h after irradiation, and the distribution and expression of IRF1 in the cells were detected using immunofluorescence. Scale bar, 2 µm. IRF1, interferon regulatory factor 1.
Figure 3. Overexpression of IRF1 inhibits the proliferation of CRC cells and increases their sensitivity to X-ray irradiation. (A) Subsequently, 0 and 4 or 6 Gy X-rays were administered to the cells at 48 h after infection. At 48 h after irradiation, EdU was used to detect the proliferation of CRC cells. Scale bar, 100 or 200 µm. (B) Subsequently, 0 and 6 Gy X-rays were administered to the cells at 48 h after infection. At 24, 48 and 72 h after irradiation, MTT was used to detect the proliferation of CRC cells. (C) After 14 days, the cells were fixed with pure methanol and then stained with Giemsa crystal violet, and the cell colony formation rate was calculated. (D) After 48 h, a flow cytometer system was used to analyze the cell cycle. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001. Ad, adenovirus expression vector; CRC, colorectal cancer; EdU, 5-ethynyl-2-deoxyuridine; IRF1, interferon regulatory factor 1; NC, negative control; OD, optical density.
western blot analysis (Fig. 2A). Notably, western blot analysis also revealed that IRF1 expression was induced by X-ray irradiation (Fig. 2B). Immunofluorescence analysis revealed that IRF1 expression in the nucleus was increased within 3 h after irradiation (Fig. 2C). In summary, IRF1 expression was induced by both IFN-γ and X-ray treatment.

**IRF1 enhances the effect of X-ray irradiation on the proliferation, apoptosis and cell cycle of CRC cells.** EdU staining was performed to investigate the effect of IRF1 on the proliferation of CRC cells. Upregulation of IRF1 decreased the proliferation rate compared with the control before or after radiation (Fig. 3A). Cell proliferation was monitored using an MTT assay, and this revealed that IRF1 upregulation could inhibit the proliferation of SW480 and CCL244 before or after radiation (Fig. 3B). The colony formation assay demonstrated that the clonogenicity of CRC cells was significantly reduced after IRF1 upregulation before or after radiation (Fig. 3C). Furthermore, the cell cycle was analyzed using a fluorescence-activated cell sorter. The results revealed that the G1 phase was significantly prolonged following upregulation of IRF1 (Fig. 3D). Overall, these results demonstrated that IRF1 upregulation inhibited proliferation, decreased colony formation efficiency and slowed the cell cycle.

Resistance to apoptosis is one of the most important radiation impairments occurring in tumors (34). To explore the effect of IRF1 on apoptosis induced by X-ray irradiation, flow cytometry analysis was performed. The results demonstrated that IRF1 upregulation significantly increased the apoptosis of cells with or without X-ray exposure (Fig. 4A). Western blot analysis demonstrated that upregulation of IRF1 increased the expression levels of Bax, caspase-3 and caspase-9, and inhibited Bcl-2 expression (Fig. 4B). This further demonstrated that IRF1 may be involved in the regulation of CRC cell apoptosis.

**IRF1 promotes radiosensitivity of CRC to X-rays in vivo.** To determine the effect of IRF1 on CRC growth in vivo, nude mice were inoculated with CCL244 cells that had been infected with Ad-NC or Ad-IRF1, or transfected with shRNA-NC or shRNA-IRF1 plasmids. Western blot analysis of IRF1 expression in CRC cells revealed that compared with the cells infected with Ad-NC, IRF1 expression in both SW480 and CCL244 cells infected with Ad-IRF1 was increased and compared with the cells transfected with shRNA-NC, IRF1 expression in both SW480 and CCL244 cells transfected with shRNA-IRF1 was reduced (Fig. S2). Nude mice inoculated with CCL244 cells infected with IRF1 overexpression adenovirus developed tumors slower than those in the control Ad-NC-infected group. By contrast, following IRF1 knockdown, the tumors grew much faster than those in other mouse groups (Fig. 5A). After the nude mice were sacrificed, tumor, liver and lung tissues were collected. The tumor volume was the smallest in the IRF1 upregulation group, and the tumor volume was the largest when IRF1 was downregulated (Fig. 5A). When observing the whole liver specimens, it was identified that the number of liver metastases was the lowest in the IRF1-upregulated group, while the number of metastases was the highest in the IRF1-downregulated group. For lung tissues, there were no nodules in the solid lung tissue observed by the naked eye. Therefore, no images were captured. When lung tissues were cut, there were isolated metastatic lung nodules in 1 mouse in the shRNA-IRF1 group (Fig. 5B and C). Further pathological tissue H&E staining revealed that the metastatic lesions in the IRF1-downregulated group were the largest, while the lesions in the IRF1-upregulated group were not obvious. In addition, pathological H&E staining of the lung revealed no metastases in the IRF1-upregulated group and obvious metastases in the IRF1-downregulated group (Fig. 5D). Tumor tissues and liver...
Figure 5. Overexpression of IRF1 promotes radiosensitivity of CRC to X-rays in vivo. (A) Tumor growth curve of each group. (B) Representative tumors from each group of mice. (C) Representative CRC metastases in the liver. Red arrows indicate lesions. Analysis of the number of liver and lung metastases in each group. (D) H&E staining of nodules in the liver and lung for CRC metastasis in each group. Red arrows indicate lesions. Scale bar, 60 μm. (E) Immunohistochemical detection of Bcl-2 expression in liver metastases and subcutaneous tumors for each group. Scale bar, 60 μm. **P<0.01, ***P<0.001 and ****P<0.0001 (shRNA-IRF1 vs. shRNA-NC/mock); ##P<0.01, ###P<0.001 and ####P<0.0001 (Ad-IRF1 vs. Ad-NC/mock). Ad, adenovirus expression vector; CRC, colorectal cancer; IRF1, interferon regulatory factor 1; NC, negative control; ns, not significant; shRNA, short hairpin RNA.
metastases were prepared for immunohistochemical staining. The results revealed that Bcl-2 protein expression in tumor tissues and liver metastases of the Ad-IRF1 group was lower than that in the Ad-Nc group, while Bcl-2 expression in the IRF1 downregulation group was the highest (Fig. 5E). These results were consistent with the results of the present in vitro experiments. In summary, the present results demonstrated that IRF1 regulation affected tumor growth, metastasis and apoptosis-related protein Bcl-2 expression in vivo.

IRF1 affects the biological behavior of CRC, likely by regulating the expression levels of interferon-induced proteins. To further explore the effect of IRF1 on the biological behavior of CRC, the present study examined the expression levels of mRNAs after IRF1 upregulation by RNA sequencing. Based on the sequencing results, the present study identified 20 mRNAs that were upregulated or downregulated, and a heat map was generated (Fig. 6A). Detailed information is included in Tables I and II. The present study identified that among the upregulated mRNAs, the expression levels of three interferon-induced proteins (IFI6, IFITM1 and IFI35) were increased significantly. Their expression was positively associated with IRF1, as demonstrated by western blot analysis (Fig. 6B).

Gene Ontology analysis, based on RNA sequencing results, indicated that IRF1 could upregulate numerous genes that are involved in the ‘type I interferon signaling pathway’, ‘response to interferon-beta’, ‘innate immune response in mucosa’ and ‘response to interferon-gamma’, suggesting that IRF1 participates in interferon-regulated tumor immunity, as aforementioned. The signaling pathway analysis also indicated that IRF1 was involved in the regulation of multiple cellular components of CRC cells, especially the cellular membrane structure, such as ‘nucleosome’, ‘nuclear nucleosome’, ‘lipid particle’, ‘apical plasma membrane’ and ‘extracellular exosome’. Furthermore, IRF1 was demonstrated to affect cellular activity at molecular levels, such as ‘insulin-like growth factor binding’, ‘phospholipase A2 activity’ and ‘protein heterodimerization activity’ in CRC cells, which may be achieved by regulating membrane structure (Fig. 6C).

### Discussion

The roles IRF1 serves in innate and adaptive tumor immunity have become a popular research topic in recent years. The effects of IRF1 on antitumor immunity depend on affecting mitochondrial dynamics (35) and glycolysis (36), regulating programmed death-ligand 1 (PD-L1) (37-40) and even priming tumor-derived exosomes (41). Lai et al (42) reported that decitabine improves the efficiency of anti-programmed cell death protein-1 (PD-1) therapy for lung cancer by activating the hypomethylation of IRF1/7 in the IFN response signaling pathway. Wu et al (43) revealed that YAP inhibits IFNc-inducible PD-L1 expression partially through microRNA-130a-mediated suppression of vestigial like family member 4 and IRF1 expression, thereby reducing the PD-L1 interaction with PD-1 to escape T-cell immune surveillance.

| ID   | Base mean control sample | NC     | Base mean case sample | Ad-IRF1 | Fold change | log2 fold change | P-value | Adjusted P-value | Gene       |
|------|--------------------------|--------|-----------------------|---------|-------------|-----------------|---------|------------------|------------|
| IFI6 | 8362.5161                | 2804.2397 | 13920.7926            | 4.9642  | 2.3116      | 0.65x10^-53    | 1.30x10^-49 | Up               |
| BST2 | 131.2432                 | 23.2430  | 239.2434              | 10.2931 | 3.6366      | 1.21x10^-33    | 4.16x10^-30 | Up               |
| TFF1 | 773.5291                 | 480.7576 | 1066.3006             | 2.2180  | 1.1492      | 7.65x10^-53    | 2.79x10^-50 | Up               |
| ISG15| 6329.8561                | 3499.2967 | 9160.4154             | 2.6178  | 1.3883      | 2.49x10^-33    | 2.79x10^-30 | Up               |
| TMEM238| 432.1279                 | 251.8244 | 612.4313              | 2.4320  | 1.2821      | 3.19x10^-30    | 3.13x10^-27 | Up               |
| RARRES3| 247.1411                 | 136.0866 | 358.1957              | 2.6321  | 1.3962      | 4.76x10^-23    | 2.49x10^-20 | Up               |
| LRRC26| 240.0970                 | 139.0721 | 341.1219              | 2.4528  | 1.2945      | 1.11x10^-19    | 3.49x10^-17 | Up               |
| NUPR1| 192.9297                 | 105.5262 | 280.3331              | 2.6565  | 1.4095      | 5.60x10^-19    | 1.60x10^-16 | Up               |
| IFTM1| 180.7444                 | 76.9545  | 284.5344              | 3.6974  | 1.8865      | 9.90x10^-18    | 2.35x10^-15 | Up               |
| C4orf48| 1337.1611               | 856.0667 | 1818.2556             | 2.1240  | 1.0868      | 1.55x10^-16    | 2.94x10^-14 | Up               |
| IFI35| 245.9523                 | 159.7153 | 332.1892              | 2.0799  | 1.0565      | 4.29x10^-14    | 5.26x10^-12 | Up               |
| SPINK4| 609.8811                | 393.3366 | 826.4257              | 2.1011  | 1.0711      | 6.25x10^-13    | 5.89x10^-11 | Up               |
| CBR3 | 210.2773                 | 138.8154 | 281.7391              | 2.0296  | 1.0212      | 7.98x10^-12    | 5.91x10^-10 | Up               |
| GADD45G| 154.6964               | 90.6289  | 218.7639              | 2.4138  | 1.2713      | 2.96x10^-11    | 1.94x10^-9  | Up               |
| MRPL23| 4537.9171               | 3004.8832 | 6070.9510             | 2.0204  | 1.0146      | 6.21x10^-11    | 3.68x10^-9  | Up               |
| MYH4 | 32.3621                  | 10.9477  | 53.7765               | 4.9121  | 2.2964      | 4.95x10^-9     | 1.73x10^-7  | Up               |
| SMIM22| 619.3599                | 411.7948 | 826.9250              | 2.0081  | 1.0058      | 6.72x10^-9     | 2.28x10^-7  | Up               |
| LGALS9| 34.6546                 | 12.7458  | 56.5634               | 4.4378  | 2.1498      | 1.10x10^-8     | 3.55x10^-7  | Up               |
| TSTA3| 174.7754                 | 109.1525 | 240.3983              | 2.2024  | 1.1391      | 7.56x10^-8     | 1.96x10^-6  | Up               |
| SHD   | 34.9248                  | 14.7999  | 55.0497               | 3.7196  | 1.8951      | 2.99x10^-7     | 6.68x10^-6  | Up               |

Ad, adenovirus expression vector; IRF1, interferon regulatory factor 1; NC, negative control.
expression levels of IRF1 were increased for different radiation capacity and abrogate the inhibitory effect of myotubularin that when IRF1 was upregulated, the proliferation rate of CRC cells was decreased, and the tumor size was smaller than that in the control sample. These findings support the hypothesis that IRF1 affects the biological behavior of CRC by regulating interferon-induced proteins. Based on the present results and the literature, it was hypothesized that IRF1 may affect the development and radiosensitivity of CRC through interferon-induced proteins. This hypothesis merits further investigation.

Table II. Significantly downregulated mRNAs following the overexpression of IRF1 according to RNA sequencing.

| ID      | Base mean | Base mean | Base mean | Log2 fold change | Adjusted P-value | Gene          |
|---------|-----------|-----------|-----------|------------------|------------------|---------------|
| CYR61   | 286.7657  | 445.3502  | 128.1812  | 0.2878           | -1.7968          | Down          |
| CTGF    | 226.0636  | 362.1031  | 90.0241   | 0.2486           | -2.0080          | Down          |
| SLC2A3  | 411.6516  | 618.3295  | 204.9737  | 0.3315           | -1.5929          | Down          |
| GPRC5A  | 861.9170  | 1180.5053 | 543.3287  | 0.4603           | -1.1195          | Down          |
| Cul116  | 432.2862  | 629.9506  | 234.6219  | 0.3724           | -1.4249          | Down          |
| TINAGL1 | 717.7809  | 986.1473  | 449.4144  | 0.4557           | -1.1338          | Down          |
| AMOTL2  | 449.3289  | 700.5839  | 198.0739  | 0.2827           | -1.8225          | Down          |
| KRT4    | 454.6213  | 609.3405  | 299.9020  | 0.4922           | -1.0228          | Down          |
| ANKRd1  | 44.2735   | 81.4814   | 7.0655    | 0.0867           | -3.5276          | Down          |
| AJUBA   | 657.5500  | 909.5501  | 405.5498  | 0.4459           | -1.1653          | Down          |
| CLIC5   | 348.2467  | 471.4531  | 225.0403  | 0.4773           | -1.0669          | Down          |
| SAMD4A  | 146.1898  | 214.0379  | 78.3416   | 0.3660           | -1.4500          | Down          |
| TAF13   | 106.3080  | 155.0966  | 57.5194   | 0.3709           | -1.4310          | Down          |
| ZNF566  | 209.2546  | 290.5804  | 127.9288  | 0.4403           | -1.1836          | Down          |
| CITED2  | 192.1906  | 259.8504  | 124.5308  | 0.4792           | -1.0612          | Down          |
| EMP1    | 303.1375  | 421.9202  | 184.3549  | 0.4369           | -1.1945          | Down          |
| GALNT3  | 306.3284  | 426.9941  | 185.6626  | 0.4348           | -1.2015          | Down          |
| PAQR3   | 161.8251  | 220.2059  | 103.4443  | 0.4698           | -1.0900          | Down          |
| ZNF540  | 118.3167  | 167.1335  | 69.4998   | 0.4158           | -1.2659          | Down          |
| GRHL3   | 87.9749   | 134.5827  | 41.3672   | 0.3074           | -1.7019          | Down          |

Ad, adenovirus expression vector; IRF1, interferon regulatory factor 1; NC, negative control.
Figure 6. Overexpression of IRF1 promotes the expression of interferon-induced proteins. (A) Heat map of the significant mRNA expression patterns in the Ad-IRF1 and Ad-NC groups. (B) Western blotting supported the RNA sequencing results. (C) Gene Ontology biological process, cellular component and molecular function analysis. Ad, adenovirus expression vector; IFI6, interferon α inducible protein 6; IFI35, interferon induced protein 35; IFITM1, interferon induced transmembrane protein 1; IRF1, interferon regulatory factor 1; NC, negative control.
In summary, the present study demonstrated that IRF1 expression was downregulated in CRC tissues and that it was positively associated with patient prognosis. IRF1 up-regulation inhibited the progression and increased the radiosensitivity of CRC likely by regulating the expression levels of interferon-induced proteins.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions
XX, KY and YW conceived and coordinated the study, designed, performed and analyzed the experiments, and drafted the manuscript. YH, Wd and cX carried out the experimental work. XX, KY and YW conceived and coordinated the study, designed, performed and analyzed the experiments, and drafted the manuscript. YH, Wd and cX carried out the experimental work. All patients provided signed, informed consent for their participation in the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate
All patients provided signed, informed consent for their participation in the study. Ethical approval for the study was obtained from the Second Hospital of Wuxi (approval no. KY‑2020‑001; Wuxi, China). The present study was approved by the Institutional Animal Care and Use Committee of Soochow University (Suzhou, China).

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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