DIRAS2 Contributes to Radiation Resistance of Renal Cell Carcinoma Via Autophagy Induction and MKK4-JNK1 Pathway Activation

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Abstract

Radiation resistance has been regarded as a main obstacle to improve the definitive treatment of renal cell carcinoma (RCC), of which clear cell RCC (ccRCC) is the most common histological type. However, the molecular mechanism underlying the radiation resistance remains largely unclear. In this study, we investigated the effect of DIRAS2 on the response to ionizing radiation (IR) in human ccRCC cells. Here, we found the expression level of DIRAS2 was significantly upregulated in human ccRCC tissues using the Oncomine platform and the Cancer Genome Atlas (TCGA) database, which was further validated by immunohistochemistry. Overexpression of DIRAS2 promoted radiation resistance of ccRCC cells based on clonogenic survival assay and enhanced the levels of radiation induced-autophagy. Moreover, inhibition of autophagy by chloroquine (CQ) pre-treatment largely eliminated the effect of DIRAS2 overexpression on radiation-resistance. Finally, molecular mechanism investigation showed that overexpression of DIRAS2 upregulated the activity of mitogen-activated protein kinase (MAPK) kinase 4 (MKK4)- c-Jun NH2-terminal kinase 1 (JNK1)-Bcl-2 pathway in response to IR. Taken together, these results indicate that DIRAS2 may confer radiation resistance on human RCC via autophagy induction through MKK4-JNK1-Bcl-2 signaling pathway.

Introduction

Renal cell carcinoma (RCC)encompasses a variety of tumors originating from renal tubular epithelial cells[1] .Worldwide, RCC is also the 7th most common tumor in men and the 9th most common tumor in women and accounts for ~2–3% of all adult malignancies[2–4]. It is classified into clear cell RCC (ccRCC), papillary RCC, and chromophobe RCC, accounting for about 85% of all the primary renal cancers. ccRCC is the most common subtype and has the highest lethallty rate. Indeed, histology of the majority (83% ~ 88%) metastatic renal cell carcinoma belongs to ccRCC[5, 6]. Surgical treatment is the first choice for RCC with localized or oligometastatic disease, and targeted therapy combined with immunotherapy is a systematic treatment for patients with surgical intolerance[7]. Radiotherapy is an effective and promising treatment strategy for solid tumors. However, a significant proportion of tumors are generally considered to be significantly resistant to radiotherapy, including RCC[8]. Currently more accurate radiotherapy techniques, such as stereotactic body radiotherapy (SBRT), have been widely used in the various early-stage carcinoma in situ and palliative treatment of advanced metastasis. However, the prognosis for patients with metastatic RCC has been poor, with a 5-year survival rate of <10%[9].Given the poor therapeutic response of metastatic renal carcinoma, we attempted to explore the potential mechanism of radiotherapy resistance of RCC to prolong and improve patient survival by modulating target molecules that influence radiotherapy sensitivity

As a member of distinct subgroup of the Ras family, the GTP-binding Ras-like protein 2 (Di-Ras2) was predominantly expressed in the brain. Despite its similarity in sequence, DIRAS2 differs from other Ras family members in biochemical and functional properties[10, 11]. DIRAS2 was first studied in attention deficit/hyperactivity disorders (ADHD), through its exact function is unclear[12]. In recent years, it has also been reported that DIRAS2 may be involved in tumor progression. DIRAS2 has been shown to be

potentially carcinogenic in ccRCC as an activator of Mitogen-activated protein kinase (MAPK) signaling pathway in the absence of the von Hippel–Lindau protein (pVHL)[13], while DIRAS2 was found to be downregulated in ovarian cancer and associated with reduced overall and disease-free survival. In murine ovarian cancer cells, DIRAS2 induced cell death via autophagy, which means DIRAS2 may also play a pivotal role in human autophagy[14].

Macro-autophagy (hereinafter referred to as autophagy) is a normal cellular process used to produce nutrients and energy in response to stress, such as ionizing radiation[15]. Autophagy plays an increasingly important role in tumor therapy. As the RAS–MAPK signaling pathway is one of the most important pathways in regulating autophagy[16], and previous studies have shown that DIRAS2 can activate this pathway in ccRCC[13]. we suppose that DIRAS2 in ccRCC may induce protective autophagy to reduce its sensitivity to radiotherapy.

As the role of DIRAS2 in radiation resistance of RCC and the underlying molecular mechanism have not been fully elucidated. Therefore, the present study mainly aimed to investigate the exact role of DIRAS2 in the regulation of radiation resistance in ccRCC. Besides, we also studied the underlying mechanism involving autophagy and the related signaling pathway.

Materials And Methods

Cell lines and culture conditions

Human RCC cell lines, 786-O and A498 were purchased from the Procell Life Science & Technology Co., Ltd., China. The cells were, respectively, maintained in Roswell Park Memorial Institute (RPMI) 1640 medium and Dulbecco's modified eagle's medium (DMEM) (Gibco, Life Technologies Inc.) supplemented with 10% fetal bovine serum (FBS) (Lonsera S711-001S), 1% penicillin–streptomycin (10,000 U/ml penicillin and 10 mg/ml streptomycin, Gibco, Life Technologies Inc., cat#15140122). All cell lines were cultured at 37 °C in humidified atmosphere containing 5% CO_2. The medium was replaced 2–3 times each week.

Cell transfection

The Lentivirus-based DIRAS2 overexpression system was purchased from GenePharma Company (Shanghai, China). Flag tag was fused to the C-terminus of DIRAS2. The lentivirus overexpressing DIRAS2 and negative control lentivirus were employed to infect the human ccRCC cell lines 786-O and A498. The stable infection cells were selected with 4 μg/ml puromycin (Solarbio, P8230). The transfection efficiency was verified by qRT-PCR and western blotting.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cultured cells using RNA-Quick Purification Kit (Esunbio) pursuant to the manufacturer’s guidelines. Then, the concentration and purity of extracted RNA were assessed by a DS 11

Spectrophotometer (DeNovix, USA). The total RNA was then reverse transcribed to cDNA using a FastKing RT Kit (with gDNase) (TIANGEN BIOTECH), and then detected by qRT-PCR with a SYBR Green Realtime PCR Master Mix (Thermo). The primer sequences are shown in Table 1. The relative mRNA expression levels were calculated using the $2^{-\Delta\Delta CT}$ method. Relative abundance of mRNA was calculated by normalization to GAPDH.

Table 1 Primer sequences in qRT-PCR

Target gene	Strand	Primer sequence
GAPDH	Forward	5'GCACCGTCAAGGCTGAGAAC3'
	Reverse	5'TGGTGAAGACGCCAGTGGA3'
DIRAS2	Forward	5'TTGCAGATCACCGACACGAC3'
	Reverse	5'CTGTCGGCTGGTAATGGAGT3'

Protein extraction and western blotting

Cells were lysed with RIPA lysis buffer (Solarbio) supplemented with PMSF (1%, v/v). The protein concentration was measured using an Enhanced BCA Protein Assay Kit (Beyotime). From each sample, 40 μg of total protein was separated by 8–12% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and transferred onto hydrophobic PVDF membrane (Millipore). Membranes were blocked in 5% skim milk powder in TBS containing Tween 20 (0.1%, v/v) for 1 h at room temperature, and then incubated with primary antibodies overnight at 4 °C. Following the primary antibody incubation, the membranes were washed in TBST and incubated with HRP-conjugated secondary antibody for 1 h at room temperature. The protein bands were detected by ECL reagent (Millipore) and quantified by Image J (National Institutes of Health).

Primary antibodies against GAPDH (ab181603) and JNK (ab199380) were purchased from Abcam. Primary antibodies against DIRAS2 (TA809398) were purchased from Origene. Primary antibodies against Bcl-2 (AF6139), p-Bcl-2 (AF3138), Beclin-1 (AF5128) were purchased from Affinity. Primary antibodies against FLAG (#14793), SQSTM1/P62 (#5114), LC3B (#3868), p-SAPK/JNK (#4668), SEK1/MKK4 (#9152), P-SEK1/MKK4 (#4514), P38 MAPK (#8690) and p-P38 MAPK (#8690) were purchased from Cell Signaling Technology Inc. HRP-linked secondary antibodies directed against rabbit or mouse IgG, respectively, were purchased from Cell Signaling Technology Inc.

Patient samples

Clinical and molecular information of ccRCC samples from TCGA datasets are showed in Supplementary Tables. The transcription level analysis of DIRAS2 gene based on the three independent datasets from Oncomine database including Jones Renal, Gumz Renaland Lenburg Renal.

Fresh samples of human ccRCC tissue and paired normal tissue were obtained during surgery at the Department of urinary surgery, Qilu Hospital of Shandong University, China. All samples were collected with the informed consent of patients and the study was approved by the Ethics Committee of Qilu Hospital of Shandong University.

Immunohistochemistry (IHC)

The human ccRCC tissues obtained from the operation were fixed in 10% formaldehyde for 24 hours and then embedded in paraffin. The paraffin slices were prepared, deparaffinized, rehydrated, and treated with 0.01 M sodium citrate (pH 6.0) for 20 min at 98 °C for antigen retrieval. Then, endogenous peroxidase activity was blocked with H2O2 (0.3%, v/v) in distilled water, and goat serum in PBS (10%, v/v) was used to block non-specific antigens. Subsequently, slices were incubated with a mouse polyclonal DIRAS2 antibody (Origene, TA809398), diluted in 1% goat serum (1:500) at 4 °C overnight. After incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 1h at room temperature, slices were incubated with 3,3'-diaminobenzidine (DAB, 25mg/ml) for 10 min. All tissue slices were counterstained with hematoxylin. Stained slides were viewed under the OLYMPUS DP27 microscope and analyzed by Image J.

Immunofluorescence

Cells on coverslips were fixed with methanol and blocked with goat serum (Origene, ZLI-9022). Then cells were incubated at 4 °C overnight with the primary antibody against LC3B (CST, #3868). After rinsing with PBS, coverslips were incubated with DyLight® 488, Goat Anti-Rabbit IgG (1:500, Abbkine, A23220), and the cell nuclei were stained with DAPI solution (Solarbio, C0065) for visualization. Three fields from each coverslip were randomly captured with Olympus DP72, and three independent experiments were performed. Quantification of LC3 puncta base on the fluorescence intensity was calculated with Image J.

Clonogenic survival assay

Cells were trypsinized to generate single-cell suspensions and seeded in six-well plates at 1000 cells per well. Then the cells were exposed to different doses of radiation (0 Gy, 2 Gy, 4 Gy, 6Gy) by a medical linear accelerator (varian 23 EX). After cultured for 7-14 days to allow cell colony formation, the cells were fixed in ethanol and stained with crystal violet for half an hour. Only colonies containing more than 50 wells were counted. The adherence rate was referred to the ratio comparing the number of colonies formed to the number of cells planted. Survival fraction (SF) is referred to the adherence rate at each dose divided by that at 0 Gy. The colony formation ability means the ratio of the SF of the transfected cells to the control cells under the same dose of radiation. The survival curves were calculated with Prism 7.0 (GraphPad Inc., La Jolla, CA, USA). The mean lethal dose (D0), quasi-threshold dose (Dq), and survival fractions at 2 Gy (SF2) were calculated by fitting the survival curves into the single-hit multitarget model ($y=1-[1-e^{(-kx)}]^N$).

Statistical analysis

The statistical analyses were performed using the GraphPad Prismsoftware (GraphPad Software Inc., La Jolla, CA). For each experiment, at least three biological replicates were conducted, and data are expressed as mean ± SD, unless otherwise specified. Statistical significance was analyzed using a two-tailed Student's t-test or repeated measures ANOVA. Values of $P < 0.05$ were considered statistically significant.

Results

1. DIRAS2 is highly expressed in human ccRCC

To investigate the role of DIRAS2 in radiation resistance we explored the expression of DIRAS2 in human clear cell renal cell carcinoma (ccRCC). Firstly, the Oncomine platform and the Cancer Genome Atlas (TCGA) database were used to analyze the transcriptional expression of DIRAS2 in different types of human cancers, mainly in ccRCC. As shown in Fig 1a-e, compared with the adjacent normal kidney tissue samples, the mRNA levels of DIRAS2 were significantly higher in ccRCC tissues. In order to verify the expression differences in the databases, we performed immunohistochemical experiments on paired ccRCC specimens, and the results in Fig 1f showed that DIRAS2 was highly expressed in tumor tissues. These data all indicated that DIRAS2 expression was upregulated in ccRCC and whether upregulated DIRAS2 expression is involved in radiation resistance of ccRCC was further studied.

2. Overexpression of DIRAS2 promotes radiation resistance of ccRCC cells

To better understand the role of DIRAS2 in radiation resistance of ccRCC in vitro, DIRAS2-overexpression cell lines were constructed by lentivirus-mediated gene expression system. The 786-O and A498 cells, both of which are von Hippel–Lindau (VHL) -mutated cell lines, were selected to ensure the overexpression of DIRAS2, as the ubiquitination and degradation of DIRAS2 can be enhanced by pVHL [13]. The results of Western blot and qRT-PCR confirmed that DIRAS2 was overexpressed successfully (Fig 2a-b). Then the cells were exposed to different doses of IR. The clonogenic assay was employed to evaluate the effect of DIRAS2 on radiation resistance. As shown in Fig 2c, compared with Lv-NC groups, the survival curves of DIRAS2 OV cells significantly shifted upward both in 786-O and A498 cell lines. The mean lethal dose (D0), quasi-threshold dose (Dq), and survival fractions at 2 Gy (SF2) were also calculated based on clonogenic assay. DIRAS2-OV cells had higher values of D0, Dq and SF2 (all $p < 0.05$) than Lv-NC cells (Fig 2d). DIRAS2 OV groups had more colonies to survive than Lv-NC cells under the same dose of IR (4 Gy) (Fig 2e). These results suggested that upregulated expression of DIRAS2 conferred radiation resistance in human ccRCC cells.

3. Human ccRCC cells overexpressing DIRAS2 exhibit enhanced autophagy in response to ionizing radiation

Since it has been reported that autophagy may play a pro-survival role under various stress conditions[17-19], including ionizing radiation[20], we overexpressed DIRAS2 in 786-O and A498 cell lines respectively and measured the expression levels of autophagy-related markers LC3. Compared with LC3-II, LC3-I was

less sensitive to antibodies and more prone to degradation under repeated freeze-thaw conditions, so LC3-II /GAPDH method was adopted in this experiment[21]. In addition, a positive control group with the addition of autophagy inhibitor chloroquine (CQ), which blocking autophagy flux by regulating the PH value of lysosome, was set up to further reflect the changes of autophagy. As shown in Fig. 3A, in both Lv-NC and DIRAS2-OV groups, after IR (6 Gy) treatment LC3-II levels increased, and the peak values reached before 8 h after IR treatment. However, overexpression of DIRAS2 promoted the conversion of LC3-I to LC3-II in both 786-O and A498 cells, and the LC3-II levels increased more significantly in the DIRAS2-OV groups than that in Lv-NC groups (Fig 3a). Furthermore, measurement of autophagy was visualized by immunofluorescence of LC3-II. As shown in Fig 3b and c, after IR treatment, a largely dispersed fluorescence distribution was both observed in the 786-O cells of Lv-NC and DIRAS2 groups (Fig 3b). Compared with Lv-NC cells, DIRAS2-OV cells exhibited more intense LC3-II-associated red fluorescence puncta (Fig 3b), and the amount of LC3 fluorescent puncta dramatically increased in DIRAS2-OV cells (Fig 3c). Consistent results were obtained in A498 in Fig S1. In conclusion, our data demonstrate that overexpression of DIRAS2 can upregulate the autophagy level in the human ccRCC cells, which is more obvious after IR treatment, suggesting that DIRAS2-enhanced autophagy may be related to radiation resistance.

4. Inhibition of autophagy by chloroquine (CQ) eliminate the effect of DIRAS2 overexpression on radiation-resistance in human ccRCC cells

Because DIRAS2 overexpression induced autophagy and enhanced the level of autophagy in response to ionizing radiation, we attempted to explore whether autophagy play a critical role on radiation-resistance induced by DIRAS2 overexpression in human ccRCC cells. The DIRAS2-OV and Lv-NC groups of 786-o and A498 cells were treated with the autophagy inhibitor chloroquine (CQ) for 12h before exposed to different dose of IR. As shown in Fig 4a, the DIRAS2 overexpression was verified by western blot analysis with anti-FLAG and anti-DIRAS2 antibody. For confirmation of autophagy inhibition, the levels of both LC3-II and P62 increased significantly compared with those in the untreated control groups (Fig 4a), which suggested that the autophagic flux was successfully blocked. To assess the cell radiation sensitivity, the clonogenic assay was performed and the survival curves were shown in Fig 4b. Only marginal difference was observed between the Lv-NC group cells with or without CQ treatment. And consistent with the results above, DIRAS2-OV groups were significantly more resistant to IR compared with Lv-NC groups both in 786-o and A498 cell lines. But under CQ treatment the survival curves of the DIRAS2-OV groups significantly shifted downward (Fig 4b). Moreover, D0, Dq and SF2 were also calculated (Fig 4c). DIRAS2-OV cells had higher values of D0, Dq and SF2 than Lv-NC cell. After pretreated with CQ, the values of D0, Dq, and SF2 of DIRAS2-OV cells reduced and were not significantly different from the values of Lv-NC cell without CQ treatment. Taken together, our data show that autophagy inhibition sensitized the DIRAS2-OV cells to IR, indicating that DIRAS2 overexpression induced radiation resistance is at least partially due to enhanced autophagy.

5. DIRAS2 enhanced the radiation-induced autophagy by activating JNK-Bcl-2 pathway

The activation of the Mitogen Activated Protein Kinase (MAPK) cascade in response to ionizing radiation (IR) plays a critical role in the sensitivity to radiotherapy[16]. And MAPK/ c-Jun NH2-terminal kinase (JNK)has also been associated with the regulation of autophagy[22].Therefore, we performed Western blot analysis of MAPK signaling activation to investigate the molecular mechanism of DIRAS2 in regulating autophagy in response to radiation. As shown in Figure 5, overexpression of DIRAS2 in 786-O and A498 cells both promoted the phosphorylation of MKK4 and JNK1, when compared with those in the Lv-NC cells. In addition, the DIRAS2 OV cells and the Lv-NC groups were exposed to IR (6 Gy), the MAPK signaling activation in each group was determined (Figure 5). After IR, the MAPK signaling activation were all enhanced in each group, while the levels of phos-MKK4 and phos-JNK1 were both increased in the DIRAS2 OV groups than in the Lv-NC groups. These data suggest that DIRAS2 may activate the MKK4-JNK1 signaling. Furthermore, the phosphorylation level of Bcl-2, which can be phosphorylated by JNK1 and then dissociates with Beclin 1 [23], upregulated after IR, and was also enhanced in the DIRAS2 OV groups than in the Lv-NC groups (Figure 5). These results suggest that DIRAS2 may activate MKK4-JNK1-Bcl-2 signaling, and then phosphorylated Bcl-2 dissociates with Beclin 1 and induces autophagy.

Conclusion And Discussion

At present, the majority of renal cancer is RCC, with high morbidity and mortality[24]. In recent years, molecular characterization of ccRCC has developed rapidly, the mechanisms by which ccRCC patients acquire radiation resistance or chemotherapy resistance remain largely unknown [25]. In addition, considering that normal tissues around cancer are relatively sensitive to radiotherapy, radiotherapy dose must be controlled within a tolerable range in clinical practice[26]. In order to improve the effectiveness of radiotherapy and fundamentally reduce the damage to normal tissues around cancer, it is necessary to explore the molecular mechanism of radiation resistance, so as to achieve the routine use of radiotherapy in clinical RCC patients. In this study, we determined the role of DIRAS2 in ccRCC response to radiotherapy, and our in vitro study found that overexpressed DIRAS2 can promote radiotherapy resistance by inducing autophagy and activating MKK4-JNK1-Bcl-2 signaling.

A significant proportion of RCC is associated with deletion of VHL tumor suppressor genes[27], pVHL, as an E3 ubiquitin ligase complex substrate recognition element, is highly associated with ccRCC by enhancing the ubiquitination and degradation of certain oncogenic related protein, including Di-Ras2[13]. Thus, a characteristic of VHL-deficient RCCs is the production of high levels of Di-Ras2 due to their extended intracellular half-life. Our immunohistochemical results also showed that Di-Ras2 was highly expressed in ccRCC. Di-ras2, as a member of RAS-related small GTPase, has been rarely studied in tumors. It has been reported that Di-ras2 is associated with autophagy regulation in ovarian cancer[14] and has a carcinogenic effect in RCC[13]. However, there are few studies on the relationship between Di-ras2 and radiation resistance of RCC. Through clonogenic survival assay, we found that the RCC cell line of DIRAS2 OV had higher radiation resistance compared with the Lv-NC cell lines. In terms of mechanism, we found that RCC cells of DIRAS2 OV could improve their resistance under IR conditions by increasing the protective autophagy level. After cells were pretreated with autophagy inhibitor CQ, the radiotherapy sensitivity of RCC cells of DIRAS2 OV was significantly enhanced.

Autophagy processes in cancer can promote survival or lead to apoptosis, depending on cellular environment and stress[28]. Autophagy plays an increasingly important role in tumor therapy. Since previous studies have shown that DIRAS2 can induce autophagy in ovarian cancer[14], we have reason to believe that DIRAS2 in RCC cell lines may also induce protective autophagy to reduce its sensitivity to radiation. To verify this view, cells in the DIRAS2-OV group and LV-NC group received or did not receive IR stimulation, and the changes of autophagy marker proteins LC3-II and P62 were detected by Western Blot. The results showed that the autophagy level of ccRCC cells significantly increased after receiving IR stimulation, and the autophagy change trend of cells in the DIRAS2-OV group was significantly higher than that in the Lv-NC group. In order to further illustrate the key role of autophagy in radiation resistance of RCC cells, we pretreated cells with autophagy inhibitor CQ, and the results of clonogenic survival assay showed that the radiation resistance of DIRAS2 OV group was weakened, and there was no significant difference between the survival curve of cells in the DIRAS2 OV group and Lv-NC group. Based on the above results, we believe that DIRAS2-OV induced protective autophagy plays a key role in the radiation resistance of ccRCC cells. In view of this, we consider that targeted therapy of autophagy may provide a new idea for enhancing radiotherapy sensitivity of RCC.

Next, we explored the relationship between DIRAS2 and signaling pathways related to autophagy. Ras-MAPK signaling pathway is one of the classic oncogenic pathways[29, 30], and DIRAS2 has also been proved to activate RAS-MAPK pathway in VHL-deficient ccRCC cell lines[13]. In addition, the activation of JNK1 in MAPK family has also been confirmed to be related to cell external stimulation, including IR[23]. Western-blot results showed that DIRAS2 could indeed activate the MEK4-JNK1 signaling pathway, and the phosphorylation of Bcl-2 which is the downstream of JNK1 could promote Beclin 1 dissociating from the Bcl-2/Beclin 1 complex to promote autophagy.

Our study for the first time revealed the relationship between DIRAS2 and ccRCC radiation resistance, and preliminarily discussed the mechanism underlying radiation resistance, providing a new idea for the wide application of targeted radiotherapy in different stages of RCC. In addition to autophagy, other mechanisms of DIRAS2 regulating radiotherapy resistance still need to be explored. Whether DIRAS2 can be used as an evaluation indicator for RCC patients before radiotherapy still needs more comprehensive and systematic clinical data

Abbreviations

ccRCC, clear cell Renal Cell Carcinoma; RCC, Renal Cell Carcinoma ; DIRAS2, DIRAS family GTPase 2 ; IHC, Immunohistochemistry; OV, Overexpression; NC, Negative Control; D0, Mean lethal dose; Dq, Quasi-threshold dose; SF2, Survival Fractions at 2 Gy; CQ, Chloroquine; IR, Ionizing Radiation; SBRT, Stereotactic Body Radiotherapy; ADHD, Attention Deficit/Hyperactivity Disorders; MAPK, Mitogen-activated Protein Kinase; pVHL, Von Hippel–Lindau protein; RPMI, Roswell Park Memorial Institute; DMEM, Dulbecco's Modified Eagle's Medium; FBS, Fetal Bovine Serum; qRT-PCR, Quantitative Real Time Polymerase Chain Reaction; WB, Western Blotting; SQSTM1/p62, Sequestosome-1; TCGA, The Cancer Genome Atlas;

MAP1LC3/LC3, Microtubule Associated Protein 1 Light Chain 3; JNK1, C-Jun N-Terminal Kinase 1; MKK4, Mitogen-activated Protein Kinase Kinase 4;

Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by [Chengfeng Cai], [Dong Yue] and [Ning Guo]. The first draft of the manuscript was written by [Chengfeng Cai] and all authors commented on previous versions of the manuscript. The final manuscript was written by [Ying He], All authors read and approved the final manuscript.

Ethics approval

This study was performed in line with the principles of the Declaration of Helsinki. Relevant retrospective studies are conducted on already available biological material, and the study was approved by the Ethics Committee of Qilu Hospital of Shandong University.

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Tables

Supplementary Tables are not available with this version

Figures

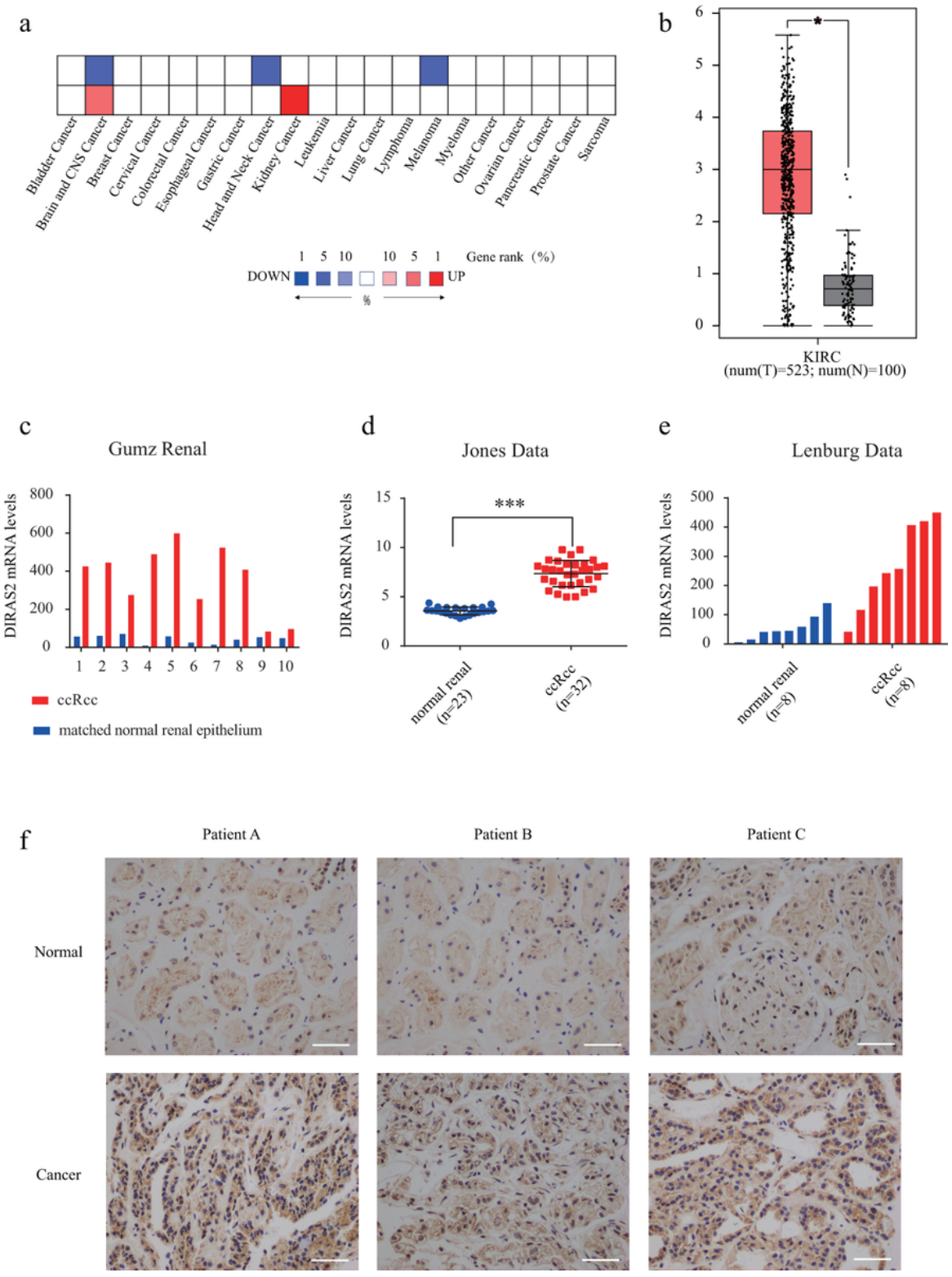
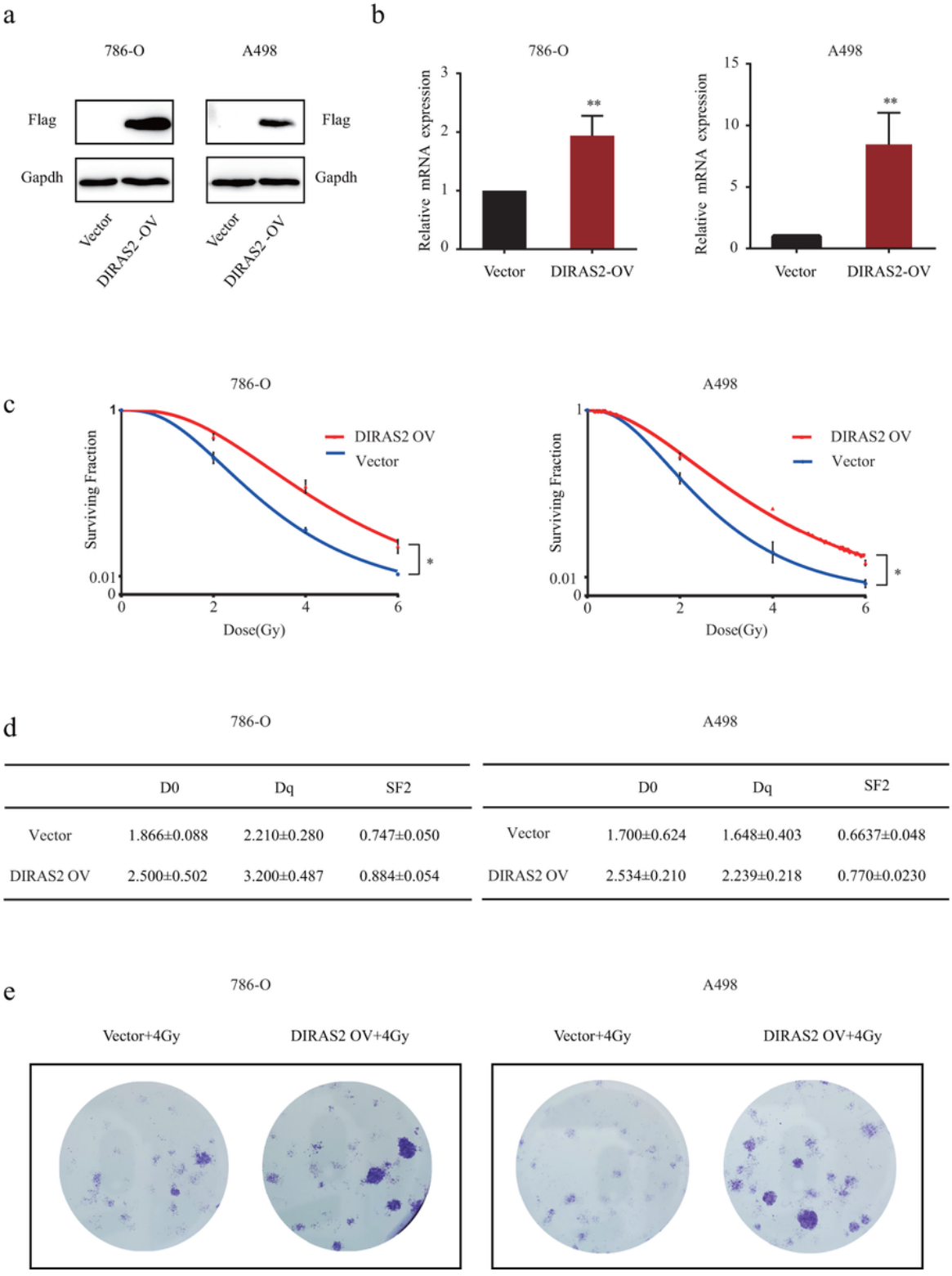


Figure 1

DIRAS2 is highly expressed in human ccRCC Analysis of DIRAS2 expression in different cancer types from Oncomine (a) and TCGA database (b). (c)-(e) Analysis of DIRAS2 expression in ccRCC versus normal tissues in three independent datasets from Oncomine database. *, p< 0.05 and ***, p<0.001. (f) Immunohistochemical staining of DIRAS2 expression in human ccRCC samples and normal tissues. Scale bar 100μm.



	D0	Dq	SF2
Vector	1.866±0.088	2.210±0.280	0.747±0.050
DIRAS2 OV	2.500±0.502	3.200±0.487	0.884±0.054

	D0	Dq	SF2
Vector	1.700±0.624	1.648±0.403	0.6637±0.048
DIRAS2 OV	2.534±0.210	2.239±0.218	0.770±0.0230

Figure 2

Overexpression of DIRAS2 increases radiation resistance in ccRCC cell lines The expression levels of DIRAS2 in 786-O and A498 cells transfected by DIRAS2 OV and Lv-NC lentivirus were verified by Western blot (a) and qRT-PCR (b). (c) The effect of DIRAS2 overexpression on radiosensitivity of 786-O and A498 cells measured by clonogenic survival assay. Cells were subjected to the indicated doses of IR (0, 2, 4, 6 Gy). (d) The mean lethal dose (D0), quasi-threshold dose (Dq), and survival fractions at 2 Gy (SF2) were

calculated using the single-hit multitarget model. The D0, Dq, and SF2 values of DIRAS2 OV groups were higher relative to Lv-NC groups, indicating the enhancement effect of DIRAS2 on radiation resistance in 786-O and A498 cells. (e) Colony formation of transfected 786-O and A498 cells exposed to 4 Gy radiation. These data were expressed as mean ± SD of three biological replicates (*, p< 0.05 and **, p<0.01.)

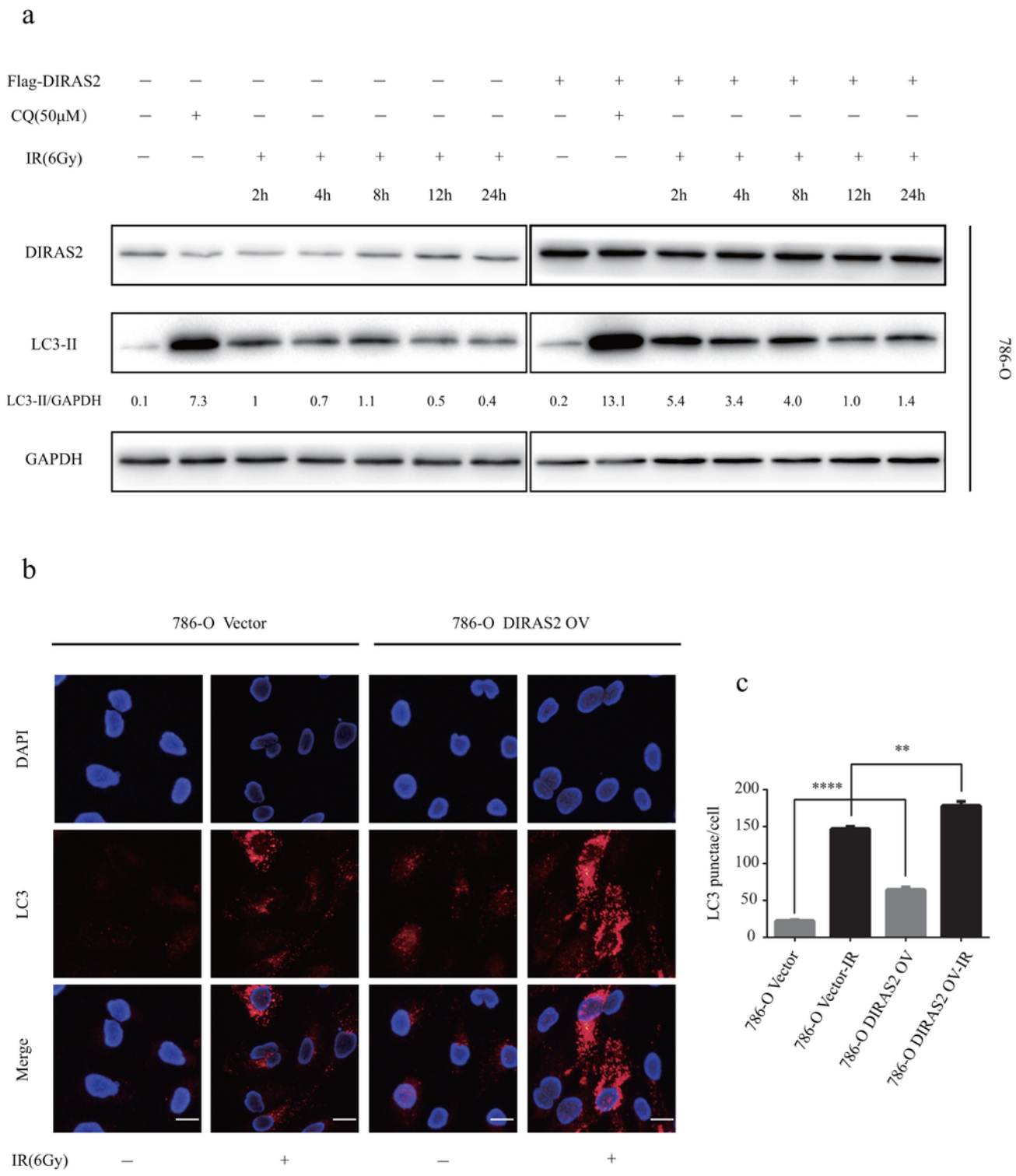
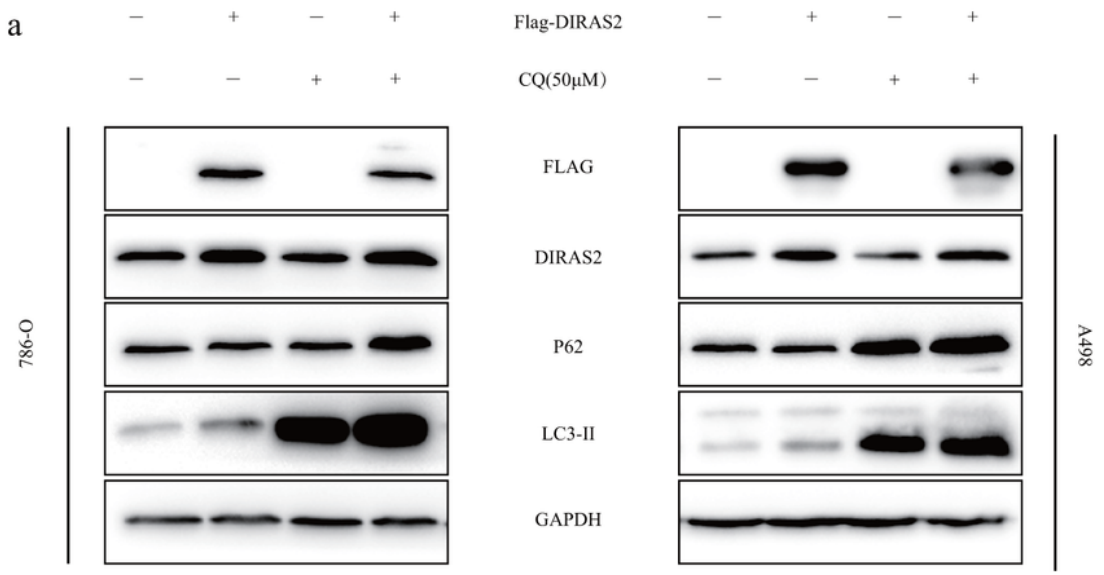
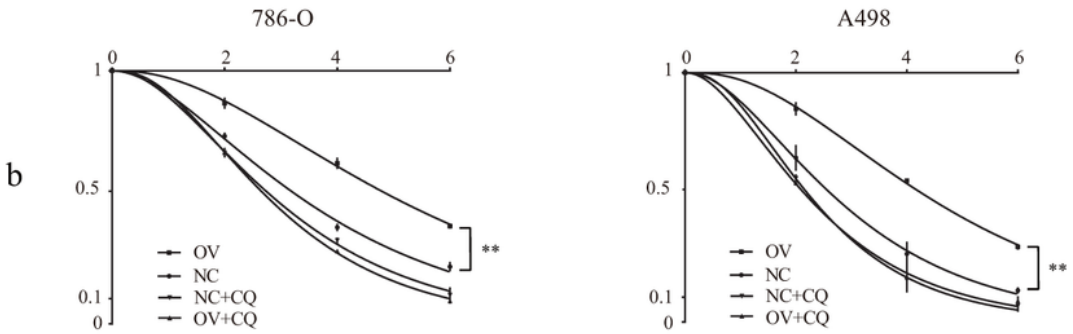


Figure 3

DIRAS2 overexpression enhances autophagy in response to ionizing radiation in ccRCC cells The 786-O cells overexpressing DIRAS2 (DIRAS2-OV), and the control cells (Lv-NC) were treated with or without IR (6 Gy) and the cell lysate was collected at the indicated time points after IR. (a) The protein levels of DIRAS2 and LC3-II were determined by Western blot analysis. Ratios of LC3-II/GAPDH were calculated by gray scale value analysis. (b) Laser confocal microscopy images showing LC3 staining in DIRAS2-OV, and Lv-NC groups of 786-O cells labelled with fluorescent antibodies to LC3 (red channel) at 2 hours post IR or mock treatment. Nuclear stained DAPI (blue channel). Scale bar 20μm. (c) Quantification of LC3 puncta from the images in (b). Data are shown as mean ± SD of three replicates. **, $p< 0.01$, and***, $p < 0.001$





c

		D0	Dq	SF2
786-O	Vector	2.683±0.124	0.832±0.030	0.731±0.011
	DIRAS2 OV	3.447±0.217	1.423±0.134	0.880±0.023
	Vector+CQ	1.815±0.048	0.815±0.046	0.679±0.018
	DIRAS2 OV+CQ	2.120±0.260	0.769±0.081	0.681±0.019
A498	Vector	1.990±0.065	0.702±0.110	0.654±0.053
	DIRAS2 OV	2.828±0.320	1.326±0.120	0.863±0.024
	Vector+CQ	1.701±0.438	0.458±0.137	0.556±0.012
	DIRAS2 OV+CQ	1.498±0.366	0.446±0.098	0.575±0.017

Figure 4

DIRAS2-induced radiation-resistance is rescued by pretreatment of autophagy inhibitor chloroquine (CQ)
DIRAS2-OV or Lv-NC cells of 786-0 and A498 were pretreated with or without 50 μM CQ for 12 hours, and then treated with indicated doses of ionizing radiation (0, 2, 4, 6 Gy). (a) The overexpression of DIRAS2 and autophagy inhibition was confirmed by Western blot analysis of Flag, DIRAS2, P62 and LC3-II expression. (b) The effect of autophagy inhibition by CQ on radiosensitivity of DIRAS2-OV or Lv-NC cells

of 786-0 and A498 measured by clonogenic survival assay. (c) The mean lethal dose (D0), quasi-threshold dose (Dq), and survival fractions at 2 Gy (SF2) were calculated using the single-hit multitarget model. Values represent the mean ± SD of three biological replicates (**, p< 0.01).

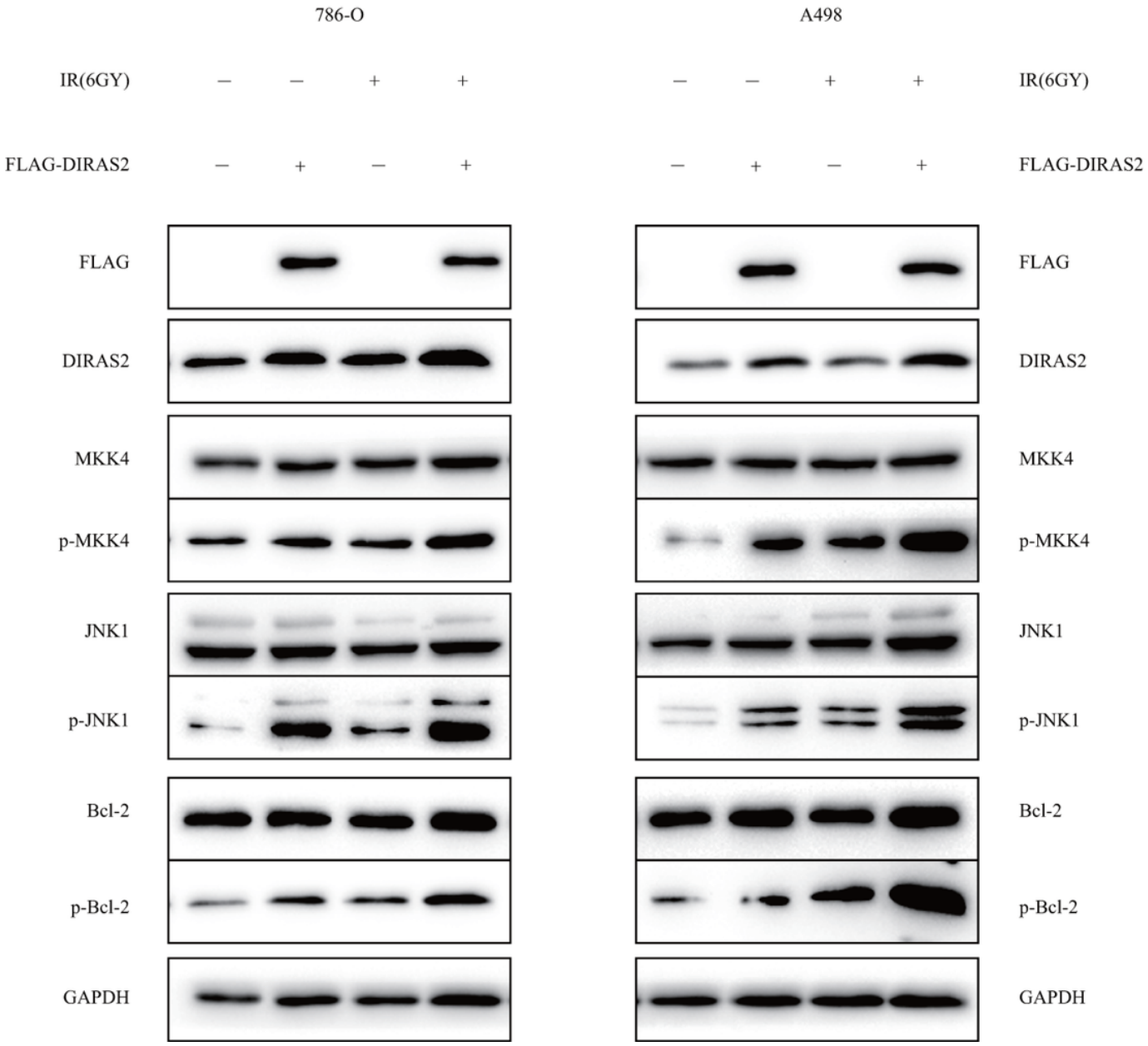


Figure 5

DIRAS2 activates MKK4-JNK1-Bcl-2 signaling in ccRCC cells DIRAS2 OV and Lv-NC groups of 786-O and A498 cells were treated with or without IR (6 Gy), the cell lysate was collected 12h later and subjected to Western blot analysis of Flag, Di-Ras2, MKK4, p-MKK4, JNK1, p-JNK1, Bcl-2, and p-Bcl-2.

Supplementary Files

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