AdipoR1 Regulates Ionizing Radiation-Induced Ferroptosis in HCC Cells Through Nrf2/xCT Pathway

Hao Feng
Wenzhou Medical University
Yi Liu
Wenzhou Medical University
Yuhan Gan
Wenzhou Medical University
Mengke Li
Wenzhou Medical University
Rui Liu
Jilin University
Lianchang Liu
Jilin University
Lan Li
Wenzhou Medical University
Huajian Chen
Wenzhou Medical University
Guanghui Li
Wenzhou Medical University
Zhujun Tian
Wenzhou Medical University
Shumei Ma
Wenzhou Medical University
Xiaodong Liu (Liuxd2014@126.com)
Wenzhou Medical University https://orcid.org/0000-0002-6879-990X

Research

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Abstract

Background: Ferroptosis is a type of cell death accompanied by iron-dependent lipid peroxidation, however, how IR-induced ferroptosis is regulated in Hepatocellular carcinoma cells (HCC) remains largely unknown. We have previously found that adiponectin receptor 1(AdipoR1) might be a prognostic biomarker for HCC after stereotactic body radiotherapy (SBRT). In this study, we aimed to elucidate the roles of AdipoR1 in radiation-induced Ferroptosis of HCC.

Methods: Human HCC cell line MHCC-97H and HepG2 and human hepatic cell lines LO2 were tested. qRT-PCR and western blotting were used to detect mRNA and protein expression respectively, colony formation assay was used to evaluate the radiosensitivity and flow cytometry was used to assess lipid peroxidation and cell death. Dual-Luciferase Reporter assay system was used to detect the transcription activity.

Results: Ionizing Radiation (IR) upregulated the expression of AdipoR1 in HCC cells and AdipoR1 knockdown could promote radiation sensitivity of HCC cells. AdipoR1 knockdown could decrease the expression of Nrf2 and Nrf2 protein stability. Nrf2 could bind to xCT promoter and promoted the transcription and expression of xCT. AdipoR1 knockdown increased significantly lipid peroxidation and ferroptosis induced by IR or Erastin respectively, which could be abolished by overexpression of Nrf2 and xCT.

Conclusion: AdipoR1 knockdown can promote radiation sensitivity of HCC cells; AdipoR1 regulates IR-induced cell death by AdipoR1-Nrf2-xCT pathway.

1. Background

Hepatocellular carcinoma (HCC) is the most common primary liver cancer and the third leading cause of cancer-related death worldwide, with around 745000 deaths reported annually[1, 2]. Most often, the HCC-related prognoses remain poor owing to the presence of underlying chronic liver disease, late diagnosis, and frequent recurrence or progression after treatment[3, 4]. Surgical resection, liver transplantation, and radiofrequency ablation (RFA) have been successfully developed as radical therapeutics for early stage HCC patients with well-preserved liver function[5]. Moreover, most patients with HCC are usually diagnosed at late stage, and usually receive local radiotherapy and chemotherapy[6, 7]. However, radiotherapy still has some inherent limitations, such as side effects and radiation resistance. It is urgent task to explore novel means that improve the radiosensitivity of HCC.

Radiotherapy uses high-energy ionizing radiation (IR) to produce DNA double-strand breaks that induce various modes of cell death, including apoptosis, necrosis, autophagy, ferroptosis and mitotic catastrophe[8]. Ferroptosis, an iron-dependent form of regulated cell death that is induced by excessive lipid peroxidation, is morphologically and mechanistically distinct from apoptosis[9–11]. It is regulated by a network revolving around glutathione peroxidase 4 (GPX4). Correspondingly, inactivation of GPX4 or xCT by genetic or pharmacologic means induces ferroptosis[9, 12, 13]. Emerging evidence supports

important roles that ferroptosis plays in tumor suppression and cancer resistance to therapy[14–18]. As the light chain encoded by xCT is a subunit unique to system Xc−, the xCT expression level often positively correlates with the activity of the antiporter[19]. The cystine/glutamate antiporter xCT (also commonly known as xCT) functions to import cystine for glutathione biosynthesis and antioxidant defense and is overexpressed in multiple human cancers. Indeed, the mechanisms of ferroptosis inducers (such as Erastin) is to inhibit system Xc−-mediated cystine import, causes depletion of intracellular GSH and subsequent iron-dependent lipid peroxidation[20, 21]. Nuclear factor E2 related factor 2 (Nrf2) is the key regulatory factor required for cells to maintain an oxidative steady state and is activated under conditions of high oxidative stress. Nrf2 can then promote target gene transcription, as well as antioxidant and anti-inflammatory protein translation by combining to the antioxidant response element (ARE) in the nucleus, which promotes cellular protection[22]. Moreover, Nrf2 exerts its anti-ferroptotic role by regulating xCT expression, knock down Nrf2 significantly decreases the level of xCT and facilitates the accumulation of lipid peroxide[23].

Adiponectin is emerging as a protein with insulin-sensitizing, anti-inflammatory and antiapoptotic functions. Adiponectin is released by adipocytes and targets a multitude of different cell types, among which prominent target cells are hepatocytes, cardiac myocytes, pancreatic β cells and podocytes[24]. Two related receptors have been cloned, AdipoR1 and AdipoR2, which may mediate some of the actions of adiponectin. Very recently, studies have shown that adiponectin receptor is a key mediator in the development and possible progression of several types of cancers, such as breast cancer, colorectal cancer and renal cell carcinoma[25, 26]. However, how adiponectin regulates the progression of HCC and its underlying mechanisms remain unknown. Therefore, our study aimed to explore the effect of AdipoR1 knockdown combined with radiotherapy on HCC through in vitro assays, investigate the potential mechanism, and provide a strong theoretical basis for exploration of clinical combinations of radiation and AdipoR1 in HCC.

2. Materials And Methods

2.1. Cell culture, IR and drug treatment

Human hepatocellular carcinoma cell lines MHCC-97H, HepG2 and normal hepatic cells LO2 were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and confirmed by STR. The cell was cultured in Dulbecco's modified Eagle's medium mixture medium (Invitrogen Inc.) supplemented with 10% fetal bovine serum (Invitrogen Inc.), and 1% penicillin/streptomycin (cat. no. 10378016, Life Technologies) in a humidified 5% CO^2, 37°C incubator. Cells were exposed to ionizing radiation (10Gy) using an X-ray generator (X-RAD 320 ix, Precision X-ray Inc., North Branford, CT, USA) at a dose rate of 3 Gy/min. The distance between the source and the target was 50 cm. cells were pretreated with 20μm Erastin (Selleck Chemicals, USA) for 24hs to induce ferroptosis.

2.2. Cell viability and cell death assays

Cell viability was determined by Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Japan) according to the manufacturer's protocol. Cells were seeded in 96-well plates (2× 10^3 cells/well) and pretreated with Erastin (20μm). CCK-8 was added to each well, and the cells were incubated for 3h. OD values were recorded at 450 nm using a microplate reader. The proliferation rate of cells was calculated by the following formula: cell viability = (OD experimental group-OD blank/OD control group-OD blank) × 100%. Trypan blue (prod. no. T8154) staining was used for observation of cell death by flow cytometry. Cells were seeded in 6-well plates (8× 10^4 cells/well) and irradiated with 10Gy. The cell supernatant and cell in the culture dish were collected and centrifuged at 500xg for 5 minutes at 4℃. The cell pellet was washed with PBS, stained with trypan blue for 3 minutes, and detected cell death by flow cytometry.

2.3. Colony formation assay and Lipid peroxidation detection

Cells were placed in 6-well plates and cultured in DMEM (Invitrogen) containing 10% FBS (Gibco) at 37°C and 5% CO^2 for 14 days. To visualize and count the colonies, the colonies were fixed with 4% paraformaldehyde for 30 min and stained with 0.2% crystal violet (Solarbio, Beijing, China) at room temperature for 15 min. The colonies containing ≥50 cells per dish were counted. Lipid peroxidation detection: Cells (2 × 10^5) in 6-well plates were incubated with 1 ml of fresh medium containing 5 μM of BODIPY 581/591C11 (Invitrogen) at 37°C in the dark for 30min. Cells were then trypsinized, washed, and resuspended in 0.2 ml of PBS for flow cytometry analysis. A minimum of 20,000 cells were analyzed per condition.

2.4. Lentiviral infections, Expression vectors and siRNAs

Lentiviral short hairpin RNA (shRNA) vector targeting AdipoR1 (pLKO.1-shAdipoR1) was constructed according to the protocol of pLKO.1-blasticidin vector (Addgene, Cambridge, MA, USA). Briefly,the forward oligo: CCGGCGTCTATTGTCATTCAGA

GAACTCGAGTTCTCTGAATGACAATAGACGTTTTTG and reverse oligo: AATTC

AAAAACGTCTATTGTCATTCAGAGAACTCGAGTTCTCTGAATGACAATAGACG were annealed and inserted into the pLKO.1-blasticidin vector. Control vector pLKO.1-shScramble was also purchased from addgene. Lentiviruses were produced in 293T cells after co-transfection of pLKO.1-shAdipoR1 or pLKO.1-shScramble, packing plasmid psPAX2 and envelope plasmid pMD2G. The supernatant containing viruses was collected 48h after transfection, filtered, and used for infecting target cells in the presence of 10μg/ml of polybrene (Sigma-Aldrich, H9268) prior to drug selection with 7μg/ml of blasticidin for one week. The xCT and Nrf2 cDNA was PCR amplified and cloned into pcDNA3.1Flag at BamHI and XhoI sites, respectively. siRNA, targeting human xCT, and the corresponding control, siRNANC were purchased from GenePharma (Shanghai, China).

2.5. Transfections and Luciferase reporter assays

Transfections were carried out using Lipofectamine 2000 (Invitrogen) according to a protocol provided by the manufacturer. Double luciferase–reporter gene determination was conducted to determine whether xCT was the direct target gene of Nrf2. Reporter plasmids of xCT promoter double luciferase was synthesized from Generalbiol (Anhui, China) and inserted into the pGL3-Basic vector. pGL3-Basic xCT promoter and pGL3-Basic vector were cotransfected into cells with PCDNA3.1-Flag Nrf2, respectively, with Lipofectamine 2000. After transfection for 48 hours, luciferase activity was determined on a Centro LB 960 Luminometer (Berthold Technologies, Germany) and the activity of renilla luciferase was used as a standardized control.

2.6. qRT-PCR

Total RNA was extracted from HCC cells with Trizol solution (TaKaRa, Dalian, China). PrimeScriptTM RTMaster Mix (TaKaRa, Dalian, China) was used for reverse transcription. qRT-PCR was carried out on a QuantStudio Real-Time PCR instrument (Thermo Fisher Scientific, USA) using SYBR Premix Ex Taq II (TaKaRa). The conditions of thermal cycling were illustrated as follows: 95°C for 30 s followed by 40 cycles at 95°C for 5 s and at 60°C for 30 s. PCR was performed using the following primers; SLC7A11/xCT forward: CCATGGGTGGAATCATATTGGA, reverse: TCAA

CGGATTTGGTCGTATTGG; AdipoR1 forward: CTCATCTACCTCTCCATCGT, rev

erse: GAACACTCCTGCTCTTGTCT; PTGS2 forward: TAGGATTCAGGGCTTTCA

CTGGCT, reverse: TGTCAGCCGACAATGAGATGTGGA; GAPDH forward: GCGT

GGGCATGTCTCTGAC, reverse: GCTGGTAATGGACCAAAGACTTC.The $2^{-\Delta\Delta Ct}$

Method was utilized to calculate the relative expression levels.

2.7. Western blot analysis

Cells were washed with PBS, collected with a cell scraper, and lysed with RIPA buffer. Total proteins (20μg) were separated on a 12% SDS-PAGE and transferred onto a PVDF membranes (Millipore). After blocking with 6% skim milk in Tris-buffered saline-tween (TBST) for 1 h at room temperature, the membranes were incubated overnight at 4°C with primary antibodies, and then after washing incubated with each secondary antibody for 1h at room temperature. The following antibodies were used: AdipoR1 (ab126611), Transferrin (ab8241), GPX4 (125066), (Abcam, Cambridge, MA, USA); xCT (#12691S), CD71 (#13113S), Nrf2 (#12721S), (Cell Signaling Technology, Danvers, MA USA); GAPDH (Proteintech, Rosemont, IL); Actin (A3853, Sigma); AdipoR1 (SC-518030, SANTA). The immunoreactions were visualized by ECL solution (Thermo Fisher Scientific, USA) analyzed using the Image J software (Bio-Rad).

2.8. Statistical analysis

SPSS 22.0 software (SPSS, Chicago, IL, USA) was utilized for statistical analyses in this study. Differences between two groups were evaluated with Student's *t*-test (two-tailed). One-way ANOVA

followed by Bonferroni post hoc tests were performed to analyze multiple groups. Survival curve was generated with Kaplan–Meier method. Experimental results are presented as mean ± SD. Data was considered as statistically significant when *p*-value was less than 0.05.

3. Result

3.1. IR upregulated the expression of AdipoR1 in HCC cells

Referring to the TCGA, AdipoR1 mRNA level was upregulated in tumor tissues compared with normal tissues (Fig. S1A). K-M curve showed that high expression level of AdipoR1 was related to poor prognosis in HCC patients (Fig. S1B). Furthermore, the basal expression of AdipoR1 in HCC cells was tested and remarkably increased compared to normal hepatic cells (Fig. 1A-B). We have previously found that adiponectin receptor 1(Adipor1) might be a prognostic biomarker for HCC after stereotactic body radiotherapy (SBRT). Next, we analyzed the expression of AdipoR1 under IR in HCC cells, 10Gy X-rays increased the protein level of AdipoR1 in both MHCC-97H and HepG2 cells compared with sham (Fig. 1C-F). Taken together, these results demonstrated that radiation upregulated the expression of AdipoR1 in HCC cells.

3.2. AdipoR1 knockdown increased IR-induced cell death in HCC cells

Radiotherapy uses high-energy ionizing radiation to produce DNA double-strand breaks that induce various modes of cell death, including apoptosis, necrosis, autophagy, and mitotic catastrophe. To investigate the effects of AdipoR1 on radiation induced cell death, we generated AdipoR1-knockdown MHCC-97H and HepG2 cells by shRNA. Effect of knockdown AdipoR1 was validated by Western blot (Fig. 2A, D). AdipoR1 knockdown increased radiation-induced cell death in both of MHCC-97H (Fig. S2B-C) and HepG2 (Fig. S2E-F) cells.

To further determine the effects of AdipoR1 on radiosensitivity, colony formation assay was performed. MHCC-97H (Fig. 2B-C) and HepG2 cells (Fig. 2E-F). Colony formation revealed that AdipoR1-knockdown significantly reduced the survival rate in HCC cells, compared with shControl group. Taken together, these results demonstrated that AdipoR1knockdown increased radiosensitivity in HCC cells.

3.3. AdipoR1 is involved in IR-induced ferroptosis in HCC cells

It has been reported that IR induces ferroptosis in cancer cells [27]. We further investigated whether AdipoR1 regulates IR-induced ferroptosis in HCC cell. The cell viability of MHCC-97H with AdiopR1 knockdown group was lower than shControl group following IR treatment. To investigate which type of cell death AdipoR1 was involved in, MHCC-97H cells with AdipoR1 knockdown or shControl were pretreated by different inhibitors of cell death, as 3-MA, ZVAD, Rapa, Fer-1, and irradiated. Compared to

DMSO group, the cell viability was significantly higher in ZVAD and Fer-1 group in shControl cells following IR treatment, but failed to change in shAdipoR1 cells, indicating IR induced apoptosis and ferroptosis in MHCC-97H cells in an AdipoR1 dependent manner (Fig. 3A). Moreover, a hallmark of ferroptosis is the accumulation of lipid peroxidation [10]. Similarly, lipid peroxidation was also up-regulated in AdipoR1 knockdown cells compared with shControl group after IR treatment (Fig. 3B-E). Apart from lipid peroxidation, ferroptosis induction is also associated with the increased expression of ferroptosis marker genes such as PTGS2[10]. Consequently, the expression of PTGS2 was increased after IR in MHCC-97H cells (Fig. 3F). AdipoR1 knockdown also increased the expression of PTGS2 (Fig. 3G). These data suggested that ferroptosis occupied a very important position in IR-induced cell death within HCC cells, and AdipoR1 acted as a key regulator of this effect.

3.4. AdipoR1 is involved in Erastin-induced ferroptosis in HCC cells

Erastin is a classic ferroptosis activator and triggers cell ferroptosis in a variety of cell types. To determine whether Erastin-induced ferroptosis in HCC cells, first the median lethal concentration LC50 of Erastin were tested in MHCC-97H and HepG2 cells (Fig. 4A-B), the LC50 values of Erastin is 40μm and 20μm respectively, for MHCC-97H for HepG2 cells. Next, the lipid peroxidation was detected and evaluated in MHCC-97H and HepG2 cells following Erastin for 48h (Fig. 4C-D), suggesting Erastin could induced ferroptosis in HCC cells. Furthermore, to investigate whether AdipoR1 is involved in Erastin-induced ferroptosis in HCC cells, as shown in Fig. 4E-I, compare with the shControl cell, AdipoR1 knockdown further reduced cell survival rate and elevated lipid peroxidation after Erastin treatment (Fig. 4E-I).

We reasoned that Erastin -induced ferroptosis might also involve other mechanisms that modulate the expression or activity of proteins involved in ferroptosis pathways. To test this hypothesis, we examined the expression level of several key proteins involved in ferroptosis pathways in response to Erastin. As shown in Fig. 4J, K, after treatment with Erastin at 20 μM for 24 h, the expression of AdipoR1was induced in MHCC-97H, decreased in HepG2, the expression of xCT was increased, decreased the expression of GPX4 in both of MHCC-97H and HepG2. Our results showed that AdipoR1 is involved in Erastin-induced ferroptosis in HCC cells.

3.5. Effect of AdipoR1 on ferroptosis-related proteins in HCC cells after IR

As previously pointed out, AdipoR1-knockdown could increase IR-induced ferroptosis in HCC cells. To study the potential mechanisms. we examined the expression levels of several key protein involved in ferroptosis in response to IR. Transferrin is the main iron-containing protein in plasma, responsible for carrying the iron absorbed by the digestive tract and the iron released by the degradation of red blood cells. Transferrin receptor 1 (CD71) is a type II transmembrane receptor and carrier protein responsible for the uptake of cellular iron through receptor-mediated endocytosis[28]. As shown in Fig. 5A, B,

AdipoR1knockdown significantly inhibited expression of xCT, CD71 and Transferrin in MHCC-97H with or without IR. AdipoR1knockdown decreased the expression of xCT, but failed to change the expression of CD71 and Transferrin in HepG2 cells with or without IR (Fig. 5C-D), Suggesting that AdipoR1 regulated the expression of xCT after IR in HCC cells.

3.6. AdipoR1 protected cells from IR-induced ferroptosis by regulating the expression of xCT

To investigate the relationship between the protein expression of AdipoR1 and xCT, we analyzed publicly available gene expression datasets and found that the AdipoR1 expression level positively correlated with the xCT level in two cohorts of hepatoma Carcinoma patients (Fig. 6A). Compared with the cells in shControl group, the xCT protein levels were dramatically decreased in the shAdipoR1group (Fig. 6B-C).This correlation is of significance given that xCT overexpression found in lung adenocarcinoma[29], colorectal cancer[30], HCC cells[31] has been shown to promote the growth of the tumors. To further verify whether AdipoR1 regulates ferroptosis via xCT in IR treatment, our results showed that xCT knockdown increased IR-induced lipid peroxidation and cell death, which could be significantly rescued by Fer-1 (Fig. 6D-H). Subsequently, xCT was overexpressed through transfected with pcDNA3.1Flag-xCT, overexpression of xCT successfully suppressed IR-induced lipid peroxidation and cell death in HCC cells (Fig. 6I-M). These results suggested that AdipoR1 protected cells from IR-induced ferroptosis by promoting the expression of xCT, which may be its key downstream target.

3.7. AdipoR1 regulated IR-induced ferroptosis by AdipoR1-Nrf2-xCT pathway

Previously, Nrf2 exert its antioxidant role in cellular protection by regulating xCT expression[23]. To further explore the molecular mechanism how AdipoR1 regulated xCT. Firstly, we determined whether AdipoR1 knockdown influenced the expression of Nrf2. As shown in Fig. 7A, AdipoR1 knockdown inhibited expression of Nrf2 and xCT compared to the in MHCC-97H. To determine whether AdipoR1 regulates the Nrf2 stability, cells were treated with protein synthesis inhibitor cycloheximide (CHX) for different periods of time. CHX treatments significantly reduced Nrf2 protein levels in AdipoR1 knockdown MHCC-97H cells compared with vector control cells (Figure 7B-C), indicating that AdipoR1 knockdown decreased NRF2 protein stability. Moreover, luciferase reporter assay determined that Nrf2 could bind to xCT promoter, and improve the transcription of xCT (Fig. 7D) Subsequently, overexpression of Nrf2 successfully reduced the IR-induced lipid peroxidation and cell death in AdipoR1 knockdown cells (Fig. 7E-H). These results suggested that AdipoR1 protected cells from IR-induced ferroptosis by promoted the expression of Nrf2, furthermore, increased the transcription and expression of xCT. Therefore, AdipoR1 might regulate IR-induced ferroptosis by AdipoR1-Nrf2-xCTpathway in HCC cells.

3.8. A schematic model demonstrating the roles of AdipoR1 in IR-induced ferroptosis

In summary, radiation increased the protein expression of AdipoR1, Furthermore, AdipoR1 promoted the expression of transcriptor factor Nrf2, Nrf2 incresed xCT transcription and expression, this directly contributed to the protective function in the early stage of radiation in HCC cells. When AdipoR1 was knock downed, the antioxidant defense was destroyed, then Lipid peroxidation increased and caused ferroptosis in HCC cells. Our finding Suggested that AdipoR1 may regulate IR-induced cell death by AdipoR1-Nrf2-xCT pathway. (Figure. 8).

4. Discussion

Previously, many studies have shown that like apoptosis, ferroptosis serves as a natural barrier to tumor development[15, 32–36]. In this study, we show that ferroptosis can also be induced by radiotherapy, one of the most widely used cancer therapies. Ferroptosis is a nonapoptotic form of cell death that can be induced by metabolic stress such as GSH depletion[10]. Mechanistically, IR induces lipid peroxidation, a hallmark of ferroptosis, through at least 2 parallel pathways. First, IR-induced ROS promote lipid peroxidation; Second, IR induces ACSL4 expression, which is critical for mediating the biosynthesis of PUFA-PLs, the type of lipids that are particularly susceptible to peroxidation[37]. Adiponectin has insulin-sensitizing, anti-inflammatory, antiatherogenic, cardioprotective effects as well as distinct effects on lipid metabolism[25, 38, 39]. Key metabolic actions include regulation of glucose and lipid metabolism through stimulation of fatty acid oxidation, suppression of hepatic glucose output, and increased insulin sensitivity in liver and skeletal muscle.

Ferrostatin-1(Fer-1), a potent and selective ferroptosis inhibitor, acts via a reductive mechanism to prevent damage to membrane lipids and thereby inhibits cell death. The IR-induced cell death could be alleviated by Fe-1, which suggests that IR could induce ferroptosis in HCC cells. AdipoR1 konckdown promoted IR induced ferroptosis in HCC cells (see Fig. 2, 3). The effect is equivalent to Erastin we have tested (see Fig. 4). Meanwhile, we propose that IR also induces an adaptive response involving xCT induction that dampens IR-induced ferroptosis and promotes cancer cell survival during radiotherapy, leading to radioresistance. This radioresistance mechanism is analogous to the pathway reactivation mechanism that leads to cancer cell resistance to targeted therapies[40] in that both resistance mechanisms restore the original downstream signaling output. Alternatively, certain genetic alterations in tumor cells (such as mutations in Keap1-Nrf2 signaling in lung or esophageal cancer) lead to aberrant expression of xCT (or presumably GPX4 in other contexts), resulting in de novo radioresistance[37]. GPX4, a glutathione peroxidase, utilizes reduced glutathione to convert lipid hydroperoxides to lipid alcohols, thereby mitigating lipid peroxidation and inhibiting ferroptosis[12, 13, 41]. Recent studies revealed that xCT overexpression inhibits ferroptosis through importing cystine, promoting GSH biosynthesis, and subsequently facilitating GPX4-mediated detoxification of lipid peroxides[10]. Ferroptosis induced by xCT or GPX4 inactivation can be largely abolished by inactivation of ACSL4, a lipid metabolism enzyme[42–44]. We have found that AdipoR1knockdown is able to significantly inhibit expression of xCT (see Fig. 5). Overexpression of xCT in AdipoR1 knockdown cells rescued the IR-induced lipid peroxidation and cell death (see Fig. 6). Interestingly, Overexpression of Nrf2 also had same result like Overexpression of xCT,

also luciferase reporter assay also determined that Nrf2 could regulate transcription of xCT (see Fig. 7). Our finding Suggested that AdipoR1 may regulate IR-induced cell death by AdipoR1-Nrf2-xCT pathway.

5. Conclusion

Together, these results suggested that AdipoR1 knockdown can promote radiation sensitivity of HCC cells; AdipoR1 regulates IR-induced cell death by AdipoR1-Nrf2-xCT pathway. For the first time, this study elucidated that AdipoR1 could function in the regulation of IR-induced ferroptosis and might provide a novel effective radiotherapy target for HCC treatment.

Abbreviations

GPX4 (glutathione peroxidase 4); GSH (glutathione); ARE (antioxidant response element); HCC (Hepatocellular carcinoma); IR (ionizing radiation); AdipoR1 (Adiponectin receptor one); AdipoR2 (Adiponectin receptor two); STR (Short tandem repeat); OD (Optical Density); SLC7A11/xCT (Solute Carrier Family 7 Member 11); CD71 (Transferrin receptor 1); TBST (Tris-buffered saline-tween); K-M (Kaplan–Meier); TCGA (The Cancer Genome Atlas); SBRT (Stereotactic body radiotherapy); 3-MA (3-Methyladenine); ZVAD (Z-VAD-FMK); RAPA (Rapamycin); Fer-1 (Ferrostatin-1); qRT-PCR (Real-Time Quantitative Reverse Transcription PCR); ROS (Reactive oxygen species); PUFA-PLs (polyunsaturated-fatty-acid–containing phospholipids).

Declarations

Acknowledgments

Not applicable.

Availability data and materials

All data generated or analyzed during this study are included in this published article.

Author Contributions

H.F. designed the study and wrote the manuscript; H.F., Y.L. and Y.G. performed cellular experiments; M.L., R.L. and L.L. (Lianchang Liu) performed RT-PCR and the study of the signaling pathway; H.C., G.L. and Z.T. analyzed the data; L.L. (Lan Li), X.L. and S.M. revised the manuscript. All authors read and approved the fnal manuscript.

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Ethics approval and consent to participate

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Consent for publication

All authors have read the manuscript and approved the fnal version.

Competing interests

The authors declare that they have no competing interests.

Author details

[1]School of Public Health and Management, Wenzhou Medical University, Wenzhou, Zhejiang 325035, China. [2] NHC Key Laboratory of Radiobiology (Jilin University), Changchun, Jilin 130021, China. [3]Key Laboratory of Watershed Science and Health of Zhejiang Province, Wenzhou Medical University, Wenzhou, China. [4]South Zhejiang Institute of Radiation Medicine and Nuclear Technology, Wenzhou, Zhejiang 325035, China. [5]The second hospital of Ji Lin University, Changchun, Jilin 130021, China.

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Figures

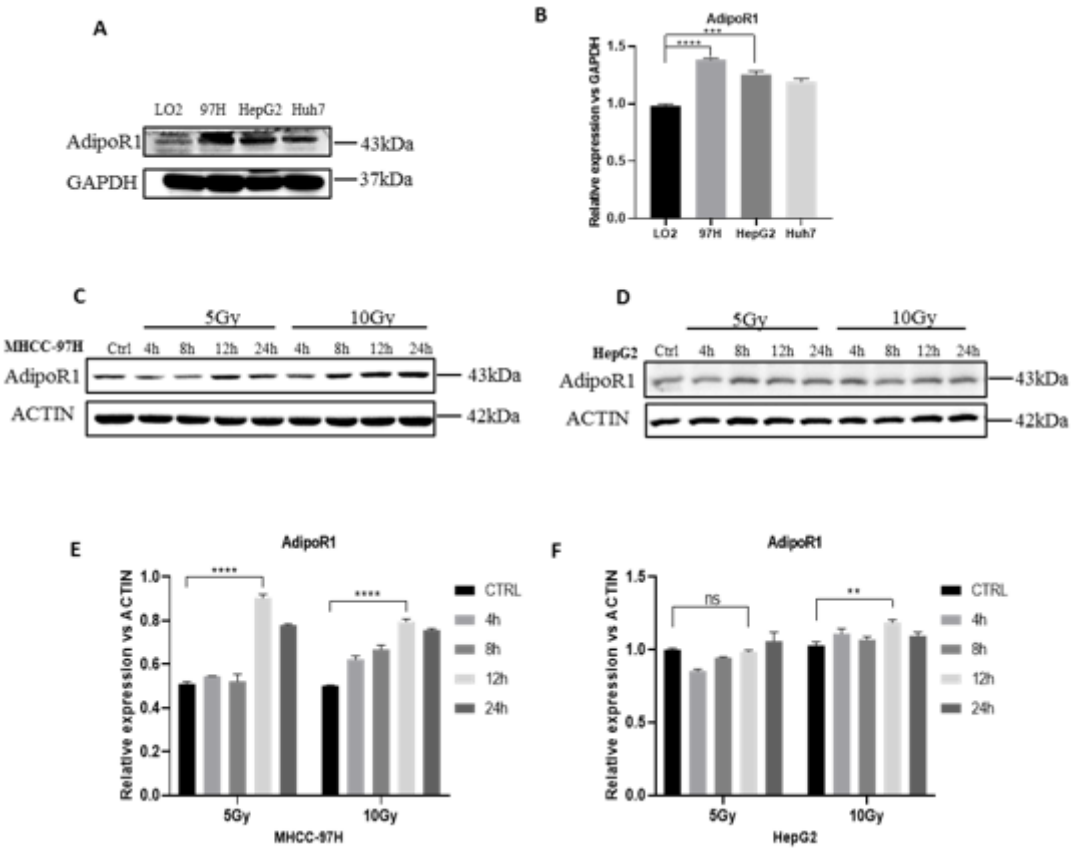

Figure 1

IR upregulated the expression of AdipoR1 in HCC cells. (A-B) Western blotting showed the basal expression level of AdipoR1 in HCC cells and normal hepatic cells. (C-F) Western blotting showed the expression level of AdipoR1 after IR (5Gy, 10Gy) in MHCC-97H and HepG2 cells.

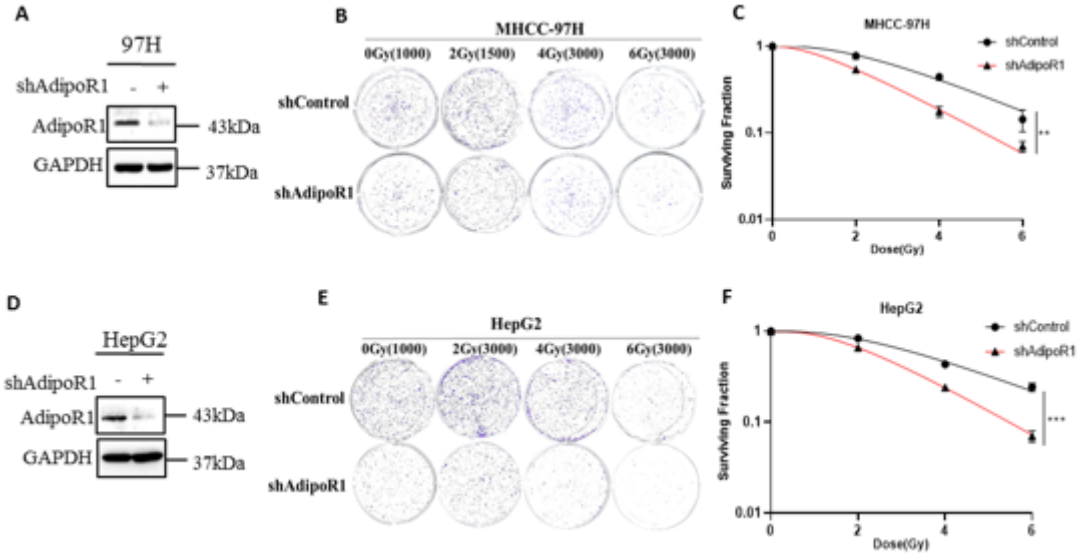

Figure 2

AdipoR1-knockdown increased radiation-induced cell deaths in HCC cells. (A, D) AdipoR1 knock down was determined by Western blot in MHCC-97H (A) and HepG2 (D) cells. (B-F) After knock down of AdipoR1, sensitization of radiation-treated MHCC-97H (B-C) and HepG2 (E-F) cells was evaluated through colony formation assay, respectively. The sensitization of radiation was measured using the multi-target, single-hit model. Data is presented as the mean ± SD, *p < 0.05, ** p < 0.01, *** p < 0.001.

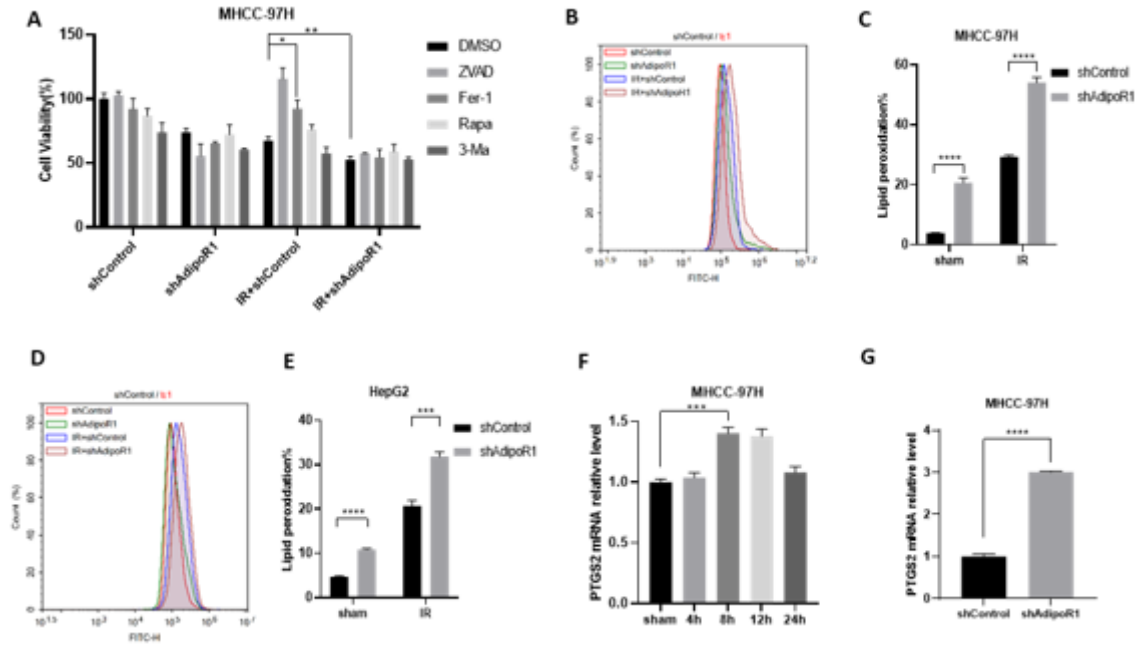

Figure 3

AdipoR1 is involved in IR-induced ferroptosis in HCC cells. (A) MHCC-97H cells, were pretreated with different inhibitors of cell death 3-MA, ZVAD, Rapa and Fer-1 and irradiated. The cell viability was detected by CCK-8 kit. (B-E) After treatment with IR (10Gy) for 48h, lipid peroxidation was assessed by

flow cytometry using C11-BODIPY in AdipoR1 knockdown MHCC-97H (B-C) and HepG2 (D-E) cells respectively. (F-G) qRT-PCR showed the mRNA level of PTGS2 in HCC cells after radiation or AdipoR1 knockdown. Data is presented as the mean ± SD, *p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

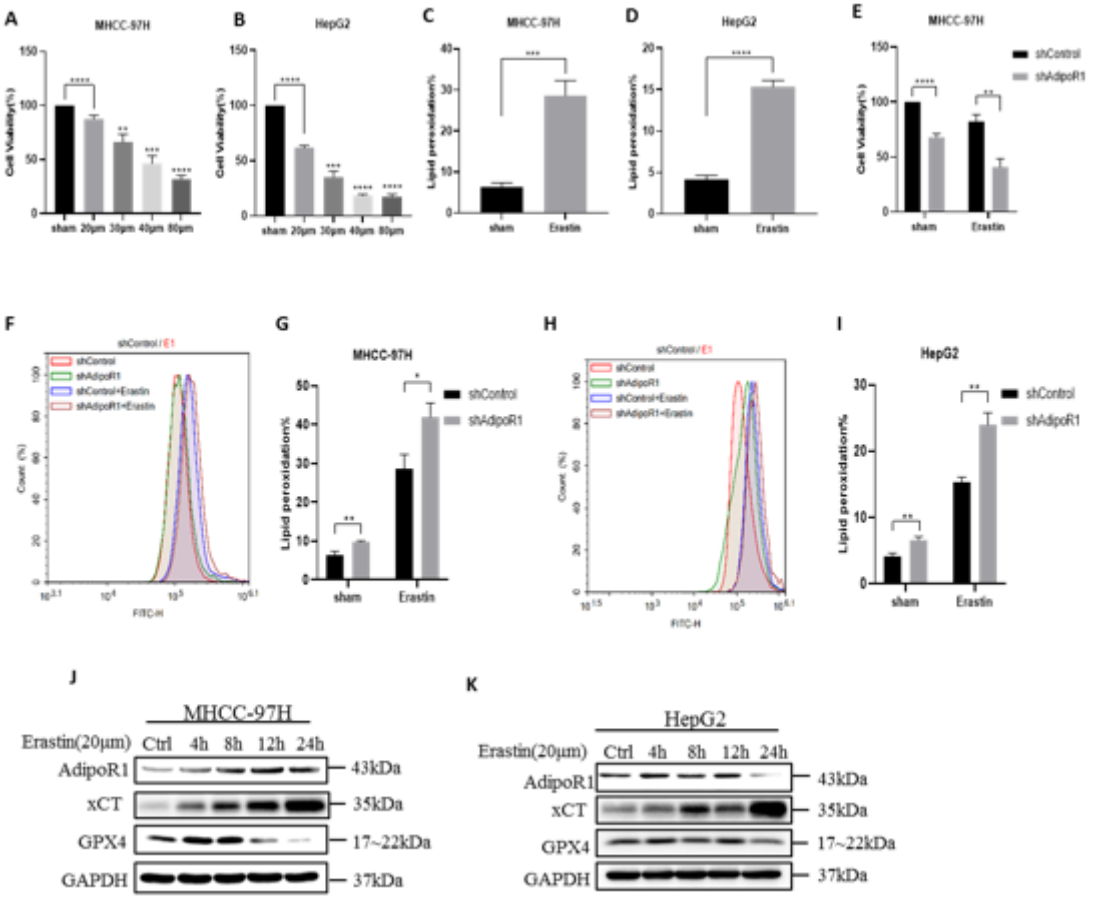

Figure 4

AdipoR1 is involved in Erastin-induced ferroptosis in HCC cells. (A-D) Lipid peroxidation and cell viability of Erastin-treated MHCC-97H and HepG2 cells were assessed by flow cytometry using C11-BODIPY (A-B, *: vs Ctrl) and CCK-8 kit respectively. (E-I) After treatment with Erastin (20μm) for 48h, lipid peroxidation and cell viability were assessed by flow cytometry using C11-BODIPY and CCK-8 kit in AdipoR1-knockdown MHCC-97H and HepG2 cells respectively. (J-K) After treatment with Erastin(20μm) for various times (4h, 8h, 12h, 24h), western blot showed the protein expression of AdipoR1, xCT, GPX4 in HCC cells. Data is presented as the mean ± SD, *p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

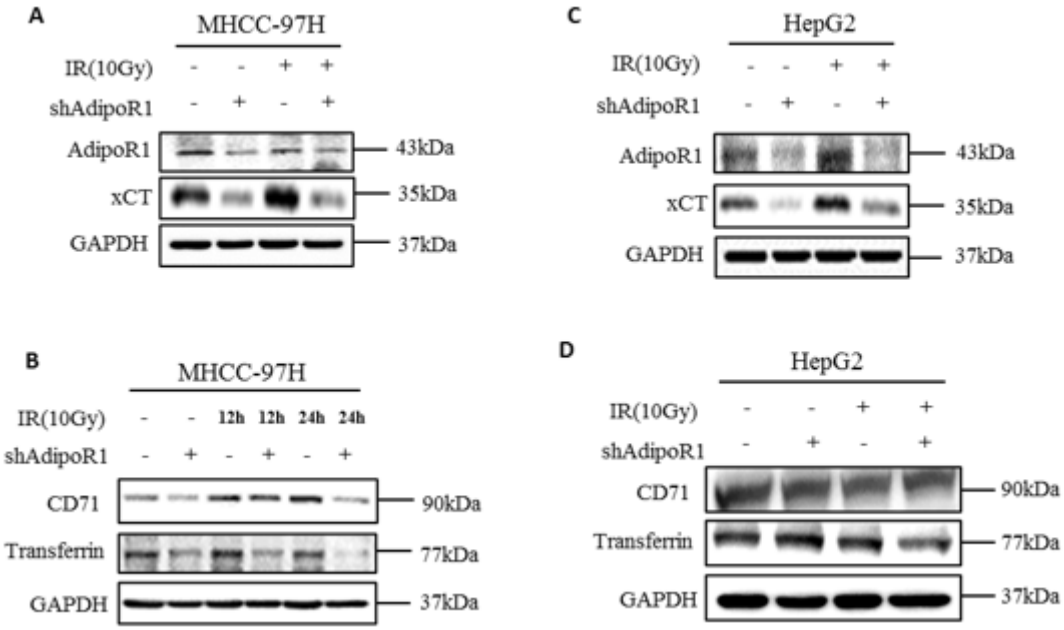

Figure 5

Effect of AdipoR1 on ferroptosis-related proteins in HCC cells after IR. (A-D) After knock down of AdipoR1 by shRNA in MHCC-97H and HepG2 cells, followed by 10Gy radiation, western blot showed the effects of AdipoR1 on the ferroptosis-related proteins. (A-B) MHCC-97H. (C-D) HepG2.

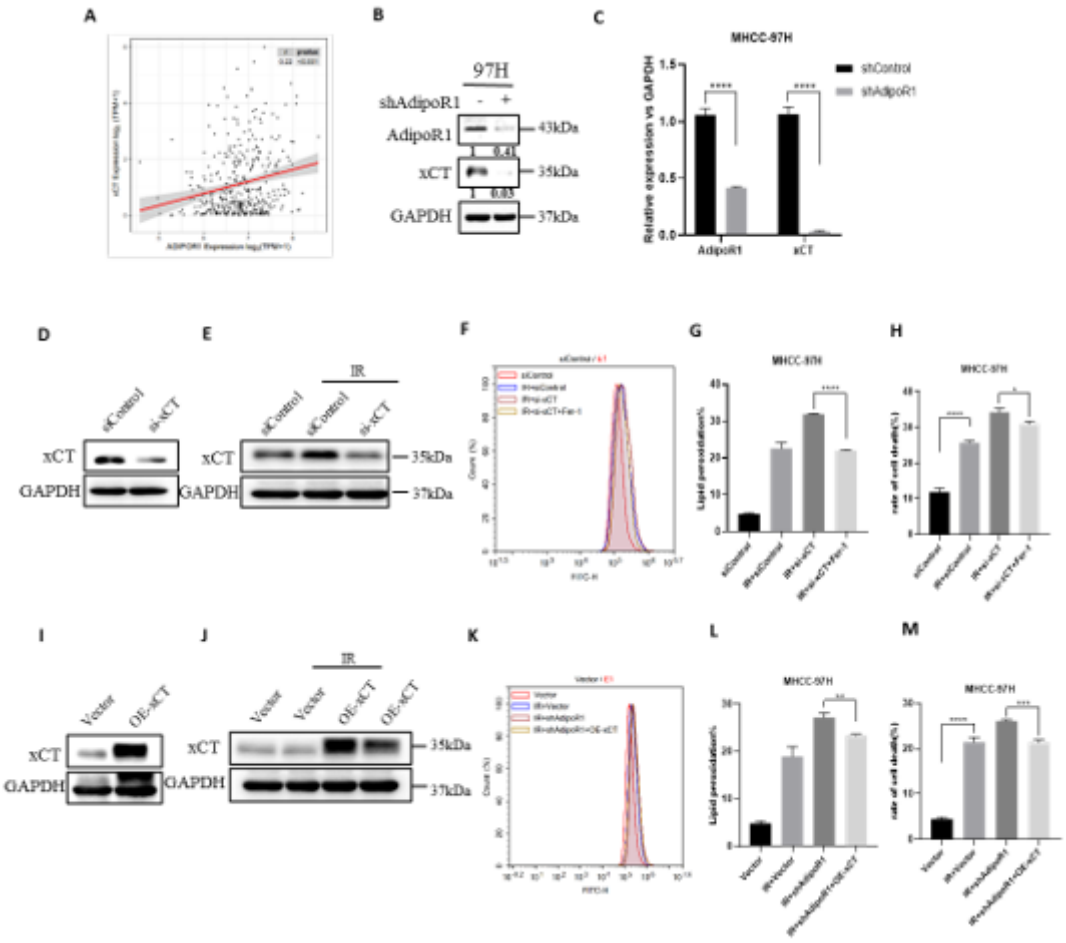

Figure 6

AdipoR1 protected cells from IR-induced ferroptosis by regulating the expression of xCT. (A) TCGA data shows that the expression level of AdipoR1 is positively correlated with xCT. (B-C) Western blot showed the levels of AdipoR1 and xCT in MHCC-97H cells after knockdown of AdipoR1 by shRNA. (D-H) xCT knockdown rendered MHCC-97H cells susceptible for ferroptosis and lipid peroxidation accumulation induce by IR. (I-M) Over-expression of xCT rescue IR-induced ferroptosis in AdipoR1 knockdown cells. MHCC-97H cells that knockdown AdipoR1 by shRNA were transfected with pcDNA3.1Flag-xCT for 48h, and then treated with IR (10Gy). Lipid peroxidation and cell death were assessed by flow cytometry using C11-BODIPY and trypan blue in AdipoR1-knockdown MHCC-97H and HepG2 cells respectively. Data is presented as the mean ± SD, *p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

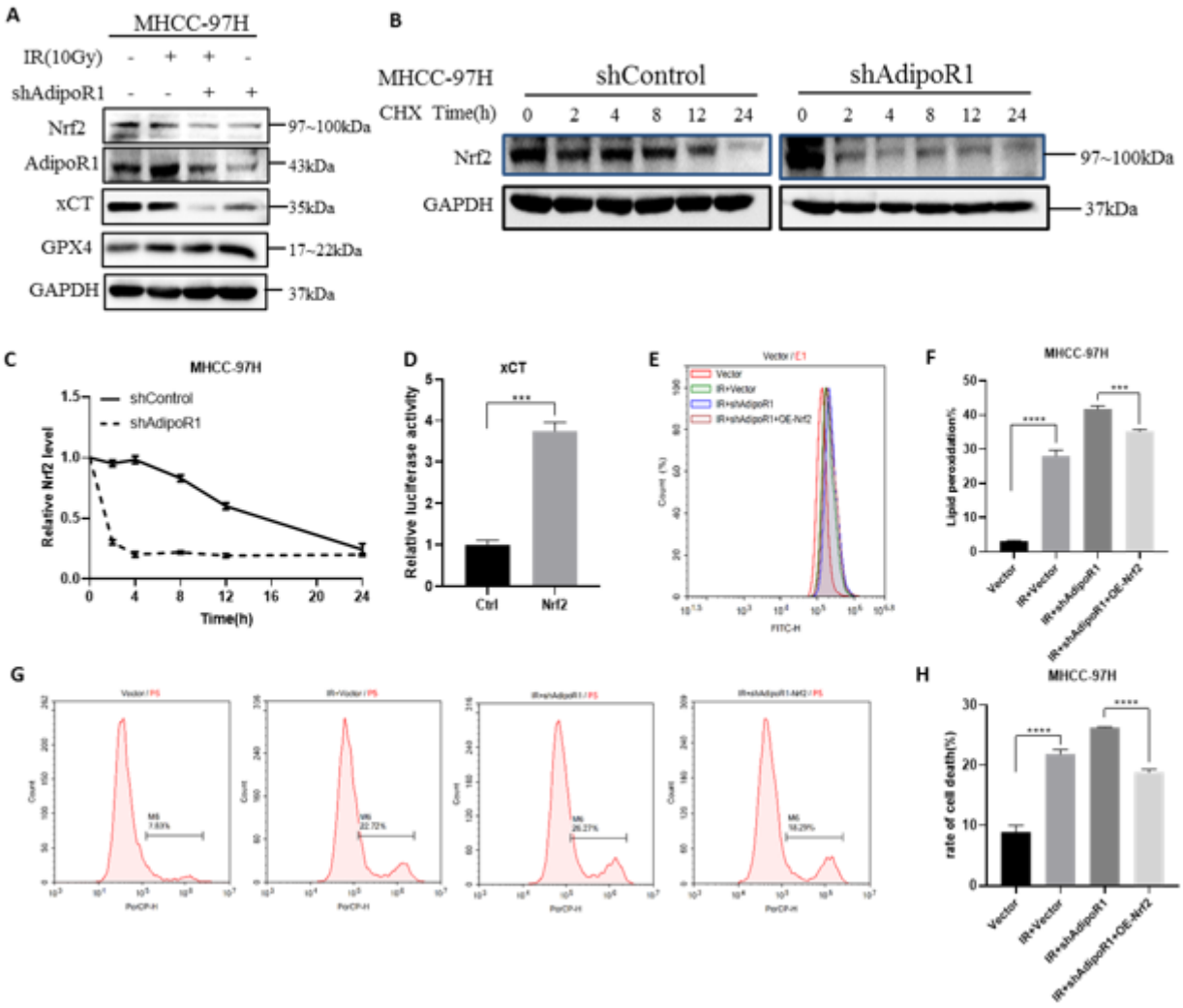

Figure 7

AdipoR1 regulated IR-induced ferroptosis by AdipoR1-Nrf2-xCT pathway. (A) After knock down of AdipoR1 by shRNA in MHCC-97H, followed by 10Gy radiation, western blot analysis showed the effects of AdipoR1 knockdown on the Nrf2 and GPX4 proteins. The results of western blot assay shown that AdipoR1 knockdown decreased the level of Nrf2 in MHCC-97H cells. (B-C) After treatment with CHX (100μg/ml) for various time in AdipoR1 knockdown MHCC-97H, western blot analysis showed the effects of AdipoR1 knockdown on the Nrf2 protein stability. (D) Effect of Nrf2 on xCT promoter double fluorescein-reporter enzyme activity in 293T cells. (E-H) Over-expression of Nrf2 rescued IR-induced ferroptosis in AdipoR1 knockdown cells. MHCC-97H cells that knockdown AdipoR1 by shRNA were transfected with pcDNA3.1Flag-Nrf2 for 48h, and then treated with IR (10Gy). Lipid peroxidation and cell death were assessed by flow cytometry using C11-BODIPY and trypan blue. Data is presented as the mean ± SD, *p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

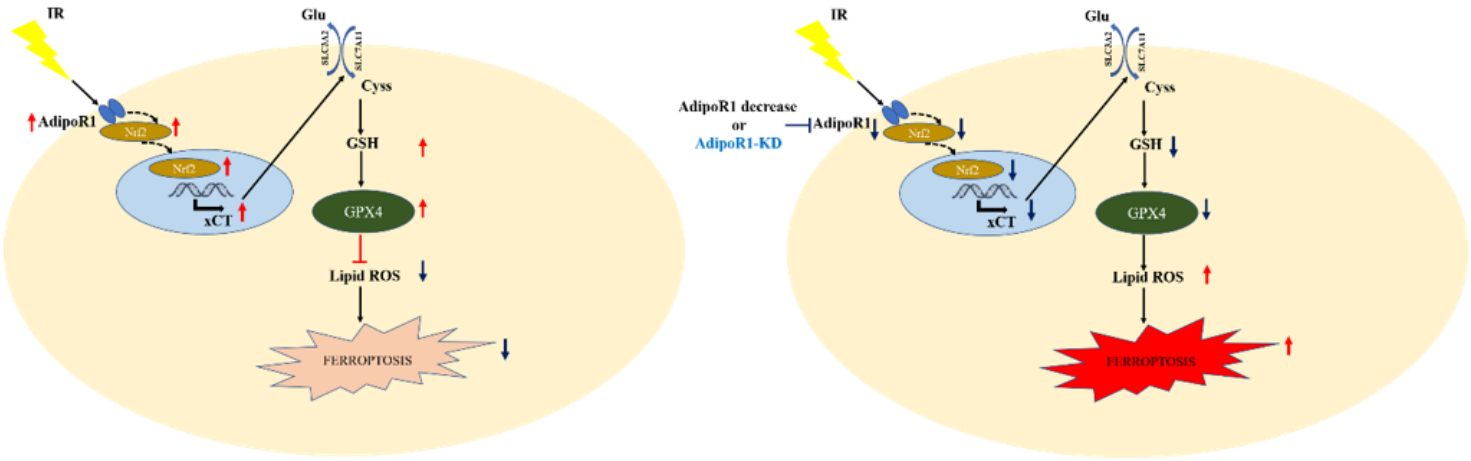

Figure 8

A schematic model demonstrating the roles of AdipoR1 in IR-induced ferroptosis.

Supplementary Files

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- FigureS1.png
- FigureS2.png