Ferroptosis as a mechanism to mediate p53 function in tumor radiosensitivity

Guang Lei1 · Yilei Zhang1 · Ting Hong2 · Xudong Zhang2 · Xiaoguang Liu1 · Chao Mao1 · Yuelong Yan1 · Pranavi Koppula1,3 · Weijie Cheng1 · Anil K. Sood4 · Jinsong Liu2 · Boyi Gan1,3

Received: 24 October 2020 / Revised: 10 March 2021 / Accepted: 12 April 2021 / Published online: 29 April 2021
© The Author(s), under exclusive licence to Springer Nature Limited 2021

Abstract
Ferroptosis, a form of regulated cell death triggered by lipid peroxidation, was recently identified as an important mechanism in radiotherapy (RT)-mediated tumor suppression and radioresistance, although the exact genetic contexts in which to target ferroptosis in RT remains to be defined. p53 is the most commonly mutated gene in human cancers and a major effector to RT. Here, we identify ferroptosis as a critical mechanism to mediate p53 function in tumor radiosensitivity. Mechanistically, RT-mediated p53 activation antagonizes RT-induced SLC7A11 expression and represses glutathione synthesis, thereby promoting RT-induced lipid peroxidation and ferroptosis. p53 deficiency promotes radioresistance in cancer cells or tumors at least partly through SLC7A11-mediated ferroptosis inhibition. Ferroptosis inducers (FINs) that inhibit SLC7A11 exert significant radiosensitizing effects in tumor organoids and patient-derived xenografts with p53 mutation or deficiency. Finally, we show that RT-induced ferroptosis correlates with p53 activation and better clinical outcomes to RT in cancer patients. Together, our study uncovers a previously unappreciated role of ferroptosis in p53-mediated radiosensitization and suggest using FINs in combination with RT to treat p53-mutant cancers.

Introduction
Radiotherapy (RT) is a primary therapeutic strategy for many cancers, including lung, esophageal, and head and neck cancers [1, 2]. RT causes DNA double-strand breaks and generates reactive oxygen species (ROS), inducing cell cycle arrest, senescence, and various cell death [3, 4]. As a major effector of RT, p53 is stabilized by RT, regulates the transcription of its target genes, and induces cell cycle arrest, senescence, and apoptosis, among other functions, thereby contributing to the therapeutic effectiveness of RT [5–7]. p53 is the most mutated tumor suppressor in human cancers, and can also be inactivated through multiple other mechanisms, such as gene deletion and epigenetic silencing [8, 9]. Although the role of p53 in determining radiosensitivity is complex and somewhat controversial, p53-deficient tumors are generally considered to be radioresistant [7, 10, 11]. How p53 regulates radiosensitivity in cancer cells remains incompletely understood, hindering our ability to therapeutically target p53-deficient cancers. Therefore, there is a significant need to identify novel agents that can overcome radioresistance in p53-deficient tumors.

Ferroptosis is an iron-dependent lipid peroxidation-induced form of regulatory cell death, which is distinct from other forms of regulated cell death, such as apoptosis, in morphology and mechanisms [12, 13]. Ferroptosis is caused by peroxidation of polyunsaturated-fatty-acid-containing phospholipids (PUFA-PLs). The GPX4-SLC7A11 signaling axis constitutes the major cellular defense mechanism against ferroptosis, wherein GPX4, a glutathione peroxidase, uses
reduced glutathione (GSH) to detoxify lipid peroxides and suppress ferroptosis [14–16], while the amino acid transporter SLC7A11 imports cystine, followed by cystine reduction to cysteine, which then provides the rate-limiting precursor for GSH synthesis (Supplementary Fig. S1) [13, 17–20]. Inactivation of GPX4 or SLC7A11, genetically or pharmacologically by ferroptosis inducers (FINs), induces ferroptosis in cancer cells or tumors [13, 17, 18]. We and others previously identified ferroptosis as a natural tumor suppression mechanism, and showed that some tumor suppressors, including p53 and BAP1, suppress tumor formation at least partly through inducing ferroptosis [21–28]. Whether ferroptosis plays any role in mediating these tumor suppressors’ functions in response to cancer therapy remains less clear.

We and others recently reported that RT can potently induce ferroptosis and that ferroptosis plays an important role in RT-induced cell death and tumor suppression both in vitro and in vivo [29–31]. However, the exact genetic contexts in which ferroptosis plays a role in RT remain unclear. Given that p53 is the most commonly mutated gene in human cancers [8] and that both RT and ferroptosis have been associated with p53 [7, 10, 11, 21, 25, 32–35], in this study we examined whether p53 regulates RT-induced ferroptosis.

**Results**

**Ferroptosis is involved in p53-mediated radiosensitization**

We examined whether ferroptosis plays any role in p53-mediated radiosensitization. Wild-type (WT) p53 restoration in p53-deficient H1299 cells significantly promoted RT-induced lipid peroxidation and ferroptosis marker gene PTGS2 expression (Fig. 1a–c). As expected, p53 restoration significantly decreased the clonogenic survival upon RT and enhanced radiosensitization in H1299 cells (Fig. 1d). Treatment with ferroptosis inhibitor ferrostatin-1 (Supplementary Fig. S1) increased the clonogenic survival upon RT; importantly, the effect of p53 restoration on radiosensitization was significantly attenuated (but not completely abolished) under ferroptosis inhibitor ferrostatin-1 treatment (Fig. 1d), suggesting that p53 promotes radiosensitization through both ferroptosis-dependent and -independent mechanisms.

Treatment with MDM2 inhibitor Nutlin or RG7112 in p53 WT A549 cells significantly increased p53 protein levels (Fig. 1e) and promoted RT-induced lipid peroxidation (Fig. 1f). Clonogenic survival assay showed that Nutlin or RG7112 treatment further decreased clonogenic survival upon RT, and that the radiosensitizing effect by these MDM2 inhibitors was significantly attenuated by ferrostatin-1 treatment (Fig. 1g), suggesting that the radiosensitization afforded by MDM2 inhibition and p53 activation is at least partly mediated by ferroptosis in A549 cells.

RT significantly induced p53 protein levels in p53 WT A549 and H460 cells (Fig. 1h, Supplementary Fig. S2a). p53 deletion in these cells by CRISPR-Cas9 (Fig. 1i, Supplementary Fig. S2b) largely abolished RT-induced lipid peroxidation and PTGS2 expression (Fig. 1j, k, Supplementary Fig. S2c, d). As expected, p53 deletion significantly decreased RT-induced cell death (Supplementary Fig. S2e), and restored clonogenic survival upon RT, leading to radioresistance; importantly, radioresistance caused by p53 deficiency was significantly attenuated by ferrostatin-1 treatment (Fig. 1l, Supplementary Fig. S2f), suggesting that p53 deficiency promotes radioresistance at least partly through inhibiting ferroptosis. This observation was further confirmed in A549 cells with p53 knockout (Supplementary Fig. S2g). We made similar observations by depleting p53 in U2OS cells (Supplementary Fig. S2h–j). Further analysis with different cell death inhibitors revealed that each of these cell death inhibitors could partially restore clonogenic survival in A549 cells upon RT (Supplementary Fig. S2k), which is consistent with our previous report [29]; these cell death inhibitors exerted marginal additional restoration effects under p53 deficient background, likely because p53 deficiency in A549 cells already potently blocked these forms of cell death induced by RT.

A recent study identified transferrin receptor protein 1 (TfR1) as a novel ferroptosis marker [36]. We found that RT significantly increased TfR1 staining in A549 cells (p53 WT) but mildly increased it in H1299 cells (p53 deficient) (Supplementary Fig. S2l); importantly, p53 deletion in A549 cells or its overexpression in H1299 cells decreased or increased RT-induced TfR1 staining, respectively (Supplementary Fig. S2m, n). Together, our data strongly suggest that p53 promotes RT-induced ferroptosis and that ferroptosis plays a role in p53-mediated radiosensitization.

As a critical effector of RT, p53 is involved in DNA damage response and repair. Consistent with previous reports that p53 promotes DNA repair upon RT [37], we found that p53 restoration in H1299 cells suppressed RT-induced H2AX phosphorylation or phospho-H2AX foci formation, a marker of DNA damage (Supplementary Fig. S3a–c). Ferrostatin-1 treatment did not affect H2AX phosphorylation or phospho-H2AX foci formation mediated by p53 in response to RT (Supplementary Fig. S3a–c). These data suggest that the role of ferroptosis in p53-mediated radiosensitization is independent of DNA damage response.
p53 promotes RT-induced ferroptosis partly through antagonizing SLC7A11 induction

Next, we sought to understand how p53 mediates RT-induced ferroptosis. Consistent with our previous observation [29], RT induced the expression of both SLC7A11 and GPX4 (Fig. 2a); while p53 deficiency did not significantly affect GPX4 levels, p53 deletion significantly increased SLC7A11 expression, which is in line with previous findings that p53 represses SLC7A11 expression [21]; notably, this phenotype was particularly pronounced upon RT treatment (Figs 2a, b). This observation was confirmed in A549 cells with p53 knockdown (Fig. S4a). We showed that p53 deficiency in U2OS cells also upregulated SLC7A11 levels (Supplementary Fig. S4b). While RT significantly induced p53 levels (Fig. 2a), RT did not induce the expression of other transcriptional regulators known to repress SLC7A11 transcription, such as BAP1 and ATF3 [26, 38] (Supplementary Fig. S4c). It should be noted that, despite p53-mediated SLC7A11 repression, RT still induces SLC7A11 expression (Fig. 2a, b). This is further discussed under Discussion.
Consistent with our recent report [29], deleting SLC7A11 in p53 knockout (KO) cells restored the reduction of PTGS2 levels and lipid peroxidation under RT caused by p53 deficiency (Fig. 2c–e), re-sensitized p53 KO cells to RT, and the effect of SLC7A11 deletion on radiosensitization in p53 KO cells was completely abrogated by ferrostatin-1 treatment (Fig. 2f). Further, knocking down SLC7A11 in p53 KO cells to the level similar to that in p53 WT cells upregulated RT-induced PTGS2 expression and re-sensitized p53 KO cells to RT (Fig. 2g–i), suggesting that SLC7A11 induction caused by p53 deficiency plays a role in radioresistance. Conversely,
p53 deficiency confers radiosensitivity partly via SLC7A11-mediated GSH synthesis

SLC7A11 is a core component of system \( x_\text{c}^- \), an amino acid transporter system that takes up extracellular cystine to promote GSH synthesis, thereby inhibiting ferroptosis (Supplementary Fig. S1) [12, 17, 18, 39]. It is known that RT causes radiolysis of cellular water and generates ROS, which subsequently deplete GSH. Consistently, we found that RT decreased GSH levels (Fig. 3a); under RT conditions, p53 deletion in A549 cells significantly restored GSH levels, and SLC7A11 deficiency largely reversed the increased GSH levels caused by p53 deletion (Fig. 3a). Similar to SLC7A11 deficiency (Fig. 2e, f), blocking GSH biosynthesis in p53 KO A549 cells by l-buthionine sulfoximine (BSO) treatment (Supplementary Figs. S1, 3b) reversed the decreased lipid peroxidation caused by p53 deficiency under RT (Fig. 3c), and re-sensitized p53 KO cells to RT (Fig. 3d). Likewise, culturing cells in cystine deprived medium increased GSH levels in p53 KO A549 cells and resensitized these cells to RT (Fig. 3e, f). Conversely, supplementing A549 cells with N-acetyl cysteine (NAC) or GSHEE (a membrane-permeable form of GSH) increased GSH levels in A549 WT cells under both basal and RT conditions, and partially restored clonogenic survival in A549 cells under RT compared with that in p53 KO A549 cells (Fig. 3g, h). We made similar observations in p53 KO U2OS cells cultured in cystine deprived medium and their WT counterparts supplemented with NAC or GSHEE (Supplementary Fig. S4d). These data suggest that p53 deficiency in A549 cells promotes radiosensitivity at least partly through SLC7A11-mediated GSH synthesis and ferroptosis inhibition.

FINs radiosensitize p53-deficient cancer cells and tumor organoids

Our above data prompted us to test whether FINs can reverse the radiosensitivity in cancer cells with p53 deficiency.
deciency, which are generally radioresistant [7, 10, 11]. As shown in Fig. 4a, b, treatment with various FINs, including erastin, sulfasalazine (class 1 FINs that block SLC7A11-mediated cystine uptake), RSL3, ML162 (class 2 FINs that inactivate GPX4), and FIN56 (a class 3 FIN that depletes GPX4 and ubiquinone) (Supplementary Fig. S1), all restored RT-induced lipid peroxidation in p53 KO cells (Fig. 4a) and reversed the radioresistance caused by p53 deficiency (Fig. 4b). Likewise, in radioresistant A549 cells (Fig. 2n, o), FIN treatment potentiated RT-induced lipid peroxidation (Fig. 4c) and significantly sensitized these cells to RT (Fig. 4d). We made similar observations in FLO-1 cells, a p53 mutant cancer cell line (Supplementary Fig. S5a, b).

GSH measurement under these FIN treatment conditions showed that treatment with erastin or sulfasalazine decreased GSH levels under basal and RT conditions, whereas treatment with RSL3, ML162, or FIN56 even increased GSH levels in p53 KO cells (Supplementary Fig. S5c). Because GPX4 utilizes GSH to detoxify lipid peroxidation (Fig. 4c) and significantly sensitized these cells to RT (Fig. 4d). We made similar observations in FLO-1 cells, a p53 mutant cancer cell line (Supplementary Fig. S5a, b).

GSH measurement under these FIN treatment conditions showed that treatment with erastin or sulfasalazine decreased GSH levels under basal and RT conditions, whereas treatment with RSL3, ML162, or FIN56 even increased GSH levels in p53 KO cells (Supplementary Fig. S5c). Because GPX4 utilizes GSH to detoxify lipid peroxidation (Fig. 4c) and significantly sensitized these cells to RT (Fig. 4d). We made similar observations in FLO-1 cells, a p53 mutant cancer cell line (Supplementary Fig. S5a, b).
peroxides, GPX4 inactivation or its protein depletion by RSL3, ML162, or FIN56 would decrease GSH consumption, leading to increased GSH levels. This phenotype was particularly pronounced under RT condition, because RT induces potent lipid peroxidation, pushing GPX4 to consume more GSH to detoxify lipid peroxides; consequently, GPX4 inactivation reserves more GSH under RT. Therefore, depending on their mechanisms of action, these FINs can either decrease or increase GSH levels, but they all promote RT-induced lipid peroxidation and sensitize p53 KO cells to RT. Radioresistant A549 cells exhibited increased GSH levels compared with parental cells, and RT decreased GSH levels in parental cells but not in RT-resistant cells (Supplementary Fig. S5d).
Fig. 4 FINs radiosensitize p53-deficient/-mutant cancer cells and tumor organoids. a Lipid peroxidation analysis in sg C and sg p53-2 A549 cells at 24 h after exposure to 6 Gy X-ray irradiation following pretreatment with DMSO, SAS, erasin, ML162, or FIN56 for 24 h. b Clonogenic survival curve of sg C and sg p53-2 A549 cells exposed to X-ray irradiation at indicated doses following indicated pretreatment for 24 h. c Lipid peroxidation analysis in parental and radiosensitive A549 cells at 24 h after exposure to 6 Gy of X-ray irradiation following pretreatment with DMSO, SAS, erasin, RLS3, ML162, or FIN56 for 24 h. d Clonogenic survival curve of parental and radioresistant A549 cells exposed to X-ray irradiation at indicated doses following indicated pretreatment for 24 h. e Representative phase-contrast images of patient-derived organoids with p53-deficiency at day 7 after exposure to 20 Gy X-ray irradiation following pretreatment with 50 μM IKE, 5 μM RSL3 or DMSO for 24 h. f Representative PI staining images of ovarian cancer patient-derived organoids with p53-deficiency at day 7 after exposure to 20 Gy X-ray irradiation following pretreatment with 50 μM IKE, 5 μM RSL3 or DMSO for 24 h. g Sensitivity of patient-derived organoids with p53-deficiency to indicated treatment. Left panel, organoid viability at day 7 after exposure to indicated combination treatments. Right panel, the dots indicated on the Fa-CI plot are shown for the combination effects of indicated treatment (combination index value <1, synergistic effect; >1, antagonistic effect). The Red dots represent 6 Gy RT + 10 μM IKE or 1 μM RSL3, the blue dots represent 10 Gy RT + 20 μM IKE or 3 μM RSL3, and the green dots represent 15 Gy RT + 40 μM IKE or 6 μM RSL3. h Cell viability of MCF10A cells treated with DMSO, erasin, or RLS3 at indicated doses for 24 h. i Cell viability of HBECs cells treated with DMSO, erasin, or RSL3 at indicated doses for 24 h. j, k Lipid peroxidation analysis in MCF10A and HBECs cells at 24 h after exposure to 6 Gy of X-ray irradiation following pretreatment with DMSO, erasin, or RLS3 for 24 h. l Clonogenic survival curve of MCF10A cells exposed to X-ray irradiation at indicated doses following indicated pretreatment for 24 h. m Clonogenic survival curve of HBECs cells exposed to X-ray irradiation at indicated doses following indicated pretreatment for 24 h. The percentage values in a, c, e, j, and k refer to the percentages of cells with lipid peroxidation measured by BODIPY™ 581/591 C11 staining followed by FACS analysis. Error bars are mean ± SD from three independent repeats. P values calculated by two-tailed unpaired Student’s t test or 2-way ANOVA (h, i).

We then tested the combination of FINs with RT in patient-derived tumor organoids, which represents a better in vitro preclinical model for therapeutic testing in cancer research [40, 41]. To this end, we established p53-deficient tumor organoids established from a patient with high-grade ovarian serous cancer, which exhibits high p53 mutation or deficiency and is considered to be radiosensitive in the clinic [42]. As shown in Fig. 4e–g, such p53-deficient tumor organoids were resistant to RT, which is consistent with clinical observations in corresponding cancer patients; notably, treatment with RSL3 or imidazole ketone erastin (IKE, a recently developed potent class 1 FIN [43]; also see Supplementary Fig. S1) significantly synergized with RT to induce cell death and reduce cell viability in these tumor organoids.

Finally, we examined whether FINs induce ferroptosis in normal cells or sensitize normal cells to RT to the same extend as in cancer cells. To this end, we compared ferroptosis sensitivities in MCF10A human breast epithelial cells (or human bronchial epithelial cells [HBECs]) and p53-deficient breast cancer BT549, MDA-MB-231, and T47D cells (or lung cancer H23, H1650, and H1299 cells). Intriguingly, MCF10A cells or HBECs exhibited less sensitivities to erastin- or RSL3-induced ferroptosis than corresponding cancer cell lines (Fig. 4h, i). Further, erastin or RSL3 only mildly induced lipid peroxidation in MCF10A cells or HBECs; while RT significantly induced lipid peroxidation in both cell lines, neither erastin nor RSL3 further increased RT-induced lipid peroxidation in these cells (Fig. 4j, k). Consistent with this, erastin or RSL3 exerted no or weak sensitizing effect to RT in these normal cell lines (Fig. 4l, m). Together, our data from different in vitro cancer models show that FINs synergize with RT to induce lipid peroxidation and can reverse the radiosensitivity in p53-deficient cancer cells and tumor organoids, and further suggest that cancer cells appear to more sensitive to FINs (or FINs in combination with RT) than corresponding normal cells.

Ferroptosis is involved in p53 deficiency-mediated radioresistance and FINs radiosensitize p53-mutant tumors in vivo

Next, we sought to determine the relevance of ferroptosis in p53 deficiency-mediated radioresistance in vivo. We found that p53 deficiency promoted A549 xenograft tumor growth, but SLC7A11 deficiency did not significantly affect tumor growth at the backdrop of p53 deficiency (Fig. 5a, b). As expected, RT strongly suppressed the growth of control xenograft tumors and p53 deletion promoted tumor radioresistance, resulting in significantly restored tumor growth under RT conditions; notably, under RT conditions, SLC7A11 deletion in p53 KO tumors largely reversed the radioresistance effect caused by p53 deficiency (Fig. 5a, b). Immunohistochemical (IHC) analyses of phospho-H2AX, cleaved caspase-3, and Ki67 in these tumor samples revealed expected results consistent with the known roles of RT and p53 in DNA damage response, apoptosis, and cell proliferation, respectively (Fig. 5c–f). Notably, SLC7A11 deficiency did not obviously affect these cellular processes under conditions with or without RT (Fig. 5c–f). Further IHC analyses of 4-hydroxy-2-noneal (4-HNE, a lipid peroxidation marker) revealed that p53 deletion largely abolished RT-induced 4-HNE levels and SLC7A11 deletion in p53-KO tumors restored 4-HNE levels (Fig. 5c, g). Together, these data suggest that, at least in this tumor model, SLC7A11 does not play a significant role in p53 deficiency-induced tumor growth but specifically mediates tumor radioresistance in p53 KO tumors, and that SLC7A11 promotes tumor radioresistance in p53 KO tumors likely through suppressing lipid peroxidation and ferroptosis.
We then tested whether inducing ferroptosis by FINs can sensitize p53-deficient/-mutant tumors to RT using xenografts derived from p53 KO A549 cells. To enable more rapid translation of our findings into clinical application, we chose sulphasalazine, an FDA-approved drug with FIN activity (Supplementary Fig. S1) [44] in our animal studies.
As shown in Fig. 5h, RT only moderately suppressed the tumor growth in p53 KO A549 xenografts, and liproxstatin-1 treatment did not significantly restore p53 KO tumor growth upon RT (likely because p53 deficiency already largely abrogated RT-induced ferroptosis in A549 cells, which is also consistent with in vitro data in these cells; see Fig. 1I). In addition, while sulfasalazine treatment alone did not obviously affect tumor growth, it dramatically sensitized these tumors to RT; notably, liproxstatin-1 almost completely abolished sulfasalazine-mediated radiosensitization effect (Fig. 5h), suggesting that sulfasalazine sensitizes p53 KO tumors to RT mainly through inducing ferroptosis.

We further tested whether combining 4-HNE and p53 levels can provide an even stronger prognosis power than 4-HNE alone to predict patient outcomes to RT. To this end, we divided the patients into two groups based on the levels of p53 and 4HNE in post-RT tumor samples: one group (15 samples) with strongly positive staining in both p53 and 4HNE, whereas the other (11 samples) with negative/mild/moderate staining in both p53 and 4HNE. Our analyses showed that, while there was no significant difference in patient baseline characteristics (such as age, sex, tumor stage, differentiation or histological type) between the two groups, responses to RT or recurrence rates in patients with strongly positive p53 and 4HNE staining were significantly better than those in patients with negative/mild/moderate p53 and 4HNE staining (Fig. 6d, Supplementary Table S1). It should be noted that p53 levels alone did not significantly correlate with these clinical responses (Supplementary Fig. S7), suggesting that the significant correlation of combined p53 and 4HNE staining with clinical response does not simply reflect a correlation of p53 levels with clinical outcomes.

Finally, disease free survival analysis also revealed that patients with strongly-positive p53 and 4-HNE staining...
survived significantly longer than those with mild/moderate p53 and 4-HNE staining (Fig. 6c), with more significant separation of survival curves between the two groups than the data shown in our previous analysis based on only 4-HNE staining (31). In summary, our data suggest that more potent RT-induced p53 activation and ferroptosis...
Fig. 6 Ferroptosis induction correlates with p53 activation and better clinical responses to RT in cancer patients. a Representative p53 and 4-HNE IHC staining images in esophageal cancer patient samples before and after radiotherapy. Scale bars represent 50 μm/20 μm. b IHC scores for 4-HNE staining in p53 negative/mild/moderate and p53 strongly-positive esophageal cancer patient samples after radiotherapy. Error bars are mean ± SD from six randomly selected magnification fields. P values calculated by 2-tailed unpaired Student’s t test. c Correlation between 4-HNE and p53 staining in patients with esophageal cancer after radiotherapy. P values calculated by Chisquared test. d Correlation between radiotherapy response and p53/4-HNE combination staining in patients with esophageal cancer after radiotherapy. P values calculated by Chisquared test. e Kaplan–Meier survival curves for esophageal cancer patients stratified by p53 and 4-HNE staining after radiotherapy. P values calculated by log-rank test. f–h The working model depicting the roles and mechanisms of ferroptosis in p53-mediated radiosensitization. See Discussion for a detailed description.

correlate with better clinical outcomes to RT in cancer patients, and that the RT-p53-ferroptosis signaling axis likely contributes to therapeutic responses to RT in cancer patients. We acknowledge that one limitation of our analysis is that we do not have p53 mutation information from these cancer patients, precluding us from correlating p53 mutation status with ferroptosis marker staining or patient outcomes; nevertheless, our study at least suggests that RT-induced p53 expression has prognosis values.

Discussion

Recently, we and others identified ferroptosis as an important part of the RT-induced cell death response and revealed that ferroptosis inactivation promotes radioresistance [29–31]. In the current study, we show that ferroptosis represents a previously unrecognized mechanism in mediating p53 function in radiosensitization. On the basis of previous studies and the findings presented in this study, we propose that there are at least three mechanisms that underlie RT-induced ferroptosis (Fig. 6f): (1) RT-induced ROS deplete GSH and promote lipid peroxidation; (2) RT alters the levels of PUFA-PLs, possibly through modulating expression and/or activity of ACSL4 and other ferroptosis regulators; (3) our current study revealed that RT activates p53, which subsequently represses SLC7A11 and further sensitizes cancer cells to ferroptosis. However, RT also induces an adaptive response to upregulate SLC7A11 expression. Under most RT conditions we have tested, it seems that this adaptive response always overrides p53-mediated repressive effect on SLC7A11, resulting in increased SLC7A11 expression as a net effect in response to RT. Nevertheless, p53-mediated SLC7A11 repression is still important in RT-induced ferroptosis, because removing this molecular brake (by p53 deficiency) leads to an even more pronounced induction of SLC7A11 under RT, resulting in ferroptosis- and radio-resistance (Fig. 6g). We further show that FIN treatment weakens the anti-ferroptosis defense systems (by inactivating SLC7A11 or GPX4) in p53-deficient cancer cells or tumors, thereby sensitizing them to RT (Fig. 6h). Our study suggests that further testing the combination of RT and FINs (such as sulfasalazine) in the treatment of p53-deficient cancers is warranted.

Our data are not in conflict with previous studies showing that p53 deficiency promotes radioresistance through multiple other mechanisms such as inhibiting apoptosis [7, 10, 11]. Indeed, our data showed that p53 regulation of radiosensitivity involves both ferroptosis-dependent and -independent mechanisms (i.e., ferroptosis inhibition significantly attenuated, but not completely abrogated, p53’s effect on radiosensitivity; see Fig. 1i, 2f). Therefore, multiple downstream mechanisms (ferroptosis, apoptosis, and others) likely underlie the role of p53 in regulating radiosensitivity.

A recent study proposed that RT induces ferroptosis through suppressing SLC7A11 expression [30], which is opposite to our observation that RT generally induces SLC7A11 expression in cancer cells, which was also reported in other studies [45]. Further analyses revealed that, in HT1080 cells, RT at different doses increased SLC7A11 expression at different time points up to 48 h; RT did decrease SLC7A11 expression at 72 h after RT (Fig. S8). Since RT can potentely induce lipid peroxidation and PTGS2 expression at 24 h after RT in HT1080 cells [29, 31] (also see this study), it is less likely that SLC7A11 repression can underlie RT-induced lipid peroxidation at or before this time point. It is possible that SLC7A11 repression could still contribute to RT-induced ferroptosis at late time points. Further studies are required to clarify these questions.

Previous studies revealed a complex role of p53 in regulating ferroptosis induced by different FINs, with both pro- and anti-ferroptosis functions depending on the context [21, 22, 25, 32–35]. Our current study proposes that, at least in the context of RT, p53 exerts a strong pro-ferroptosis function. We also compared ferroptosis sensitivities of p53 WT and KO A549 cells to different FINs, including erastin, RSL3 and tert-butyl hydroperoxide (TBH). Our results showed that p53 deficiency in A549 cells promoted ferroptosis resistance to TBH and erastin, but not to RSL3 (Supplementary Fig. S9a, b). Our data, therefore, appear to be consistent with the results published from Gu lab [22]. It should be noted that parental and radioresistant A549 cells did not exhibit apparent differences in ferroptosis sensitivity to erastin or RSL3 (Fig. S9c). This is not entirely surprising, because unlike p53 deficient cells, these radioresistant cells still express p53 but exhibit defective p53 signaling circuitry under RT; as a result, SLC7A11 levels were not significantly changed between parental and radioresistant
cells under basal conditions but were significantly upregulated in radioresistant cells compared with parental cells under RT conditions (Fig. 2a). Consequently, these radioresistant cells exhibit ferroptosis resistance under RT conditions, but not under RSL3 or erastin treatment. Of note, although we propose that p53 promotes RT-induced ferroptosis partly through suppressing SLC7A11 expression and GSH synthesis, we do not rule out the potential involvement of other SLC7A11-independent or SLC7A11-dependent/GSH-independent mechanisms mediating p53 function in RT-induced ferroptosis.

Materials and methods

Cell line-derived xenograft and patient-derived xenograft (PDX) models

Cell line xenograft and PDX experiments were conducted as previously described [29, 46–48]. Female 4- to 6-week-old athymic nude mice (Foxn1<sup>nu</sup>/Foxn1<sup>nu</sup>) and NOD scid gamma (NSG) mice were obtained from the Experimental Radiation Oncology Breeding Core Facility at MD Anderson Cancer Center. All animal studies were performed under the approval of the Institutional Animal Care and Use Committee (IACUC) of The University of Texas MD Anderson Cancer Center and the protocol is in full in line with IACUC policies. See “Supplementary Material and methods” for detailed information.

Cell lines and organoids

A549, H460, H1299, H23, H1650, T47D, BT549, U2OS and HEK293T cell lines were purchased from American Type Culture Collection (2011–2017). HBECs, MCF10A, MDA-MB-231 and FLO-1 cell line was obtained from MD Anderson Cancer Center. Ovarian cancer organoids were cultured in appropriate organoid medium (OC/OCwnt/OSE/FT medium) and changed to fresh medium every 3–4 days as previously described [49]. CRISPR KO cells were generated as previously described [50]. See “Supplementary Material and methods” for detailed information.

Constructs

The primer sequences used in this study are listed in IACUCTable S2. See “Supplementary material and methods” for details.

Patient samples

The procedure of patient sample collection was reviewed and approved by the Ethics Committee of Hunan Cancer Hospital & The Affiliated Cancer Hospital of Xiangya School Of Medicine, Central South University, and The University of Texas MD Anderson Cancer Center, and informed consent was obtained from all subjects. See “Supplementary Material and methods” for detailed information.

Statistical analyses

Statistical analyses were performed with GraphPad Prism 8 or SPSS 25.0 software using unpaired Student’s t test, chi-squared test, or log-rank test. Data are presented as means ± standard deviation (SD) from three independent experiments or biological replicates. Statistical significance (P values) are as follows: *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; n.s., non-significant.

Acknowledgements

This research was supported by Institutional Research Fund and RO Strategic Initiatives (ROSI) Boot Walk Seed Award from The University of Texas MD Anderson Cancer Center, KC180131 from Department of Defense Kidney Cancer Research Program, and R01CA181196, R01CA244144, and R01CA247992 from the National Institutes of Health (to BG); P50 CA217685, American Cancer Society, and MD Anderson Moon Shot Program in Ovarian Cancer (to AKS). BG was an Andrew Sabin Family Fellow. PK is supported by CPRIT Research Training Grant (RP170067) and Dr. John J. Kopchick Research Award from The University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences. This research has also been supported by the National Institutes of Health Cancer Center Support Grant P30CA016672 to The University of Texas MD Anderson Cancer Center.

Author contributions

GL performed most of the experiments with assistance from YZ, TH, XL, CM, WC, PK, and YY; JL, AKS, TH, and XD provided ovarian cancer organoids; BG designed and supervised the experiments; BG and GL wrote most of the manuscript with assistance from YZ, TH, XL, CM, WC, PK, and YY; JL, AKS, TH, and XD provided ovarian cancer organoids; BG designed and supervised the experiments; BG and GL wrote most of the manuscript with assistance from other co-authors; all authors commented on the manuscript.

Compliance with ethical standards

Conflict of interest AKS declares the following competing of interests: consulting (Merck, Kiyatec); shareholder (BioPath); and research support (M-Trap). Other authors declare no competing interests.

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

References

1. Delaney G, Jacob S, Featherstone C, Barton M. The role of radiotherapy in cancer treatment: estimating optimal utilization from a review of evidence-based clinical guidelines. Cancer. 2005;104:1129–37.
2. Jaffray DA. Image-guided radiotherapy: from current concept to future perspectives. Nat Rev Clin Oncol. 2012;9:688–99.
3. Baidoo KE, Yong K, Brechbiel MW. Molecular pathways: targeted alpha-particle radiation therapy. Clin Cancer Res. 2013;19:530–7.
4. Azzam EI, Jay-Gerin JP, Pain D. Ionizing radiation-induced metabolic oxidative stress and prolonged cell injury. Cancer Lett. 2012;327:48–60.

5. Fei P, El-Deiry WS. P53 and radiation responses. Oncogene. 2003;22:5774–83.

6. Gudkov AV, Komarova EA. The role of p53 in determining sensitivity to radiotherapy. Nat Rev Cancer. 2003;3:117–29.

7. Voussden KH, Prives C. Blinded by the light: the growing complexity of p53. Cell. 2009;137:413–31.

8. Muller PA, Voussden KH. p53 mutations in cancer. Nat Cell Biol. 2013;15:2–8.

9. Bykov VJN, Eriksson SE, Bianchi J, Wiman KG. Targeting mutant p53 for efficient cancer therapy. Nat Rev Cancer. 2018;18:89–102.

10. Lee JM, Bernstein A. p53 mutations increase resistance to ionizing radiation. Proc Natl Acad Sci USA. 1993;90:5742–6.

11. Lowe SW, Bodis S, McClatchey A, Remington L, Ruley HE, Fisher DE, et al. p53 status and the efficacy of cancer therapy in vivo. Science. 1994;266:807–10.

12. Dixon SJ, Lemberg KM, Lamprecht MR, Skouta R, Zaitsev EM, Gleason CE, et al. Ferroptosis: an iron-dependent form of non-apoptotic cell death. Cell. 2012;149:1060–72.

13. Stockwell BR, Friedmann Angeli JP, Bayir H, Bush AI, Conrad M, Dixon SJ, et al. Ferroptosis: a regulated cell death nexus linking metabolism, redox biology, and disease. Cell. 2017;171:273–85.

14. Seibt TM, Proneth B, Conrad M. Role of GPX4 in ferroptosis and its pharmacological implication. Free Radic Biol Med. 2019;133:144–52.

15. Friedmann Angeli JP, Schneider M, Proneth B, Tyurina YY, Tyurin VA, Hammond VJ, et al. Inactivation of the ferroptosis regulator Gpx4 triggers acute renal failure in mice. Nat Cell Biol. 2014;16:1180–91.

16. Yang WS, SriRamaratnam R, Welsch ME, Shimada K, Skouta R, Viswanathan VS, et al. Regulation of ferroptotic cancer cell death by GPX4. Cell. 2014;156:317–31.

17. Koppula P, Zhang Y, Zhuang L, Gan B. Amino acid transporter SLC7A11/xCT at the crossroads of regulating redox homeostasis and nutrient dependency of cancer. Cancer Commun. 2018;38:12.

18. Conrad M, Sato H. The oxidative stress-inducible cystine/glutamate antiporter, system x (c) (–): cystine supplier and beyond. Amino Acids. 2012;42:231–46.

19. Koppula P, Zhang L, Gan B. Cystine transporter SLC7A11/xCT in cancer: ferroptosis, nutrient dependency, and cancer therapy. Protein Cell 2020; 1–22. https://10.1007/s13238-020-00789-5.

20. Liu X, Zhang Y, Zhuang L, Olszewski K, Gan B. NADPH debt drives redox bankruptcy: SLC7A11/xCT-mediated cystine uptake as a double-edge sword in cellular redox regulation. Genes Dis. 2020. https://doi.org/10.1016/j.gendis.2020.11.010.

21. Jiang L, Kon N, Li T, Wang SJ, Su T, Hibshoosh H, et al. Ferroptosis as a p53-mediated activity during tumour suppression. Nature. 2015;520:57–62.

22. Chu B, Kon N, Chen D, Li T, Liu, Jiang Y, et al. ALOX12 is required for p53-mediated tumour suppression through a distinct ferroptosis pathway. Nat Cell Biol. 2019;21:579–91.

23. Gan B. DUBbing ferroptosis in cancer cells. Cancer Res. 2019;79:1749–50.

24. Liu T, Jiang L, Tavana O, Gu W. The deubiquitylase OTUB1 mediates ferroptosis via stabilization of SLC7A11. Cancer Res. 2019;79:1913–24.

25. Jennis M, Kung CP, Basu S, Budina-Kolometa J, Leu J, Kaku S, et al. An African-specific polymorphism in the TP53 gene impairs p53 tumor suppressor function in a mouse model. Genes Dev. 2016;30:918–30.
47. Xiao ZD, Han L, Lee H, Zhuang L, Zhang Y, Baddour J, et al. Energy stress-induced IncRNA FILNC1 represses c-Myc-mediated energy metabolism and inhibits renal tumor development. Nat Commun. 2017;8:783.

48. Liu X, Olszewski K, Zhang Y, Lim EW, Shi J, Zhang X, et al. Cystine transporter regulation of pentose phosphate pathway dependency and disulfide stress exposes a targetable metabolic vulnerability in cancer. Nat Cell Biol. 2020;22:476–86.

49. Kopper O, de Witte CJ, Lohmussaar K, Valle-Inclan JE, Hami N, Kester L, et al. An organoid platform for ovarian cancer captures intra- and interpatient heterogeneity. Nat Med. 2019;25:838–49.

50. Zhang Y, Shi J, Liu X, Xiao Z, Lei G, Lee H, et al. H2A monoubiquitination links glucose availability to epigenetic regulation of the endoplasmic reticulum stress response and cancer cell death. Cancer Res. 2020;80:2243–56.