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Research Paper

Hypoxia-induced autophagy as an additional mechanism in human osteosarcoma radioresistance

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ABSTRACT

Osteosarcoma (OS) responds poorly to radiotherapy, but the mechanism is unclear. We found OS tumor tissues expressed high level of protein HIF-1α, a common biological marker indicative of hypoxia. It is known that hypoxic cells are generally radioresistant because of reduced production of irradiation-induced DNA-damaging reactive oxygen species (ROS) in the anaerobic condition. Here we report another mechanism how hypoxia induces radioresistance. In MG-63 human osteosarcoma cells, hypoxic pretreatment increased the cellular survival in irradiation. These hypoxia-exposed cells displayed compartmental recruitment of GFP-tagged LC3 and expression of protein LC3-II, and restored the radiosensitivity upon autophagy inhibition. The following immunohistochemistry of OS tumor tissue sections revealed upregulated LC3 expression in a correlation with HIF-1α protein level, implying the possibly causative link between hypoxia and autophagy. Further studies in MG-63 cells demonstrated hypoxic pretreatment reduced cellular and mitochondrial ROS production during irradiation, while inhibition of autophagy re-elicited them. Taken together, our study suggests hypoxia can confer cells resistance to irradiation through activated autophagy to accelerate the clearance of cellular ROS products. This might exist in human osteosarcoma as an additional mechanism for radioresistance.

1. Introduction

Osteosarcoma (OS) is the most common type of primary bone cancer that mainly affects younger populations [1,2]. Current therapies combining surgery with chemotherapy (doxorubicin and cisplatin with or without methotrexate) yield 60–70% of the 5-year survival rate. However, the effective cure for patients with metastatic or relapsed osteosarcoma is still challenging [3]. Therefore, improvement of the existing therapies and exploitation of other approaches are highly anticipated.

Radiotherapy is an alternative combinatory therapy for OS. The incorporation of radiotherapy significantly improved the efficiency of chemotherapy by certain anticancer drugs (e.g., ifosfamide, cisplatin, HDMTX, etc.) [4], which even led to a long-term remission in some patients [5]. Locally complete cure could also be observed in unresectable or partially resected cases by radiotherapy when applied at high intensity [6]. Nevertheless, OS is generally considered radioresistant with poorly understood mechanism [7].

In this study, we found HIF-1α was overexpressed in human OS tissues. HIF proteins are often indicators of hypoxia which is common in solid tumors like OS where blood supply in the microenvironment is usually limited [8–11]. In cancer stem cells, HIF proteins promote tumor aggressiveness and confer resistance to certain therapies including irradiation [12–15].

The mechanism that tumor with hypoxia has reduced sensitive to radiotherapy is well studied [16]. It is known that irradiation generates free radicals on DNA. At the normal condition, these radicals can be fixed by oxygen (O_2) to generate DNA-damaging ROS products which will initiate cellular death. However, this death-inducing effect is compromised when the oxygen availability is low in hypoxic cells and ROS production is therefore limited [17].

Here, we found an additional mechanism that involves

Abbreviations: HIF-1α, hypoxia-inducible factor 1-alpha; ROS, reactive oxygen species; OS, osteosarcoma; OC, osteochondroma; LC3, microtubule-associated protein-1 light chain 3; CQ, chloroquine; 3-MA, 3-methyladenine

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autophagy in the mechanism of OS radioresistance, which is independent of oxygen at the time during irradiation. Autophagy is a process in which subcellular organelles or complex of proteins are sequestrated by intracellular membranes and then fused with lysosomes for degradation. This process is an important to eliminate damaged cellular components and maintain cellular survival [18]. Autophagy has been evidenced to be involved in cancer [19–21], and recent studies suggest its contribution to radioresistance in various tumors. Lomonaco et al. have found the induction of autophagy contributes to the radioresistance of glioma stem cells [22]. The Rodemann group also reported that autophagy also caused resistance to ionizing radiation in breast cancer cell lines [23]. The similar phenomenon was additionally evidenced in pancreatic cancer cells [24]. Another study also thoroughly support the role of autophagy in mediating radioresistance [25].

In this study, we propose that autophagy induced by hypoxia is another important mechanism that accounts for the radioresistance of OS.

2. Materials and methods

2.1. Patient samples

Histopathologically confirmed paraffin-embedded tissue sections from 89 osteosarcoma (51 males and 38 females) and 28 age-matched osteochondroma patients (16 males and 12 females) were recruited from the Fourth Hospital of Hebei Medical University. Clinical stages were evaluated according to the 2002 American Joint Committee on Cancer (AJCC). This study complied with the Declaration of Helsinki and was approved by the Human Ethics and Research Ethics Committees of the hospital. Written informed consents were obtained from all patients.

2.2. Tissue section and cell immunostaining

Paraffin-embedded tissue sections (4 μm) were incubated sequentially with primary antibodies and HRP-conjugated secondary antibodies. The signal was developed by EnVisionTM Peroxidase/DAB detection kit (Dako, UK). For immunocytochemical staining, MG-63 cells were washed with PBS and then received common processes like fixation (4% paraformaldehyde), permeabilization, blocking, and antibody incubation. Antibodies used in this study included anti-HIF-1α (Abcam, USA), anti-LC3 (Novus Biologicals, USA) and anti-γH2AX (Cell Signaling, USA). DAPI, Hoechst 33,258 and dichlorofluorescin diacetate (DCF-DA) were purchased from Sigma-Aldrich of USA. MitoSOX Red was from Thermo Fisher Scientific of USA.

2.3. Cell culture and irradiation procedure

Human MG-63 osteosarcoma cells were cultured in the DMEM medium (10% FBS, 50 U/ml penicillin, 50 μg/ml gentamicin, 2.5 μg/ml amphotericin B, 1% glutamine and 2% HEPES) at 37 °C in atmosphere with 5% CO_2. ELEKTA Synergy Linear Accelerator (Cravoley, UK) was used to treat the cells at 6 Gy (350 cGy/min) unless otherwise indicated. Culture medium was replaced with fresh medium without serum or antibiotics at 6 h before irradiation. Cellular viability was measured by the trypan blue exclusion method.

2.4. Western blot

Cell lysate with equal amount of protein was resolved by SDS-PAGE, and then transferred to NC membrane. After being blocked by 5% nonfat milk, the membrane was incubated with primary and secondary antibodies sequentially. Signals were developed by Pico Chemiluminescent Substrate (Thermo Fisher Scientific, USA) on films.

2.5. Statistical analysis

ANOVA, Tukey's test, and regression analysis were performed by software SPSS 21.0.

3. Results

3.1. HIF-1α expression is increased in osteosarcoma and is associated with the survival rate

It is established that hypoxia is common in most solid tumors due to limited blood supply in the microenvironment. This low oxygen condition and cellular adaptive responses often cause tumor aggressiveness and resistance to treatments including irradiation. Osteosarcoma (OS) is commonly known to be radioresistant. To determine whether radioresistance of this solid tumor could possibly involve hypoxia, we recruited osteosarcoma tissues from 89 cases to stain the typical hypoxia marker, HIF-1α, by immunohistochemistry, using 28 control samples from osteochondroma (OC), the most common benign bone tumor.

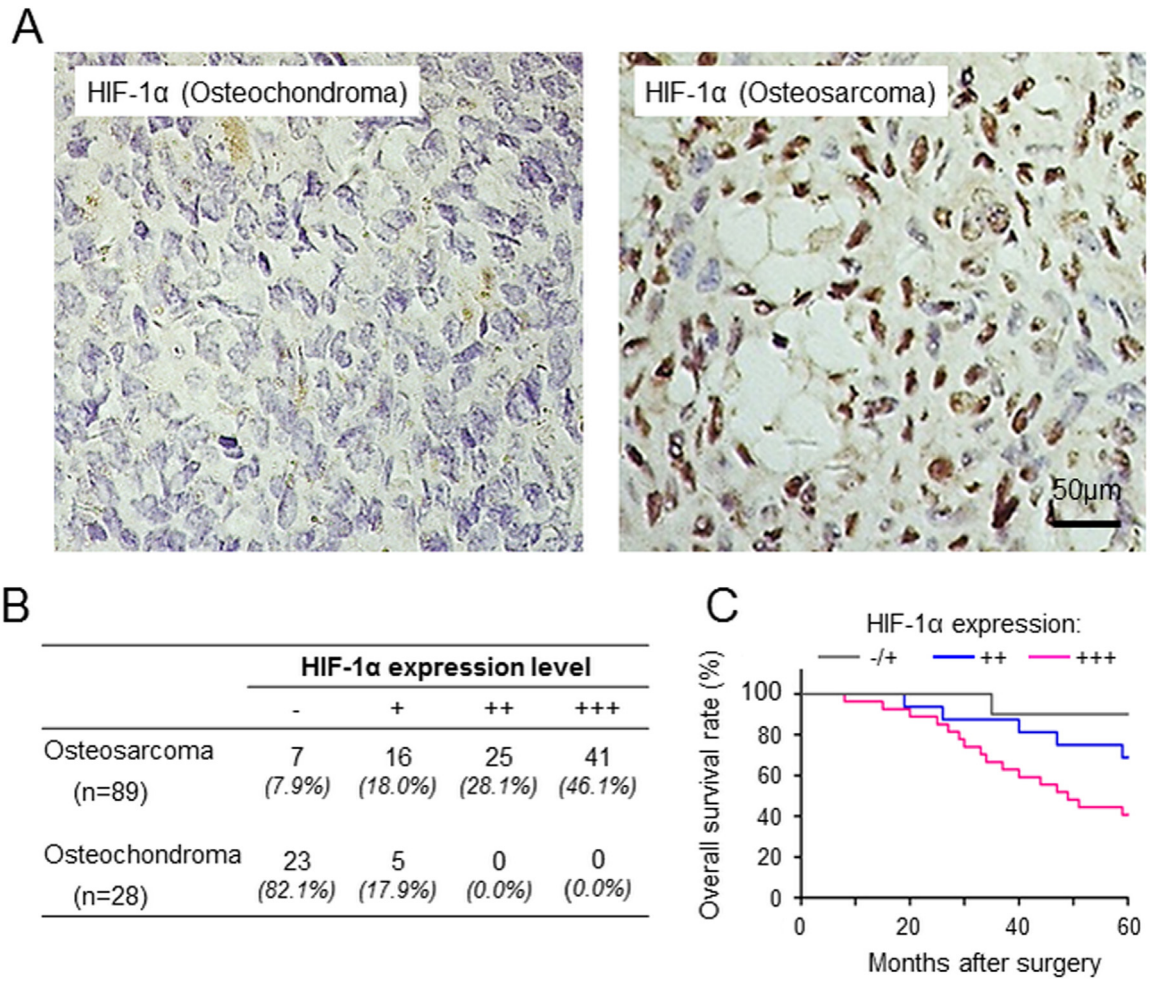
When compared to OC controls, most OS tissue samples expressed higher level of HIF-1α. Much more cells demonstrated positive staining and had stronger intensity in OS sections (Fig. 1A). Because the staining intensity was largely correlated with the number of positively stained cells, we simply counted the number of cells with observable staining and calculated the percentage of HIF-1α positive cells to grade the expression level ranges. 5%, 15% and 45% were used as the cutoff values for expression ranges of "−", "+", "++" and "+++"accordingly. We found 82 out of 89 (92.1%) OS sections expressing HIF-1α in positive ranges ["+": 16 (18.0%); "++": 25 (28.1%); and "+++": 41 (46.1%)] (Fig. 1B). In contrast, most OC samples have no or relatively low HIF1α expression ["−": 23 (82.1%); "+": 5(17.9%)].

HIF-1α expression in cancer often results from hypoxia and predicts poor prognosis because it is involved in tumor aggressiveness and intractability such as chemoresistance, radioresistance, angiogenesis, vasculogenesis, invasiveness and metastasis [9,26]. We therefore looked into the case medical history records and found the overall survival rate of these patients was correlated with HIF-1α expression: cases in the "+++" range had significantly lower survival rate than those in the "− or +", "++" ranges (Fig. 1C, Kaplan-Meier curve, the log rank test, $p=0.019$). "The positive correlation of HIF-1α expression with the post-operative treatment (mainly chemotherapy) indicates HIF-1α expressed in the tumor tissue exerts a biological effect. Because HIF-1α can contribute to resistance of both chemotherapy and irradiation [9,17,27,28], therefore although none of these patients received irradiation after surgery, the poorer chemotherapeutic efficiency on patients with higher HIF-1α expression might implicate a insensitive response of these cases to irradiation as well".

3.2. Hypoxia pretreatment protects osteosarcoma cells from irradiation

It is commonly known that hypoxic cells generally are less sensitive to irradiation because of insufficient oxygen to generate toxic ROS. We found another mechanism how hypoxia leads to radioresistance in a cellular model. This mechanism requires hypoxia not during the irradiation, but prior to the irradiation.

The human osteosarcoma cell line MG-63 was used to demonstrate in this study. We first determined the optimal



	HIF-1α expression level			
	-	+	++	+++
Osteosarcoma (n=89)	7 (7.9%)	16 (18.0%)	25 (28.1%)	41 (46.1%)
Osteochondroma (n=28)	23 (82.1%)	5 (17.9%)	0 (0.0%)	0 (0.0%)

Fig. 1. HIF-1α expression in osteosarcoma tissues and association with survival rate after surgical resection. (A) HIF-1α protein expression by immunohistochemistry in osteochondroma (OC) and osteosarcoma (OS) tumor tissues. (B) Summary of HIF-1α expression in OS and OC tissues. Expression level is graded based on the percentage of positively stained cells. "−": < 5%; "+": 5–15%; "++": 15–45%; "+++": > 45%. For each sample section, 5 view fields under the microscope were chosen. Three were from the region with the average number of total cells, and two other were from the densest or sparsest region respectively. (C) The overall survival rate of OS patients with different HIF-1a expression range. Kaplan-Meier curve, the log rank test, $p=0.019$.

irradiation intensity by applying different doses to cells, and found the one that caused nearly 50% cellular death is 5.6 Gy (Fig. 2A). We therefore chose 6 Gy in this study. DNA damage was verified in cells receiving irradiation at this dose by immunocytochemical staining of gamma-H2AX (Fig. 2B). Besides, we also confirmed on the Western blot that 1% O_2, the typical experimental condition to induce hypoxia, elicited the expression of HIF-1α (Fig. 2C), indicating the successful induction of cellular hypoxic response under this oxygen condition.

When preincubated in 1% O_2 for 24 h, cells showed reduced death by irradiation as compared to those without hypoxic pretreatment under the microscope (Fig. 2D). Overall, the cell survival rate evaluated by the trypan blue exclusion method was 46.5% under the irradiation, but increased to 72.4% significantly by hypoxic exposure prior to irradiation (Fig. 2E), suggesting hypoxic pretreatment introduced cellular tolerance to irradiation.

3.3. Hypoxic treatment induces autophagy in MG-63 cells

Autophagy regulates tumorigenesis and is involved in radioresistance in cancer therapy [21,29,30]. Autophagy can also be induced by hypoxia, which in turn contributes to the reduced sensitivity to therapeutic irradiation [28,31,32]. We first examined whether autophagy could similarly be activated in osteosarcoma cells by hypoxia by expressing GFP tagged protein LC3 in MG-63 cells to trace the morphological change of autophagy. LC3, the Microtubule-associated protein 1 A/1B-light chain 3, is a common marker for autophagic activation [33]. When autophagy begins, the cytosolic form of LC3 (LC3-I) is conjugated to phosphatidylethanolamine to become LC3-phosphatidylethanolamine conjugate (LC3-II), which is recruited to autophagosomal membranes. Detection of LC3 by immunoblotting or immunofluorescence is generally considered a reliable method for monitoring autophagy and autophagy-related processes.

Under 1% O_2 treatment for 24 h, the green signals were recruited from even distribution to localized speckles that resembled typical sequestering compartments during autophagosome formation (Fig. 2F). The Western blot also demonstrated the expression of LC3-II, the featured modification of LC3 required for autophagosome maturation (Fig. 2G). These suggest hypoxic treatment is able to induce the activation of autophagy in osteosarcoma cells.

To determine whether this hypoxia induced autophagy was possibly involved in reduced cellular sensitivity to irradiation, we added two different autophagy inhibitors, 10 μM chloroquine (CQ) and 2 mM 3-methyladenine (3-MA), into the cell culture medium 2 h before the irradiation. Without treatments, the survival rate under irradiation with the pretreatment of 1% O_2 was 75.1%. However, this was reduced to 52.5% and 49.6% by CQ and 3-MA respectively, which was close to the 48.3% under irradiation when

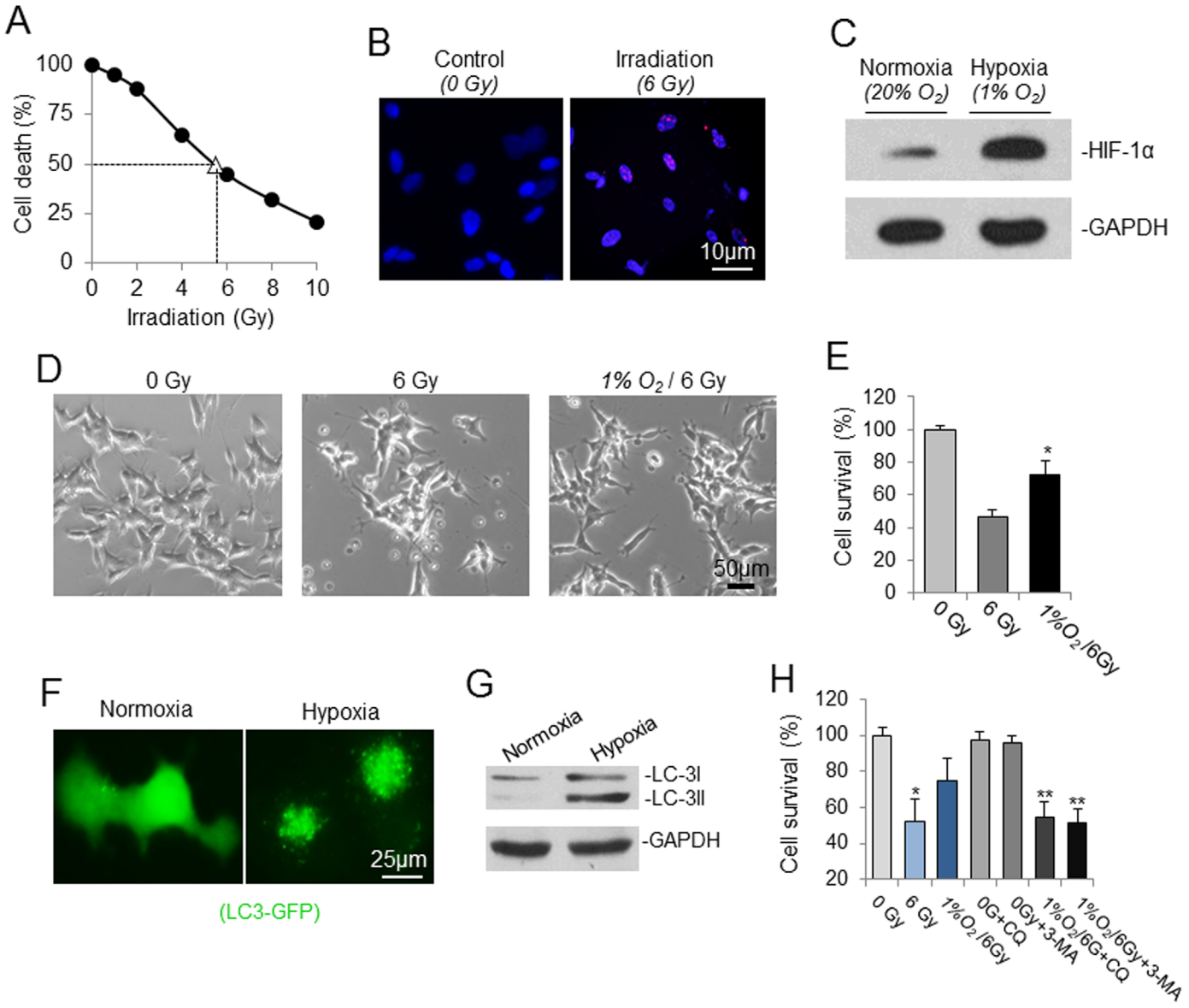


Fig. 2. Hypoxia pretreatment mediates radioresistance and induces autophagy in osteosarcoma cells. (A) MG-63 cell death rate by irradiation at different dosages. The dosage that causes 50% of cell death is about 5.6 Gy. (B) Immunofluorescence of gamma-H2AX indicates DNA damage induced by irradiation in MG-63 cells. (C) Western blot to demonstrate HIF-1α induction by the hypoxic treatment. (D) Reduced cellular death from irradiation by hypoxic pretreatment. (E) Trypan Blue staining to assess the cellular survival at different irradiation and hypoxic conditions. Error bar is standard deviation; one-way ANOVA ($p < 0.05$) and Tukey's tests were used (both $^{*}p < 0.05$). (F) Morphological change of MG-63 cells expressing GFP-tagged LC3 in hypoxic treatment (1% O_2) for 24 h. (G) Activated autophagy of MG-63 cells in hypoxia as assessed by LC3-II. (H) Autophagy inhibition abolishes protective effect of hypoxic pretreatment. CQ: Chloroquine, 10 μM; 3-MA: 3-methyladenine, 2 mM. Error bar is S.D.; one-way ANOVA ($p < 0.05$) followed by Tukey's tests ($^{*}p < 0.05$ and both $^{**}p < 0.05$).

no hypoxia or autophagy inhibition was applied (Fig. 2H). It is notable that these two drugs did not show significant toxic effect on the cells in this experiment.

Taken together, these results suggest the hypoxia can induce autophagy to protect cells from irradiation, implying a possible novel mechanism of radioresistance in human osteosarcoma.

3.4. LC3 expression is correlated with HIF-1α in osteosarcoma tissues

To examine whether the activated autophagy also exists in the osteosarcoma tissues, we stained the protein LC3 by immunohistochemistry. Tissue sections from 15 OS cases with different HIF-1α expression in "+", "++" or "+++" ranges were selected. LC3 staining was generally stronger in samples expressing higher HIF-1α (Fig. 3A), indicating an upregulated autophagic activation in these tissues. If autophagy is induced by hypoxia, then presumably there is a correlation between LC3 and HIF-1α expressions. Indeed, their relative abundances derived from their immunostainings have demonstrated a correlation of "$R^2 = 0.4407$" with a significance in the regression analysis ($p = 0.0070$) (Fig. 3B). These results imply that the hypoxia in the osteosarcoma tissues have probably activated the autophagy.

3.5. Hypoxia-induced autophagy reduces ROS production during irradiation

The ionizing radiation used in radiotherapy kills cells through ROS [17]. The irradiation introduced radicals on the DNA (DNA) are fixed by O_2 to form superoxide which causes DNA double-strand breaks to initiate the cellular death processes. Therefore, ROS production is the key event in the mechanism of radiotherapy.

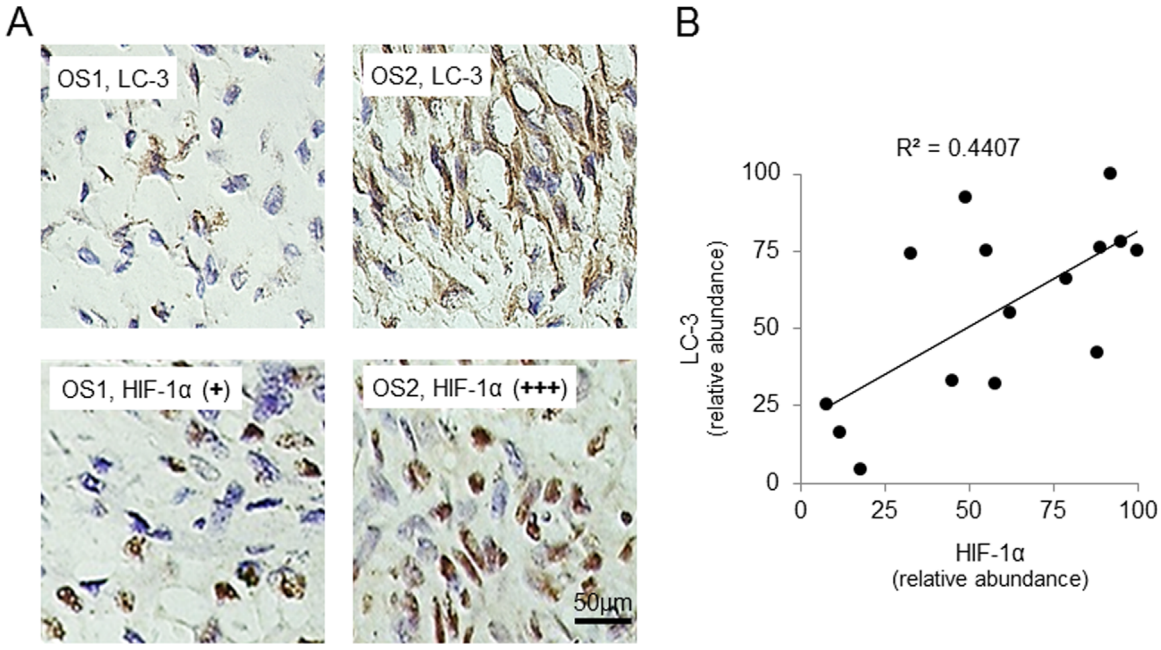


Fig. 3. Elevated autophagy in osteosarcoma. (A) Immunohistochemistry of HIF-1α and LC3-II in osteosarcoma tissues from two different cases that express high or low protein level of HIF-1α. (B) HIF-1α expression correlates with LC3-II in osteosarcoma tissues. Tissue sections from 15 OS cases with different HIF-1α expression levels were selected. LC3 relative abundance was calculated as follows. Multiple images (up to 10) were initially taken from different regions that contained dense, average or sparse cells. One image from each of the three ranges that had similar cell number among all 15 samples were finally chosen: 15 images in each range with no more than 50% difference in cell number, 45 images in total. The overall staining intensity (mixed nuclear and LC3 signals) was quantified by the software Image J from multiple areas (http://imagej.nih.gov/ij/), and then averaged and normalized to the cell number. Three images for each case were further averaged, and the final value was considered the abundance of LC3 of this case. The case with the highest LC3 abundance was considered 100%, and all other 14 cases were normalized to this case to have "relative abundance" result values which were used in this correlation curve. The percentage of stained cells was used for HIF-1α expression abundance, and the highest expression was used for normalization to yield "relative abundance". Regression analysis, $p=0.0070$.

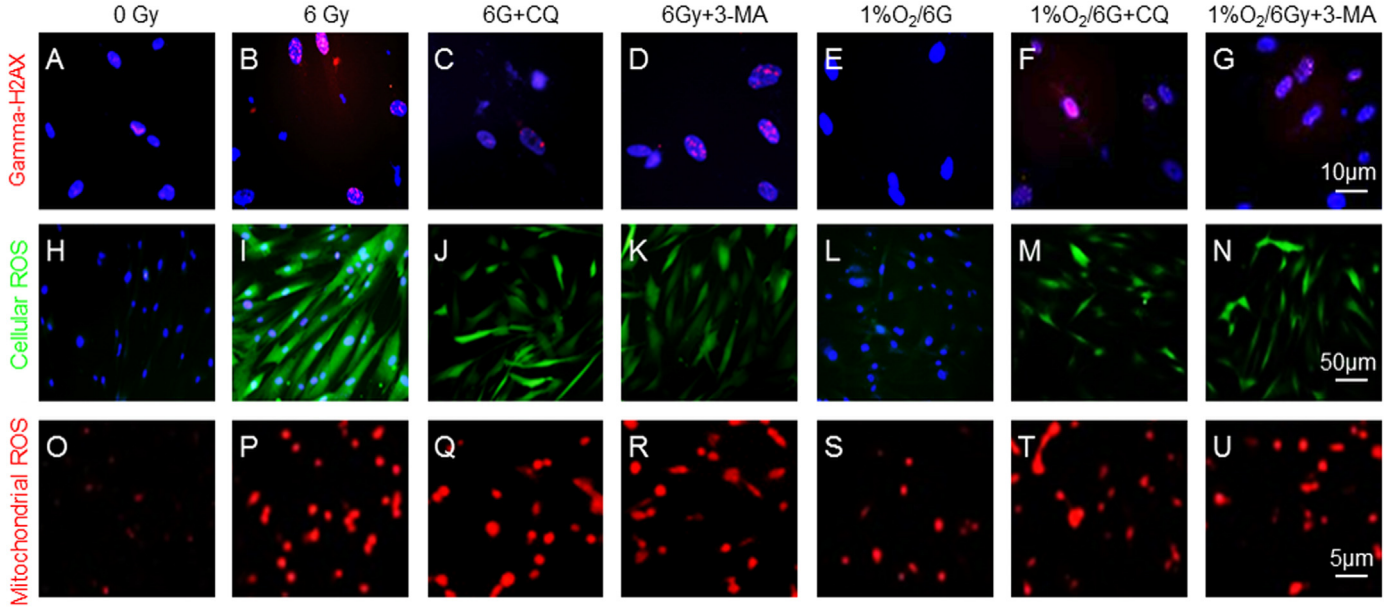


Fig. 4. Hypoxia-induced autophagy reduces irradiation-induced ROS production. (A-G): DNA damage indicated by immunestaining of gamma-H2AX and DAPI. Cellular ROS generation (H-N) and mitochondrial ROS generation (O-U) under different treatments by dichlorofluorescin diacetate (DCF-DA) staining and MitoSOX Red respectively. MG-63 Cells received treatments as indicated in the figures and fixed for immunestaining at 6 h- after irradiation. CQ, 10 μM; 3-MA, 2 mM. The scale bars for the top (A-G), middle (H-N) and bottom (O-U) layers are 10 μm, 50 μm and 5 μm respectively.

To determine whether ROS is involved in the autophagy-mediated protective effect on cellular death upon irradiation, we examined the cellular and mitochondrial ROS production in MG-63 cells during irradiation under different treatments using gamma-H2AX (DNA double-strand maker), dichlorofluorescin diacetate (cellular ROS marker) and MitoSOX Red (mitochondrial ROS marker). As expected, cells displayed DNA damage and increased both cellular and mitochondrial ROS by irradiation (Fig. 4A–D, H–K and O–R); and these alterations were restored by the pretreatment of 24 h' incubation in 1% O_2 prior to irradiation (Fig. 4E, L and S). However, this protective effect was abolished by both autophagy inhibitors (10 μM CQ and 2 mM 3-MA), as the DNA damage and both cytoplasmic and mitochondrial ROS products reappeared (Fig. 4F-G, M-N and T-U). These observations suggest that the

cellular radioresistance medicated by hypoxia-induced autophagy is probably through accelerated clearance of ROS products during irradiation.

4. Discussion

In this study, we found HIF-1α overexpression and possibly hypoxia-induced autophagic activation in human osteosarcoma tissues. Hypoxic cells are known to be less sensitive to radiotherapy because of reduced generation of DNA-damaging ROS during irradiation when low oxygen is present. In addition to this common mechanism, we have found hypoxia confers radioresistance by inducing autophagy which can accelerate scavenging toxic ROS products. Both of these mechanisms are probably involved in the radioresistance of human osteosarcoma tissues.

Insights from studies on cancer stem cells which often demonstrate resistance to irradiation include: 1) prolonged S-phase in cell cycle or more population of cells in this phase as mitotic cells are more sensitive to irradiation; 2) increased DNA repair activity; 3) enhanced ROS scavenging capacity and upregulated HIF-1α; and 4) rescuing cues from stromal environment [12]. Here it is very clear that accelerated ROS clearance and activated hypoxic response are among common mechanisms for radioresistance. We have found in this study that both of these are present in the human osteosarcoma, although other mechanism mentioned here might be existent as well.

It is established that hypoxia can induce autophagy [32,34–37]. We evidenced the autophagic activation in the cultured human osteosarcoma MG-63 cells after incubation in 1% O_2 for 24 h. The concomitantly elevated LC3 protein levels with HIF-1α high expression on OS tissue sections also indicates hypoxia might have activated autophagy in human OS tissues.

A recent publication also supports our study [38]. This report demonstrated that irradiation induced ROS accumulation which led to DNA damage in mesenchymal stem cells. However, this toxic effect was reduced by autophagic induction, supporting the notion that autophagy has an important role in conferring cells the tolerance to irradiation. Consistently, the hypoxia-induced autophagy has also been evidence in other radioresistant cancers [23,28,39].

How autophagy is activated in OS needs be further studied. Proteins and pathways like HIF-1α, BNIP3, MAP1LC3B, ATG5, ATF4, AMPK, etc., have been reported to have mechanistic roles in hypoxia-induced autophagic activation [32,40–44]. The recent advancement of next generation sequencing technologies might reveal more specific clues by comprehensive analyses of the entire molecular profiles from the clinical OS tissues with proper controls [45–50].

In summary, we have found hypoxia-induced autophagy might contribute to radioresistance in osteosarcoma as an additional mechanism. Therefore, the pharmacological inhibition of autophagy might improve the efficiency of radiotherapy in human osteosarcoma treatments.

Conflict of interest

The authors declare no competing financial interests.

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