

ORIGINAL ARTICLE

Ionizing radiation induces autophagy in human oral squamous cell carcinoma

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Summary

Purpose: Irradiation-induced autophagy has been reported in several types of cancers, however, the relationship between irradiation and autophagy in human oral squamous cell carcinoma (OSCC) has not yet been described. In this study we investigated the induction of autophagy in cell lines by exposing them to ionizing irradiation.

Methods: Human OSCC OC3 and SAS cell lines were used in this study. Cell viability and induction of autophagy were determined under irradiation treatment. The GFP-LC3 puncta formation and the levels of LC3-II as indicators of autophagy were detected by fluorescence microscopy and Western blot method. The signaling pathways involved in irradiation-mediated autophagy were also determined by Western blot method.

Results: Irradiation decreased cell viability only in OC3 cells, while autophagic machinery and related signaling pathways were found to be elevated after irradiation in OC3 and SAS cells. However, autophagic degradation determined by the reduction of p62 levels was only found in OC3 cells, suggesting autophagosome accumulation took place in SAS cells. In addition, irradiation accompanied with rapamycin treatment elevated autophagy formation and induced death of OC3 cells.

Conclusions: These results suggested that induction of autophagy might provide an advantageous strategy to increase the anticancer effects of radiotherapy in patients with OSCCs.

Key words: autophagy, human oral squamous cell carcinoma, irradiation

Introduction

Oral cancer (OC) is a common malignancy worldwide and the fourth leading cause of cancer-related deaths [1,2]. Its incidence is one of the fastest increasing ones in Taiwan, with annual incidence increasing from 5.12 per 10⁵ men and 1.54 per 10⁵ women in 1982 to 27.04 per 10⁵ men and 3.17 per 10⁵ women in 2001, marking a 5.3-fold increase in men and a 2-fold increase in women in 2 decades [3]. In United States, United Kingdom,

Brazil, and several other countries, the incidence and mortality of OSCC have remained stable or declined during the last 2-4 decades [4-6]. Among OCs, OSCC is the most common neoplasia and frequently occurs within the oral cavity (cheek, gum, and tongue) [7]. Cigarette smoking and alcohol drinking are two major risk factors of oral carcinogenesis [8]. In developed countries, exposure to one or both of these factors accounts for more than 75% of all OCs, and further studies suggest that the combined effect is synergistic rather than

additive [9,10]. In addition, OC is closely associated with betel quid chewing in Taiwan and other countries in Southeastern Asia [8,11].

Surgery and radiation treatment, in combination with chemotherapeutic agents, are the typical therapeutic strategies for OCs [12,13]. Surgery is the preferred initial approach in patients with major tumors in the oral cavity. Adjuvant postoperative radiotherapy is also recommended to increase the locoregional tumor control [14].

In clinical practice, radiotherapy provides high cure rates in various human malignancies. Irradiation causes irreparable DNA strand break and induces apoptosis through activation of intracellular proteases in normal and cancer cells [15,16]. However, an additional cell death mechanism called autophagy plays important roles in case when radiotherapy is used, as shown in a recent report [17]. Apoptosis is characterized by cell rounding, membrane blebbing, chromatin condensation, nuclear fragmentation, and DNA laddering, and is classified as type I programmed cell death [18]. Autophagic cell death is characterized by membrane blebbing, autophagosome vacuole formation, and partial chromatin condensation, and is classified as type II programmed cell death [18]. Previous studies have demonstrated that autophagy plays an important role in embryonic development and reports have shown that radiation treatment induces autophagy in both normal and cancer cells [17,19]. However, the relationship between autophagy and irradiation in OC has not yet been described.

This study was undertaken to investigate whether exposure to ionizing radiation could induce autophagy in human OSCCs.

Methods

Cell culture

Human OSCC cell lines derived from betel quid chewing patients (OC3) were provided by Dr Lin Shu-Chun [20]. The cell line derived from non-betel quid chewing patients (SAS) was purchased from Japanese Collection of Research Bioresources (JCRB, Osaka, Japan). The OC3 cells were cultured in a medium containing keratinocyte serum-free medium (Gibco, Grand Island, NY, USA) and DMEM (Gibco) in a 1:1 ratio. The SAS cells were cultured in DMEM medium. All media were supplemented with 10% fetal bovine serum (FBS; Gibco), penicillin (100 IU) and streptomycin (100 µg/ml). Cells were cultured at 37°C in a atmosphere containing 5% CO₂.

Western blotting

After treatment, cell lysate was harvested by mammalian protein extraction reagent (M-PER, Thermo Fisher Scientific, Waltham, MA) containing 0.1% protease inhibitor cocktail and incubated on ice for 5 min. The protein concentration in the supernatant was quantified using a BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA). The protein samples were resolved on SDS-PAGE gels and electrophoretically transferred to PVDF membranes. The primary antibodies LC3 (ABGENT, San Diego, CA, USA), p62 (ABGENT), phospho-Akt (Cell Signaling Technology, Danvers, MA, USA), phospho-mTOR (Cell Signaling Technology), and GAPDH (GeneTex, Irvine, CA, USA) were used to identify protein levels. A BioSpectrum Imaging System was used to determine relative protein levels (UVP, Upland, CA, USA).

GFP-LC3 puncta determination

The CO3 and SAS cells (1x10⁵) were incubated in a 25T flask. The plasmid pEGFP1-LC3 (provided by Drs. Yoshimori and Mizushima) [21] was transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After 24 h transfection, cells were treated with or without irradiation or rapamycin, and then fixed in ice cold 4% formaldehyde/PBS. Punctate GFP-LC3 was observed using fluorescence microscope (Olympus, Tokyo, Japan).

Estimation of cell viability

The OSCC cells (1x10⁵) were cultured in a 25T flask. After treatment with or without chemicals, or irradiation, cells were trypsinized at the indicated times, stained with 0.5% trypan blue, and the viable cells were counted. Cells without irradiation and/or DMSO treatments were used as normal (or negative) control and the viable cells in all of the groups were compared with the formula shown below:

Cell viability (%) = 100% × (viable cells in each group/viable cells in normal group)

Source of irradiation

The cells were irradiated using a Varian Clinac IX linear accelerator with VARiS software, Version 7.1 (Varian Medical System, Palo Alto, CA, USA), with doses indicated in the Figures.

Statistics

Data were presented as mean±standard deviation for the indicated number of separate experiments. Student's *t*-test was performed for comparisons; if less than 30, the Mann-Whitney U test was used. Statistical significance was defined as a *p*-value less than 0.05 in all tests.

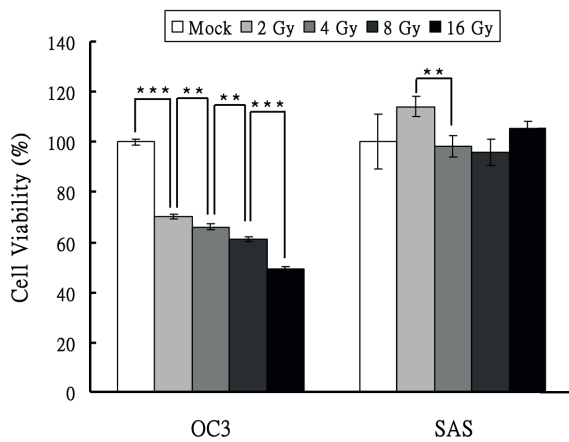


Figure 1. Ionizing irradiation decreases the viability of human OSCC. Human OSCC cell lines OC3 and SAS were irradiated with different doses. The inhibition of cell proliferation was determined by counting the number of viable cells using staining with trypan blue exclusion assay after 24-h treatment. Values represent mean \pm SEM of three independent experiments. ** $p < 0.01$, *** $p < 0.001$.

Results

Ionizing irradiation decreases viability of human OC3 cells

The human OSCC cell lines OC3 and SAS were used to evaluate the effects of ionizing radiation on cell viability. Following treatment with various doses of irradiation, cell viability was evaluated by the trypan blue test. As shown in Figure 1, the number of viable OC3 cells decreased significantly after irradiation in a dose-dependent manner after one-step irradiation treatment (Figure 1 and supplementary data 1A). However, SAS cells resisted to ionizing irradiation-induced cell death (Figure 1). After prolonged incubation (48h), these OSCC cell lines showed partial recovery (supplementary data 1B). In conclusion, treatment with ionizing irradiation decreased cell viability in OC3 cells, but not in SAS cells.

Irradiation increases autophagosome formation in human OSCC cells

The prolonged incubation study suggested that a protective mechanism might be triggered after irradiation. It is known that autophagosome is formed together with the modification of LC3 into a membrane-bound form, LC3-II, which further anchors on the autophagosome during autophagy formation [21]. To evaluate whether autophagy took place in the OSCC cells after irradiation, we observed the expression of LC3-II in autophago-

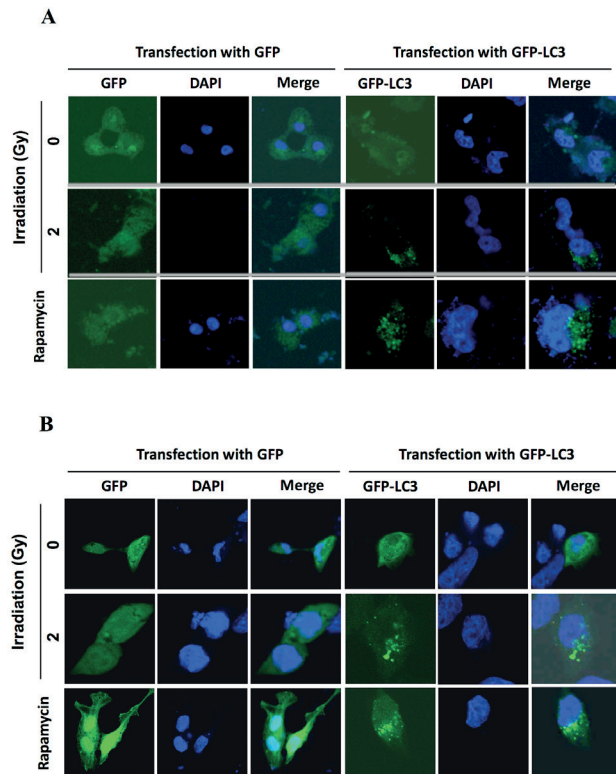


Figure 2. Ionizing irradiation-induced autophagosome formation in human OSCC. **A:** OC3 and **B:** SAS cells were transfected with GFP or GFP-LC3 constructions and then treated with or without irradiation. The GFP-LC3 puncta were analyzed using confocal microscopy. Rapamycin treatment provided a positive control to induce autophagy. The results shown here are the representative images of three independent experiments.

some and/or autophagy activation after irradiation. The pEGFPC1-GFP-LC3 was first transfected into OC3 and SAS cells and an irradiation dose of 2 Gy was delivered. The GFP-LC3-associated punctate autophagosome was evaluated using a confocal microscope (Figure 2). The pEGFPC1 vector transfection was performed and used as negative control (Figure 2), and treatment with rapamycin was used as positive control. In GFP transfected cells, there was no punctuated autophagosome expression regardless the treatment with irradiation or rapamycin, while both irradiation and rapamycin treatment increased punctate formation in GFP-LC3 transfected cells (Figures 2A and 2B). To further characterize whether LC3-I was processed into LC3-II after irradiation, we analyzed this transition by Western blot. The results showed that different doses of irradiation resulted in slight increase of LC3-I to LC3-II switch in SAS cells, while irradiation induced strong switch of LC3-I to LC3-II in OC3 cells (Figure 3A). Inter-

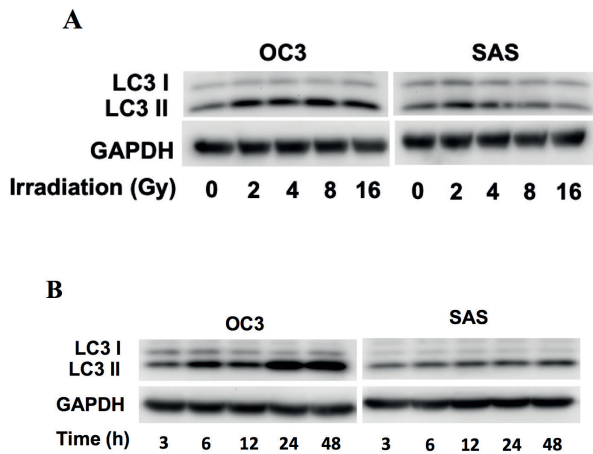


Figure 3. Ionizing irradiation treatment induced switch expression of LC3-I to LC3-II in OSCC. The OC3 and SAS cells were irradiated with **A:** different doses, and the levels of LC3-I/LC3-II were evaluated after 24-h treatment by Western blot. **B:** Cells were irradiated with 2 Gy at the indicated period of time and the levels of LC3-I/LC3-II were evaluated by Western blotting. GAPDH was used as a loading control. The results shown here are the representative Western blot results of three independent experiments.

estingly, a mild and transient increase in LC3-II levels was observed in SAS cells after irradiation. However irradiation induced a fast and prolonged LC3-II upregulation in OC3 cells (Figure 3B). Altogether, these results suggested that induction of autophagy in OC3 and SAS cells was triggered by irradiation. Of note, OC3 cells were more susceptible to irradiation than SAS cells to induce autophagy.

Signaling pathways involved in irradiation-induced autophagy in human OSCC

The PI3K/Akt/mTOR pathway has been reported to play an important role in multiple factors-induced autophagy activation. To elucidate the involvement of this pathway in ionizing irradiation-induced autophagy in OSCC, the OC3 and SAS cells were treated with irradiation and the levels of p-Akt, p-mTOR, LC3, and p62 were analyzed using Western blotting. As shown in Figure 4, the levels of p-Akt and p-mTOR were slightly decreased in SAS cells after irradiation. However, irradiation increased the levels of p-Akt but reduced the levels of p-mTOR in OC3 cells (Figure 4). These results suggested involvement of the mTOR pathway in ionizing irradiation-induced autophagy in OC3 and SAS cells. It also implied that the upstream pathways involved in irradiation-

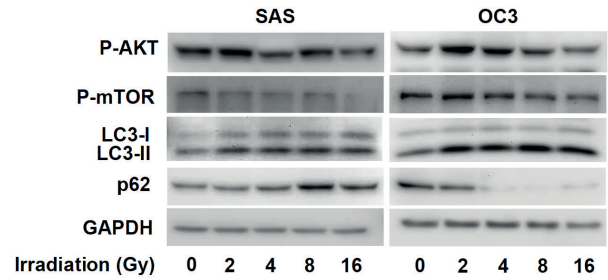


Figure 4. Signaling pathways involved in irradiation-induced autophagy in human OSCC. Cells were irradiated with the indicated dosage for 24 h and the levels of phospho-Akt, phospho-mTOR, LC3, and p62 were evaluated by Western blotting. GAPDH was used as a loading control. The results shown here are the representative Western blot results of three independent experiments.

tion-induced autophagy were different in SAS and OC3 cells. The p62 protein, an ubiquitin binding protein which was shown to be involved in autophagic degradation, increased in SAS cells after higher doses of irradiation, while it decreased in OC3 cells. This result suggested the occurrence of autophagic degradation in OC3 cells (Figure 4). Irradiated SAS cells, however, showing evidence of reduced PI3k/Akt/mTOR signaling pathway and induction of autophagy, suggested autophagosome accumulation in SAS cells (Figure 4). These results indicated that irradiation-induced autophagy occurred through different pathways in various OSCC cells.

Increasing formation of autophagy reduces viability in OC3 cells

It has been reported that activation of autophagy resulted in cell death in multiple cancer cell types. In this study, we demonstrated that autophagy was elevated in OC3 and SAS cells. However, p62 degradation (autophagic degradation) was only found in the OC3 cells. Moreover, irradiation didn't reduce cell viability in SAS cells (Figure 1). We speculated that irradiation-mediated autophagic cell death may be involved in OC3 cells. The present study further elucidated the effects of rapamycin on autophagy formation accompanied with irradiation on OC3 cell death. Cells were treated with or without irradiation and/or rapamycin, and cell viability was evaluated by trypan blue exclusion. Treatment of rapamycin resulted in increase of LC3-II in OC3 cells in a dose-dependent manner (Figure 5A). Combined treatment of rapamycin and irradiation to OC3 cells resulted in further decrease of cell viability

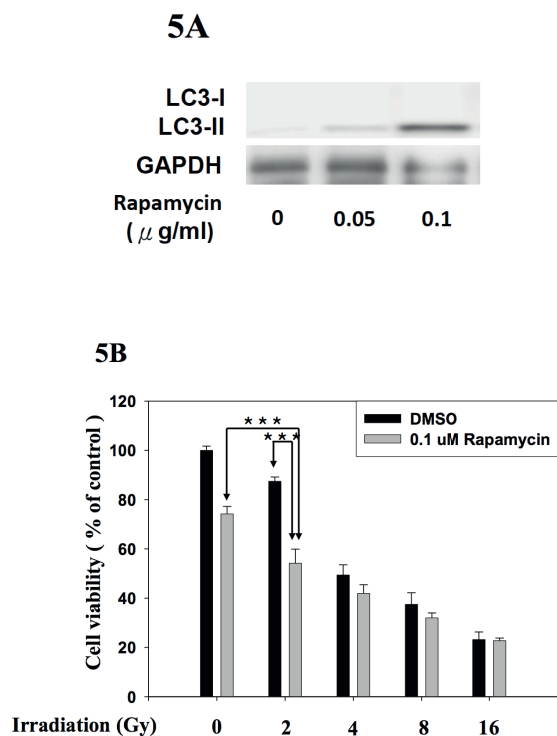
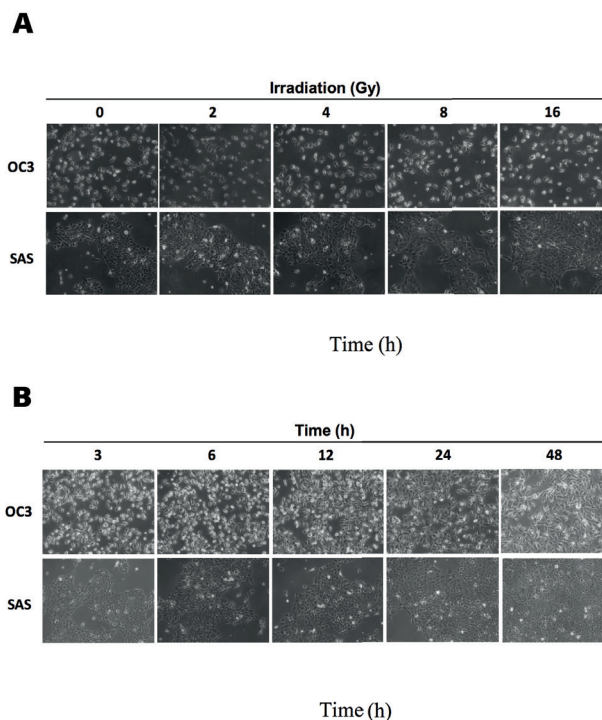


Figure 5. Increase of the formation of autophagy reduces viability in OC3 cells. **A:** The OC3 cells were treated with different doses of rapamycin for 24 h, and the induction of switch expression of LC3-I/LC3-II was assessed by Western blotting. **B:** Cells treated with or without rapamycin and/or ionizing irradiation for 24 h; the cell viability was evaluated by counting cells by trypan blue exclusion assay. DMSO was used as negative control. *** p<0.001.

at an irradiation dosage of 2 Gy (Figure 5B). These results suggested synergistic anticancer effects of irradiation and induction of autophagy in OSCC cells.

Discussion

Several studies have reported irradiation-induced apoptosis in multiple tumor types [22]. Others described DNA damage and aberration of signaling pathways (such as PKC and MAPK) after radiotherapy, and their involvement in irradiation-mediated apoptosis [22-25]. Irradiation-induced autophagic cell death has been reported in malignant glioma, breast and prostate cancer cells [17,26,27]. The present study is the first to report that ionizing irradiation treatment induced autophagy in human OSCC cell lines. After irradiation, loss of cell viability was found in OC3 cells, but not in SAS cells (Figure 1). In clinical practice,



Supplementary data 1. Irradiation decreased cell growth of human OSCC. Human OSCC cell lines OC3 and SAS were irradiated with **A:** different doses for 24 h and the cell morphology was studied by microscopy and **B:** with a 2 Gy dose treatment and the cell morphology was studied by microscopy at the indicated time periods

we have observed more favorable prognosis in non-betel quid chewing OSCC patients following radiation therapy compared to betel quid chewing OSCC patients (unpublished data). The findings of the present study were, thus, similar to these clinical observations. However, the possibility of genetic diversity among the Taiwanese and Japanese OSCC patients cannot be excluded. We also discovered that a one-step irradiation resulted in growth inhibition but showed recovery following prolonged incubation in all cell lines (supplementary data 1B). In clinical practice, radiotherapy is usually delivered in low dosages repeatedly to increase the therapeutic effect. The findings of this study could, therefore, explain why multiple radiation fractions are more effective than single-fraction for antitumor response.

In the formation of autophagy, the autophagosome (a double-membrane vesicle) is upregulated and LC3-II anchors on the autophagosome membrane. The autophagosome further fuses with a lysosome to form an autophagolysosome, and then an autolysosome. At the same time, LC3-II is degraded in the autolysosome. The levels of

LC3-II can, therefore, be used as a marker of autophagosome formation and induction of autophagy [28]. In the present study, we discovered GFP-LC3 puncta formation in ionizing irradiation-treated OC3 and SAS cells but not in GFP-transfected cells (Figure 2). These findings suggested that irradiation increased autophagosome formation in both OC3 and SAS cells. The Western blot results confirmed the upregulation of LC3-II in irradiated OC3 and SAS cells in a time-dependent manner (Figure 3B) but the OC3 was more susceptible to irradiation than SAS cells. Here, we demonstrated that autophagy was activated after ionizing irradiation treatment in human OSCC.

Several reports have described that attenuation of class I PI3K/Akt/mTOR pathway promotes the activation of autophagy [29]. It is also known that class III phosphatidylinositol 3-kinase (C3-PI3K) complex, which is composed of Vps34, Vps15 (also called p150), and beclin 1 (Atg6 in yeast), is involved in vesicle nucleation processes for phagophore assembly and autophagy induction [30,31]. Other molecules have reported involvement in the regulation of autophagy, such as 5'-AMP-activated protein kinase (AMPK), phosphorylated eukaryotic initiation factor 2 α (eIF2 α), p53, inositol-requiring enzyme-1 (IRE-1), c-jun-N-terminal kinase 1 (JNK1), inositoltriphosphate receptor (IP3R), and intracellular calcium [32]. The present study's results indicated the involvement of the mTOR pathway in irradiation-induced autophagy in human OSCC. The activation of Akt, one of the upstream of mTOR pathway, was reduced in SAS cells during irradiation, while OC3 cells did not show this response, indicating the involvement of different upstream pathways in irradiation-induced autophagy in various human OSCCs. Also the involvement of signaling pathways other than the mTOR pathway in irradiation-mediated autophagy in human OSCC cannot be excluded. Although OC3 and SAS cells both displayed evidence of induced autophagy after irradiation, degradation of p62 protein only occurred in OC3 cells (Figure 4). This result suggested that autophagolysosome and autolysosome formation and autophagy-induced degradation occurred in the OC3 cell line, but not in SAS cells. Further investigation is required to confirm this observation. Further studies are also needed to determine

whether the blocking of autolysosome formation occurred in SAS cells following irradiation. On the other hand, autophagy was induced in both OC3 and SAS cells; however, irradiation-mediated growth inhibition did not occur in SAS cells (Figure 1). The possible contribution of irradiation-mediated autophagic degradation to growth inhibition also warrants further investigation.

Autophagy has reported to play roles in anticancer therapy in multiple cancers [33-36]. Previous studies have described that chemoagent-induced autophagy increases the antitumor effects in human OSCC therapy [37,38]. Other previous investigations have also shown that irradiation-mediated autophagy increased anticancer activity in multiple cancer types [17,39-43]. The chemoagent-induced autophagy provokes sensitization of cells to irradiation and increases the anticancer effects of radiotherapy [17,27]. On the other hand, autophagy is also reported to reduce or resist anticancer effects during radiation treatment [40,44,45]. Nevertheless, whether irradiation can increase autophagy and the roles of irradiation-mediated autophagy in OSCCs are still unrevealed. In the present study, the results indicated that ionizing irradiation inhibited OC3 cell growth in a dose-dependent manner and rapamycin-induced autophagy further increased the inhibition of cell growth (Figure 5B). This finding demonstrated the synergistic effects of rapamycin and irradiation in suppressing of tumor growth.

In conclusion, the present study demonstrated that irradiation exposure increased autophagy through an mTOR-related pathway in human OSCC. Promoting autophagy by rapamycin could enhance the effects of radiotherapy. Application of autophagy therapies could, therefore, provide a novel approach in future cancer radiotherapeutics.

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