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Original Article

CDK4/6 inhibitor enhances the radiosensitization of esophageal squamous cell carcinoma (ESCC) by activating autophagy signaling via the suppression of mTOR

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Abstract: Objective: To investigate the radiosensitizing effect of cyclin D-cyclin dependent kinase (CDK) 4/6 inhibitor palbociclib on esophageal squamous cell carcinoma (ESCC) and its underlying mechanisms. Methods: The effect of palbociclib on ESCC cell radiosensitivity was detected by cell counting kit-8 (CCK-8) and clonogenic assay. γH2AX immunofluorescent staining was used to assess the repair of DNA damage induced by radiation. The expression of DNA repair proteins were examined by western blotting. Subsequently, immunoblotting and autophagy inhibitors were used to evaluate the underlying mechanisms of palbociclib triggered radiosensitization. Finally, the xenografts of ESCC were established to study the radiosensitizing effect of palbociclib *in vivo*. Results: Palbociclib combined with irradiation significantly inhibited the cell viability of ESCC *in vitro*. The expression level of γH2AX showed that radiation induced DNA damage repair was inhibited by palbociclib treatment. Palbociclib also suppressed the expression of RAD51 and phosphorylated DNA-dependent protein kinase catalytic subunit (p-DNA-PKcs) after irradiation. Mechanically, palbociclib enhanced the radiosensitization of ESCC by activating autophagy via suppression of mammalian target of rapamycin (mTOR). Inhibition of autophagy using chloroquine could partially reverse the radiation enhancing effect of palbociclib. Lastly, the xenografted tumor experiment confirmed the radiosensitizing effect of palbociclib in ESCC *in vivo*. Conclusion: Our results showed that palbociclib improved the radiosensitivity of ESCC *in vivo* and *in vitro*, and thus it may be a promising radiosensitization agent for the treatment of ESCC.

Keywords: CDK4/6 inhibitor, radiosensitization, esophageal squamous cell carcinoma, autophagy

Introduction

Cell cycle dysregulation is a hallmark of cancer, which promotes the proliferation of cancer cells [1, 2]. Cyclin D-cyclin dependent kinase (CDK) 4 and CDK6 are serine/threonine kinases that control the transition of cells from G1 to S phase through phosphorylation of the retinoblastoma (Rb) protein. Rb phosphorylation further promotes the release of E2 factor (E2F) family transcription factors which then transcribes the genes needed to enter S phase [3]. However, CDK4 and CDK6 are frequently altered in human cancer, resulting in the sustained proliferation of cancer cells [4, 5]. Targeting CDK4/6 activity by small molecule inhibitors has been considered as an attractive approach for cancer treatment for a long time [6, 7].

Palbociclib is an oral, highly specific and reversible CDK4/6 inhibitor, which can "turn off" CDK4 and CDK6 kinases and dephosphorylate Rb, resulting in G1 phase cell cycle arrest and proliferation inhibition in various types of cancer [8, 9]. The anticancer effect of palbociclib has been widely studied in preclinical studies in a series of human malignancies, including esophageal cancer [10-13]. However, the clinical trial have shown that palbociclib monother-

Table 1. List of materials used in the study

Material	Manufacturer
Ec109 cell line	BnBio, China
KYSE-150 cell line	BnBio, China
KYSE-70 cell line	BnBio, China
KYSE-30 cell line	Sigma-Aldrich, USA
RPMI-1640 medium	Gibco, USA
DMEM medium	Gibco, USA
MEM medium	Gibco, USA
10% fetal bovine serum	Gibco, USA
Dimethyl sulfoxide (DMSO)	Biotopped, China
Palbociclib (PD 0332991)	Sigma-Aldrich, USA
CCK-8 kit	Beyotime, China
RPA70, XRCC-2, XRCC-3, XRCC-4, Ligase IV, Ku70, p-DNA-PKcs, DNA-PKcs, GSDME-N, γ-H2AX antibody	Abcam, UK
RAD51, c-PARP, LC3-I/II, p-mTOR, mTOR antibody	Cell Signaling, USA
β-actin, Ki-67 antibody	Bioss, China
Enhanced chemoluminescence (ECL)	Thermo Fisher Scientific, USA
Goat anti-rabbit antibody	ZSGB-Bio, China
Athymic nude mice	CAS, China
DAB system	Dako, Denmark

apy failed to achieve the desired therapeutic effect in patients with esophageal cancer [14]. Therefore, developing a novel combination strategy with palbociclib is a possible way for successful treatment of esophageal cancer in the future.

Radiotherapy or combined chemotherapy is the main treatment for patients with advanced esophageal cancer. However, the response rate of some patients is still not optimal [15, 16]. Therefore, we are trying to determine whether palbociclib combined with irradiation is more effective than either treatment modality alone in esophageal cancer, as well as the underlying mechanisms of this phenomenon are also what we want to know [17].

In the present study, we evaluated the anticancer effect of CDK4/6 inhibitor palbociclib combined with irradiation on ESCC *in vivo* and *in vitro*. In addition, the potential mechanisms of the combined application of palbociclib and irradiation were also explored. We found that palbociclib combined with irradiation synergistically inhibited the proliferation of esophageal cancer *in vitro* and *in vivo*. The radiosensitizing effect of palbociclib was related to the sustained inhibition of mammalian target of rapamycin (mTOR) signal transduction and subsequent activation of autophagy. These results indicate that palbociclib is a promising radiosensitization candidate for the treatment of esophageal cancer, and it is reasonable to evaluate the role of palbociclib as a radiation modulator in clinical trials.

Materials and methods

Cell culture and treatment

Ec109 cells were maintained in RPMI-1640 medium. KYSE-150 cells were maintained in DMEM medium. KYSE-30 and KYSE-70 cells were maintained in MEM medium. All media was supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. All cell lines were incubated at 37°C in a humidified atmosphere containing 5% CO_2. The original solution of palbociclib (PD 0332991) was reconstituted in dimethyl sulfoxide (DMSO) and stored at -20°C until further use. The radiation was done with the Varian linear accelerator as previously described [18]. The materials used in this study are shown in **Table 1.**

Cell counting kit-8 (CCK-8) assay

The viability of ESCC cells were detected using CCK-8 kit according to the manufacturer's instructions. Cells were seeded in 96-well plates with 4000 cells per well, and treated with irradiation (IR), IR+palbociclib, or palbociclib alone. The control group was exposed to

the same concentration of DMSO as palbociclib. CCK-8 assay was performed at 72 hours after treatment. A total of 100 μl 10% CCK-8 solution was added to each well. After incubation at 37°C for 2 hours, the absorbance was measured at 450 nm with Synergy microplate reader.

Colony formation assay

Cells were seeded in 6-well plates and treated with IR or IR+palbociclib. After treatment, cells were then cultured in complete medium at 37°C in 5% CO_2 for 14 days. Finally, the monolayer of cells was fixed with 4% paraformaldehyde and stained with 0.2% crystal violet for 20 minutes. After washing, the number of colonies containing 50 or more cells was counted. The survival curve was fitted by linear-quadratic model according to the number of colonies.

Western blotting

Cell samples were lysed in a radioimmunoprecipitation (RIPA) buffer containing proteinase inhibitors and phosphatase inhibitors. The protein concentration of the lysates was determined by bicinchoninic acid (BCA) protein detection kit. Equal amount of protein was resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were transferred to polyvinylidene fluoride (PVDF) membrane. The membranes were then exposed to 5% skim milk at room temperature for 1 hour. After that, the membranes were incubated with primary antibodies for RPA70, XRCC-2, XRCC-3, XRCC-4, Ligase IV, Ku70, RAD51, p-DNA-PKcs, DNA-PKcs, c-PARP, GSDME-N, LC3-I/II, p-mTOR, mTOR, and β-actin, overnight at 4°C. Subsequently, the membranes were washed with tris-buffered saline (TBS) plus Tween 20 (TBST), and then incubated with secondary antibody at room temperature for 1 hour. Specific bands were visualized with the enhanced chemoluminescence liquid and detected by an imaging system (Bio-Rad, USA). The relative expression levels of protein were normalized against β-actin as the internal control.

Immunofluorescence

Cancer cells grown on chamber slides were fixed with 4% paraformaldehyde for 20 minutes at room temperature and permeabilized for 20 minutes in 1% Triton X-100. After washing, the slides were stained with antiphosphate agent γH2AX at 4°C overnight. The slides were then incubated with goat anti-rabbit antibody in dark at 37°C for 2 hours. In order to visualize the nuclei, cells were stained by 4', 6-diamidino-2-phenylindole (DAPI) diluted in phosphate-buffered saline (PBS). Finally, a digital image of the slides was analyzed using a confocal microscope (Olympus, Japan). The experiment was carried out in triplicate.

Tumor xenograft study

Six-week-old female athymic nude mice were used. A total of 2×10^6 Ec109 cells were suspended in 100 μl of PBS and subcutaneously injected into the left axilla of mice. When tumor volume was close to 100 mm^3, the mice were randomly divided into four groups: vehicle control, IR, IR+palbociclib, and palbociclib alone. There were 6 mice in each group. Palbociclib was dissolved in sterile-filtered sodium lactate buffer. The solution was then administered orally at a dose of 75 mg/kg three times every other day. Tumors were irradiated by Varian linear accelerator once, and the dose was 6 Gy (Unit of absorbed irradiation dose). Two weeks after treatment, all mice were sacrificed, and the tumors were dissected for further analysis. The volume of tumor was measured with caliper every week and calculated by the formula: volume $=0.5 \times (\text{length} \times \text{width}^2)$. This study was approved by the Ethics Committee of the General Hospital of Ningxia Medical University (2019-170) and carried out in accordance with the guidelines of the International Association for Experimental Animal Care Assessment and Certification.

Immunohistochemistry

Immunohistochemical staining was performed on 4 μm formalin-fixed and paraffin-embedded xenografts tissue. Following deparaffinization, the slides were rehydrated with a series of graded alcohols. Antigen retrieval was carried out by high pressure for 20 minutes at 100°C. Endogenous peroxidase activity was quenched with 3% H_2O_2. Afterwards, the slides were incubated with the indicated primary antibodies at 4°C overnight. The antigen-antibody binding was visualized using the DAB system accord-

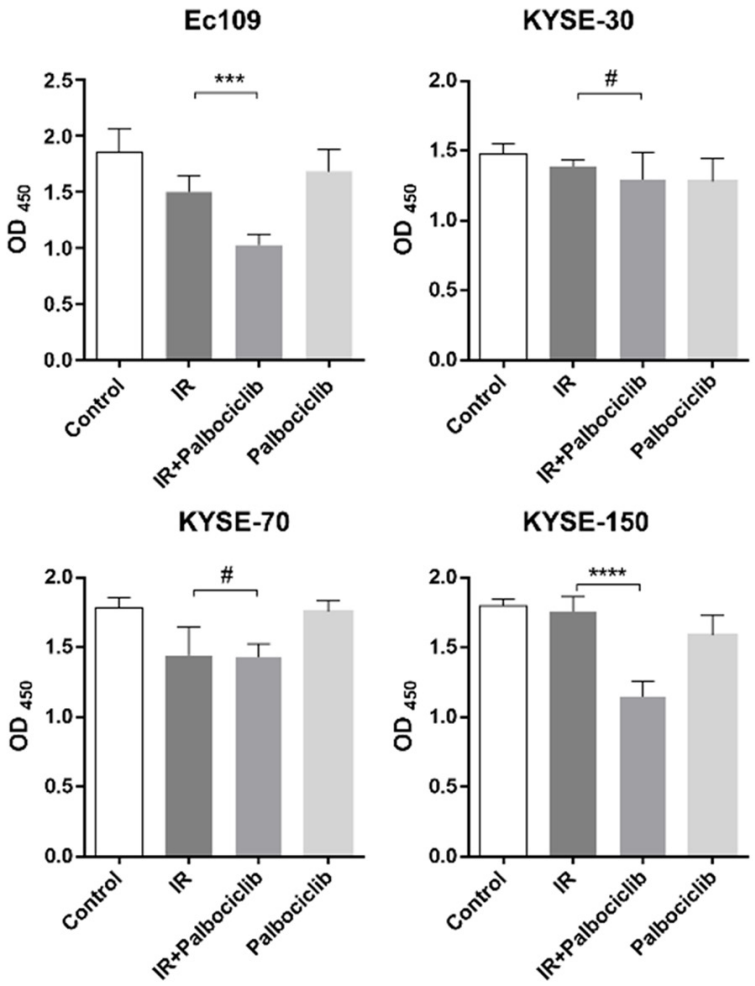

Figure 1. Palbociclib enhanced the sensitivity of esophageal squamous cell carcinoma (ESCC) cells to irradiation (IR). The cell viability of Ec109, KYSE-30, KYSE-70, and KYSE-150 cells after exposure to different treatment regimens were detected by CCK-8 assay. All results are presented as the mean ± SEM from three repeated experiments. The differences among the groups were obtained using Analysis of Variance (ANOVA). #No statistical significant, IR VS IR+Palbociclib; ***P<0.001, IR VS IR+Palbociclib; ****P<0.0001, IR VS IR+Palbociclib.

ing to the manufacturer's instructions. Finally, slides were counterstained with hematoxylin, dehydrated, and mounted. The following primary antibodies were used: LC3-II and Ki-67.

Statistical analysis

Data were shown as mean ± SEM. Student's t test or one-way analysis of variance (ANOVA) was used to determine the significance among groups. SPSS 22.0 software (Armonk, USA) and GraphPad Prism 8.0 software (San Diego, USA) were used for statistical analysis. *P* value less than 0.05 was considered significant.

Results

Palbociclib enhances radiosensitivity of ESCC cells in vitro

To evaluate the radiation enhancement effect of CDK4/6 inhibition on ESCC cell lines, we first performed CCK-8 assay. Four ESCC cell lines were treated with IR (single dose of 4 Gy), palbociclib (10 μM), IR+palbociclib (pretreated one hour prior to IR), and the cell viability was determined by CCK-8 assay. The results showed that palbociclib at a concentration of 10 μM increased the radiosensitivity of Ec109 and KYSE-150 cells. However, palbociclib failed to enhance the lethal effect of radiation on KYSE-30 and KYSE-70 cells (**Figure 1**). In order to further confirm the radiosesitizing role of palbociclib, we investigated the proliferation ability of the Ec109 and KYSE-150 cells which treated with IR+palbociclib through colony formation assays. The combined regimen more potently restrained the survival of clone formation in Ec109 and KYSE-150 cells (**Figure 2**). Collectively, these data suggest that palbociclib is a potential radiation modifier for ESCC cells.

Palbociclib inhibits radiation-induced DNA damage repair in ESCC cells

DNA damage repair exerts a critical role in the regulation of cancer radiosensitivity [19]. Next, we performed γH2AX immunofluorescence staining to evaluate the effect of palbociclib on the radiation-induced DNA damage repair in ESCC cells. The intensity of γH2AX foci of esophageal cancer cells exposed to IR was detected at different time points. **Figure 3** shows the γH2AX kinetics of Ec109 and KYSE-150 cells treated with IR or IR+palbociclib. In Ec109 and KYSE-150 cells, the positive rate of

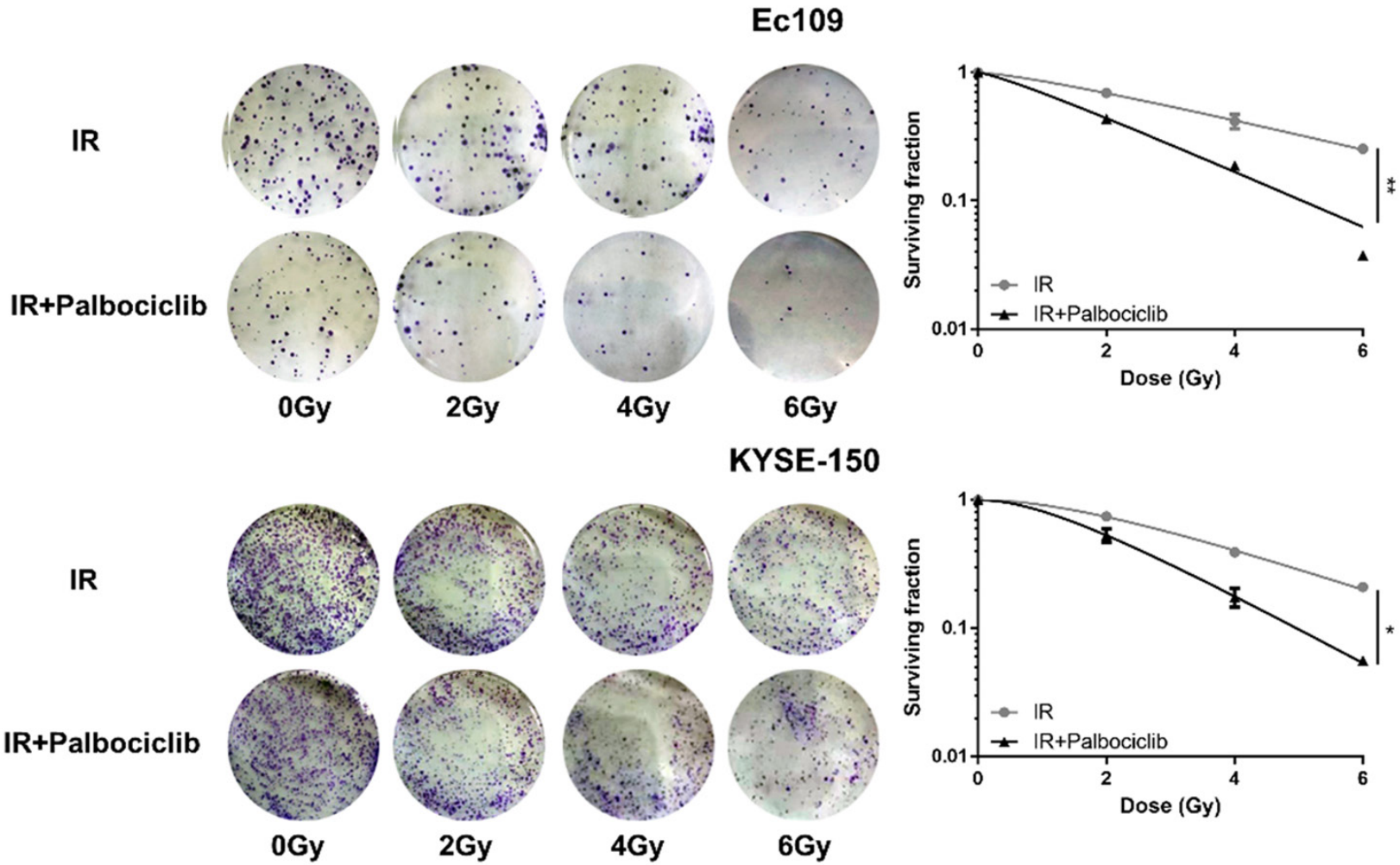

Figure 2. Colony formation of irradiated Ec109 and KYSE-150 esophageal squamous cell carcinoma (ESCC) cells with or without treatment with palbociclib were analyzed. The representative pictures of clone formation assay and cell survival curves are given. All results are presented as the mean ± SEM from three repeated experiments. The differences in the two groups were obtained using Student's t-test. *P<0.05, IR VS IR+Palbociclib; **P<0.01, IR VS IR+Palbociclib.

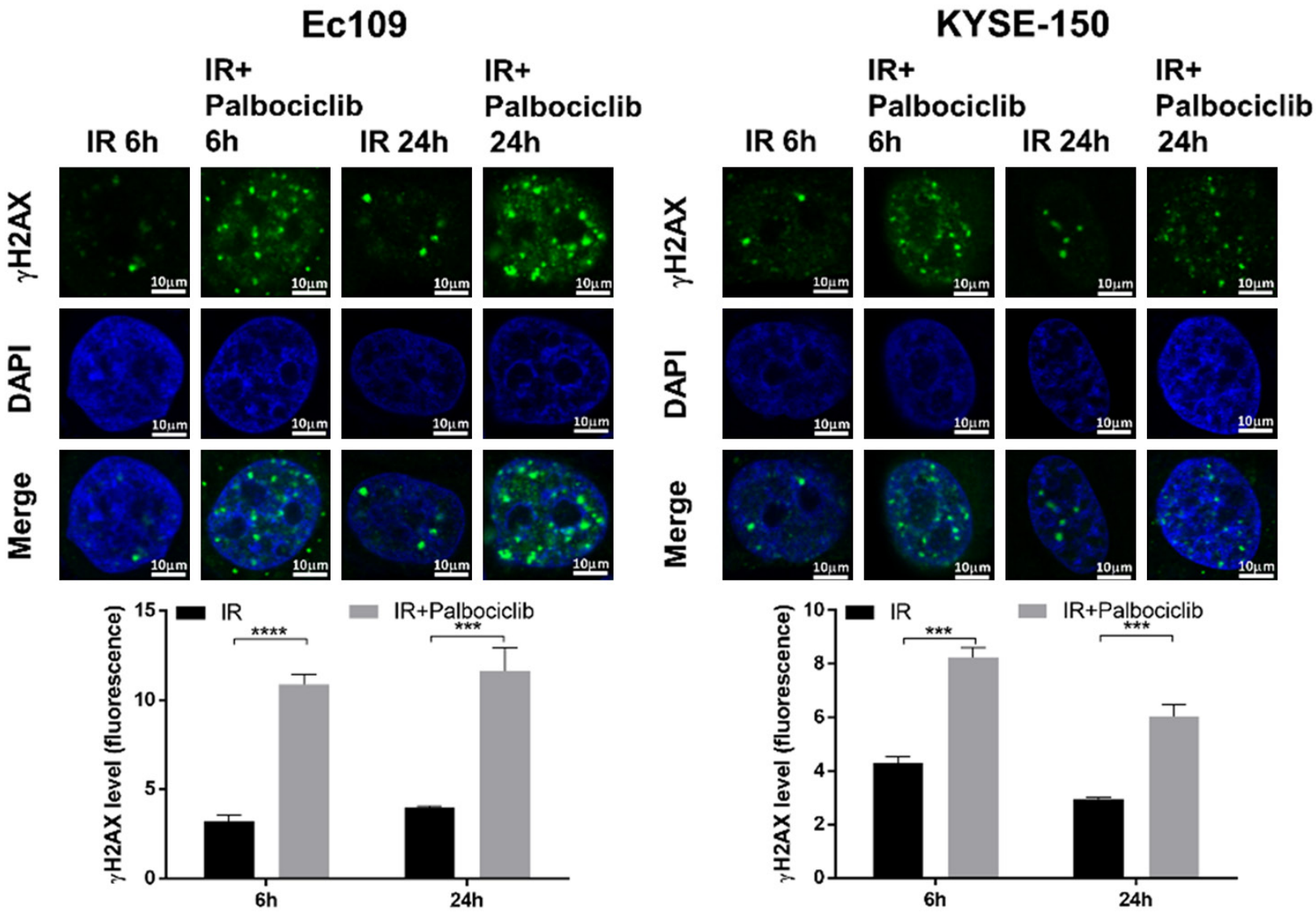

Figure 3. Palbociclib enhanced the irradiation (IR)-induced DNA damage in esophageal squamous cell carcinoma (ESCC) cells. Immunofluorescence analysis was used to evaluate the expression of γH2AX (green) in Ec109 and KYSE-150 cells treated with IR and IR+palbociclib for 6 hours and 24 hours. The nuclei were detected by DAPI. All results are presented as the mean ± SEM from three repeated experiments. The differences in the two groups were obtained using Student's t-test. ***P<0.001, IR VS IR+Palbociclib; ****P<0.0001, IR VS IR+Palbociclib.

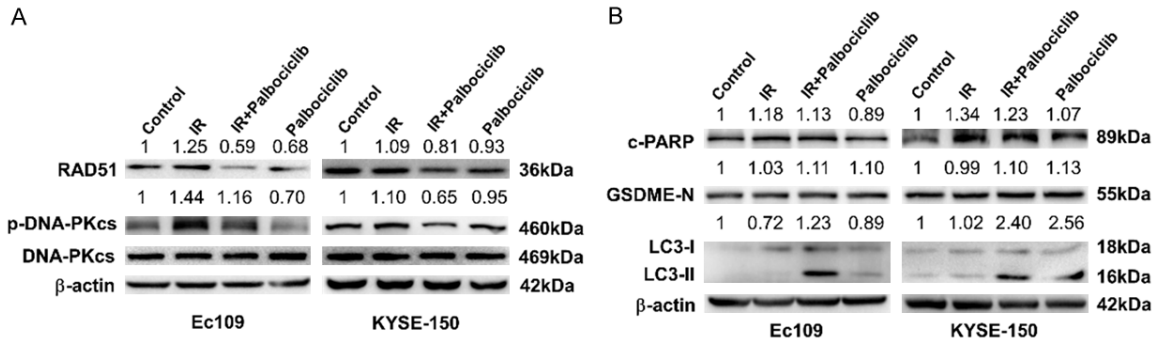

Figure 4. Palbociclib enhanced the radiosensitization of esophageal squamous cell carcinoma (ESCC) through impairing DNA damage repair proteins and activating autophagy. The key proteins in homologous recombination (HR) and non-homologous DNA end joining (NHEJ) DNA repair pathway were analyzed by western blotting. The results showed that compared with IR, IR+palbociclib could significantly inhibit the protein expression of RAD51 and p-DNA-PKcs (A). Western blotting was used to analyze the key proteins in different processes of cell death, such as apoptosis, autophagy, and pyroptosis. The results showed that IR+palbociclib could significantly transform LC-3 I into LC-3 II (B).

γH2AX immunocytochemistry was significantly increased at 6 hours and 24 hours after IR compared with radiation alone. These data suggest that the combined use of IR and palbociclib leads to the accumulation of DNA damage, which may contribute to the enhancement of radiosensitivity.

To further evaluate the influence of palbociclib on radiation induced DNA damage repair, western blotting was used to detect the protein expression levels of RPA70, XRCC-2, XRCC-3, XRCC-4, Ligase IV, Ku70, RAD51, and p-DNA-PKcs, the key proteins of DNA repair after IR or IR+palbociclib management. Compared with IR alone, the combination of palbociclib and IR decreased the expression of RAD51 and p-DNA-PKcs in esophageal cancer cells (**Figure 4A**). These results suggest that palbociclib inhibits both homologous recombination (HR) and non-homologous DNA end joining (NHEJ) DNA repair pathway and leads to the radiation sensitization.

Palbociclib increases the radiosensitivity of ESCC cells by inducing autophagy

In order to further explore the potential mechanisms of radiosensitization induced by palbociclib in ESCC cells, different subroutines of regulated cell death, including apoptosis, autophagy, and pyroptosis, that may occur in response to IR+palbociclib were evaluated by western blotting. Compared with IR group, IR+palbociclib could transform LC-3 I into LC-3 II, but there was no significant difference in protein expression level of cleaved poly (ADP-ribose) polymerase (c-PARP) and N-terminal of Gasdermin E (GSDME-N) between IR group and IR+palbociclib group (**Figure 4B**). Many reports have revealed that the mTOR signaling pathway was the main negative regulator of autophagy [20, 21]. In the subsequent study, western blotting was used to verify whether mTOR was involved in autophagy induced by palbociclib. The results showed that compared with IR group, the expression of p-mTOR protein in IR+palbociclib group was down-regulated, but the total amount of mTOR protein had no significant change (**Figure 5A**). These results suggest that IR+palbociclib kills ESCC cells by preferentially stimulating cellular autophagy.

The role of autophagy in regulating palbociclib-mediated radiosensitivity has been further confirmed in rescue experiments. Firstly, the effect of autophagy inhibitor chloroquine (CQ) on the IR+palbociclib-induced death of ESCC cells was measured with CCK-8 assays. Compared with IR+palbociclib group, the addition of 20 μM CQ could significantly improve ESCC cell viability (**Figure 5B**). Therefore, we chose 20 μM CQ for clonogenic survival assays to further confirm the effect of autophagy on palbociclib-mediated radiosensitivity of ESCC cells. The results showed that 20 μM CQ treatment partially restored the clone survival rate of ESCC cells decreased by IR+palbociclib exposure (**Figure 5C**). In summary, our results suggest that the radiosensitization induced by pal-

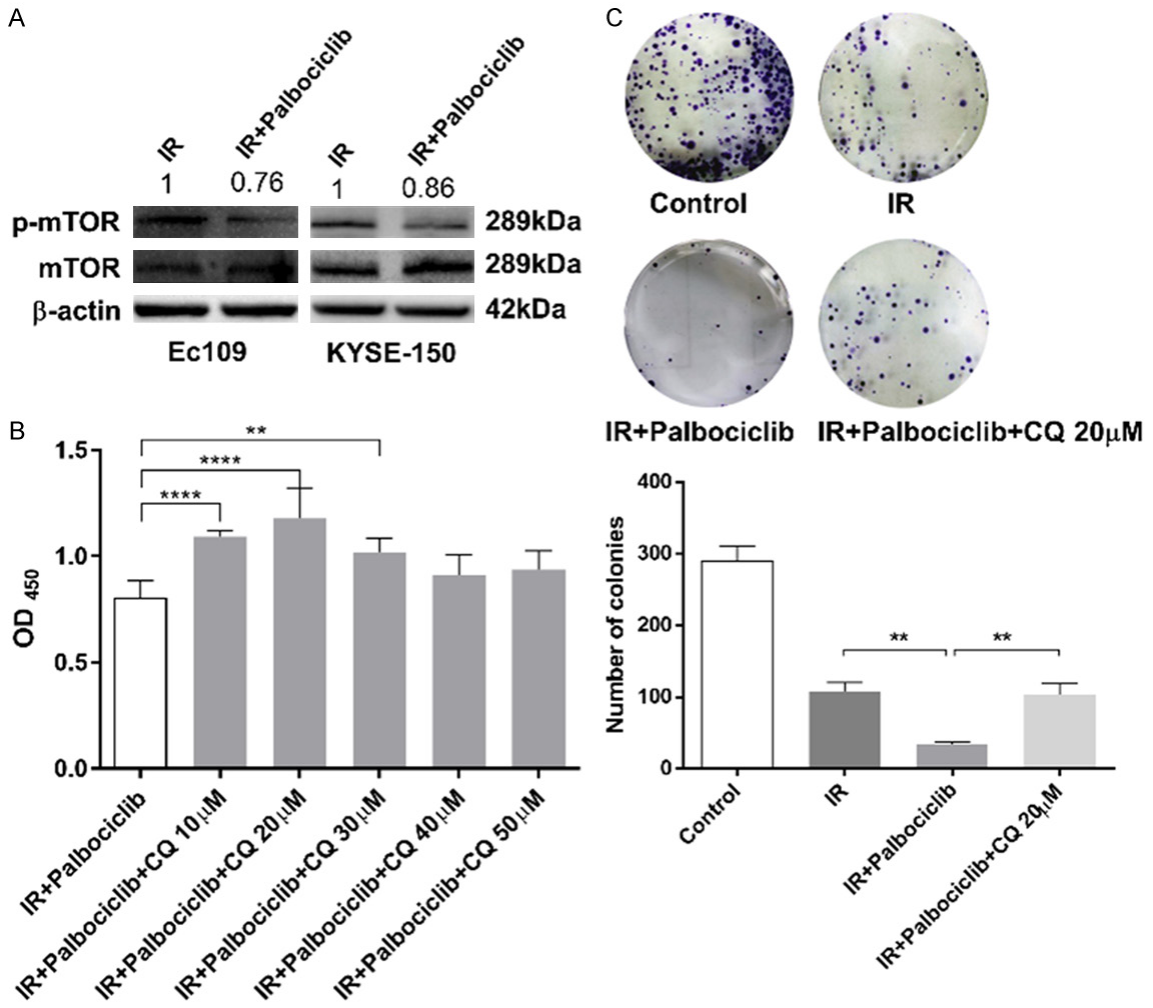

Figure 5. Palbociclib induced-esophageal squamous cell carcinoma (ESCC) radiosensitization could be partially reversed by autophagy inhibitor. Western blot analysis showed that compared with IR, IR+palbociclib significantly inhibited the expression of p-mTOR (A). CCK-8 assay was used to detect the cell viability after the combination of IR+palbociclib and different concentrations of chloroquine (CQ) (B). Twenty μM CQ was selected for further study. Representative pictures of clone formation assay when combined with CQ and IR+palbociclib (C). All results are presented as the mean ± SEM from three repeated experiments. The differences among the groups were obtained using Analysis of Variance (ANOVA). ****P<0.0001, IR+Palbociclib VS IR+Palbociclib+CQ 10 μM; ****P<0.0001, IR+Palbociclib VS IR+Palbociclib+CQ 20 μM; **P<0.01, IR+Palbociclib VS IR+Palbociclib+CQ 30 μM; **P<0.01, IR VS IR+Palbociclib; **P<0.01, IR+Palbociclib VS IR+Palbociclib+CQ 20 μM.

bociclib is at least partially due to the activation of autophagy.

Palbociclib enhances the radiosensitivity of ESCC xenografts

Finally, we evaluated the efficacy of palbociclib combined with radiotherapy in xenografts. Athymic nude mice bearing Ec109 tumor xenografts were used for tumor growth delay assay. When the tumor was palpable (100 mm^3), the mice were randomly divided into control, IR, IR+palbociclib, or palbociclib alone group. As shown in **Figure 6**, in combination with radiation, palbociclib produced a significant radiosensitization effect compared with radiation alone. Furthermore, detection of the Ki-67 proliferation marker by IHC showed that the proliferation of Ec109 cells was slightly decreased in the tumors of the IR group, while the combination treatment of IR and palbociclib exerted a remarkable effect. In addition, tumors in the IR+palbociclib-treated animals demonstrated an increase in the expression level of LC-3 II compared with those from the IR-treated mice. These results suggested that palbociclib can

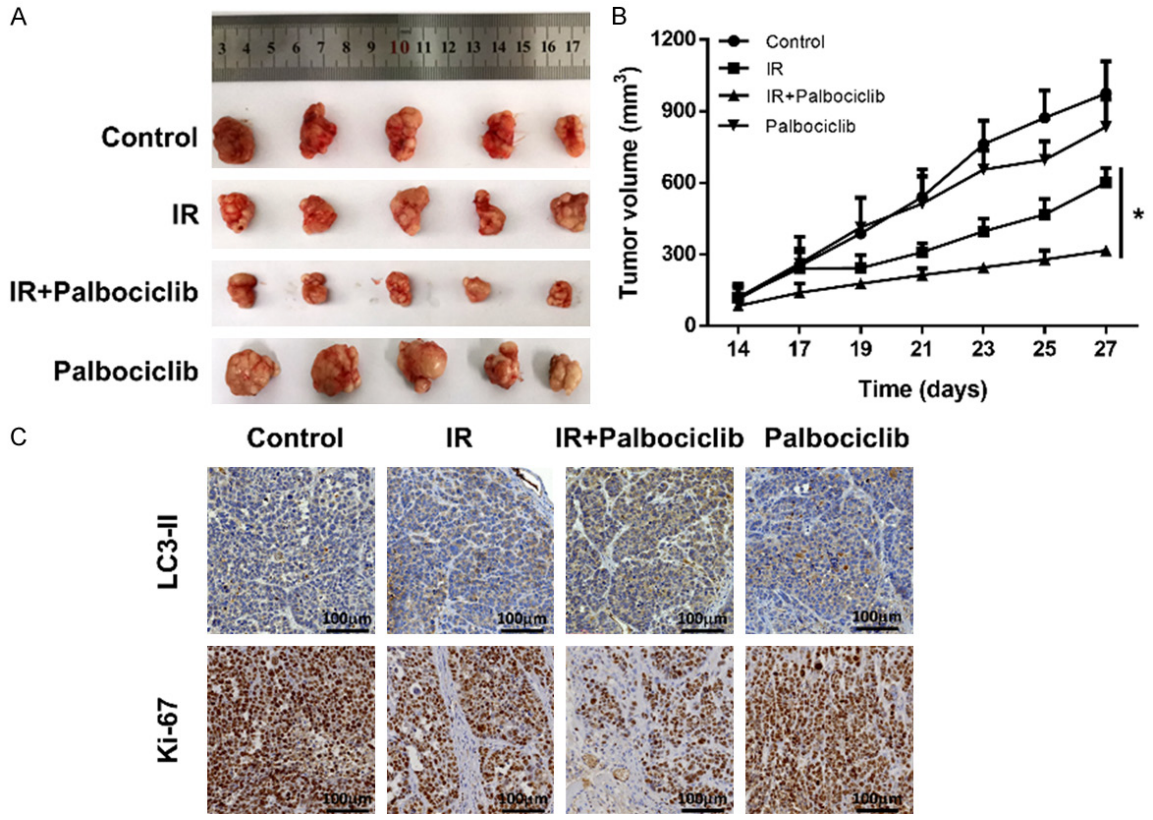

Figure 6. Palbociclib sensitized esophageal squamous cell carcinoma (ESCC) xenografts to radiotherapy. The growth images of Ec109 tumor in nude mice in control, IR, IR+palbociclib, and palbociclib alone group (A). The growth curves of Ec109 tumor in nude mice in control, IR, IR+palbociclib, and palbociclib alone group (B). Immunohistochemical staining of formalin-fixed, paraffin-embedded tumor tissue for LC3-II and Ki-67 (C). All results are presented as the mean ± SEM of 5 mice in each group. The differences among the groups were obtained using Analysis of Variance (ANOVA). *P<0.05, IR VS IR+Palbociclib.

effectively enhance the radiosensitivity of ESCC, reduce tumor burden through activating autophagy.

Discussion

CDK4/6 exerts a critical role in the proliferation of esophageal cancer cells. Targeting CDK4/6 is considered to be a feasible way for the treatment of esophageal cancer. In fact, several CDK4/6 specific inhibitors have been developed, including palbociclib, abemaciclib, and riociclib, which are being intensively explored in human cancers in pre-clinical studies and clinical trials. Recently, the anticancer effect of CDK4/6 specific inhibitors on ESCC has been confirmed *in vivo* and *in vitro*. Chen et al. [10] found that palbociclib inhibited the growth of ESCC cells and induced mitochondrial-dependent apoptosis. In addition, palbociclib had a synergistic effect with cisplatin or 5-fluorouracil. Wang et al. [12] demonstrated that CDK4/6 inhibitor SHR6390 could inhibit the proliferation of ESCC cells *in vitro* and tumor growth in a patient-derived tumor xenograft (PDX) model. Mechanically, SHR6390 effectively suppressed the phosphorylation of Rb and arrested the cell cycle at G1 phase. Moreover, SHR6390 combined with paclitaxel or cisplatin had synergistic inhibitory effect on Eca 9706 xenografts which showed relative lower sensitivity of SHR6390 alone. Zhou et al. [13] revealed that a CDK4/6 inhibitor could prevent the resistance of epidermal growth factor receptor (EGFR) inhibitors in ESCC with EGFR amplification *in vitro* and *in vivo*. Su et al. [22] confirmed cyclin dependent kinase inhibitor 2A/2B (CDKN2A/2B) deletion was a biomarker for predicting the sensitivity of CDK4/6 inhibitors in PDX model of ESCC. However, the radiosensitization effect of CDK4/6 inhibitors on ESCC has not been widely studied. Al-

though Li et al. [23] reported that targeting both yes1 associated transcriptional regulator (YAP1) and CDK6 pathways could overcome radiation resistance in esophageal cancer.

In the present study, the combined use of palbociclib and IR significantly inhibited the viability of Ec109 and KYSE-150 cells. However, no radiosensitization effect was observed in KYSE-30 and KYSE-70 cells, which may be related to the status of p53 and Rb in these two cell lines. In order to further explore the mechanisms of radiosensitization of palbociclib, the positive rate of γH2AX and the expression level of a key DNA damage response protein were tested. The results showed that the immunocytochemical positive rate of γH2AX after IR+palbociclib treatment was significantly higher than that in the IR group. In addition, the combined action of CDK4/6 inhibition and IR led to the impairment of HR and NHEJ. Thus, the impact of palbociclib on the repair of DNA damage may be one of the mechanisms of its radiosensitization effect. Although we focused on CDK4/6 inhibition and the DNA damage response, other mechanisms may also contribute to radiosensitization phenotype. Subsequently, we evaluated the subroutines that regulated cell death induced by the combined therapy. The results showed that the combination of palbociclib and IR mainly induced autophagy, which had also been confirmed in rescue studies.

Autophagy is generally regarded as a physiological phenomenon that protects cells from changes in the intracellular or extracellular environment [24-28]. Radiotherapy is an effective and common strategy for the treatment of various types of cancer. It is well known that the outcome of radiotherapy can be regulated by activation of autophagy [29]. A large number of pharmaceutical studies have shown that promoting autophagy through a variety of ways can improve the results of cancer radiotherapy. Similar to radiotherapy, CDK4/6 inhibitors can also induce autophagy. Iriyama et al. [30] observed that CDK4/6 inhibitor abemaciclib induced autophagy in multiple myeloma cell lines in a dose-dependent manner. Valenzuela et al. [31] studied the inhibitory effect of CDK4/6 inhibitors on gastric cancer cell lines. Inhibition of CDK4/6 in gastric cancer cells led to the induction of autophagy which was dependent on the status of pRb and p53. Vijayaraghavan et al. [32] further revealed that an intact G1/S transition was a reliable predictive biomarker for the combined use of CDK4 and autophagy inhibitors. Okada et al. [33] found that the combination of CDK4 and an autophagy inhibitor could further induce apoptosis. Autophagy and apoptosis are different types of programmed cell death. Recent studies have shown that there is a complex interaction between autophagy and apoptosis. In some cases, autophagy induced by anticancer therapy can directly lead to cell death, but in other situations, autophagy can induce cell death by mediating apoptosis [34, 35]. In this study, we demonstrated that palbociclib mediated the radiosensitization of ESCC by activating autophagy. Autophagy inhibitor CQ could reverse the radiosensitivity of esophageal cancer cells caused by CDK4/6 inhibition. More importantly, *in vivo* experiments also confirmed that palbociclib could improve the radiosensitivity of ESCC cells in nude mice by activating autophagy, which was consistent with our results *in vitro*. Autophagy itself or apoptosis mediated by autophagy may contribute to cell death triggered by CDK4/6 inhibitors and radiotherapy. Therefore, palbociclib enhances the radiosensitivity of ESCC by mediating DNA damage and cellular autophagy, and is expected to become a radiosensitizer for ESCC.

Conclusion

In conclusion, our results show that compared with the use of IR alone, the combined use of IR and palbociclib could significantly inhibit the cell viability of ESCC. Palbociclib combined with IR causes DNA damage repair disorders and induction of autophagy. The combination of CDK4/6 inhibition and IR is a potential and effective strategy for radiosensitization of esophageal cancer, which warrants clinical investigation.

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Disclosure of conflict of interest

None.

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References

[1] Suski JM, Braun M, Strmiska V and Sicinski P. Targeting cell-cycle machinery in cancer. Cancer Cell 2021; 39: 759-778.

[2] Schwartz GK and Shah MA. Targeting the cell cycle: a new approach to cancer therapy. J Clin Oncol 2005; 23: 9408-9421.

[3] Goel S, DeCristo MJ, McAllister SS and Zhao JJ. CDK4/6 inhibition in cancer: beyond cell cycle arrest. Trends Cell Biol 2018; 28: 911-925.

[4] O'Leary B, Finn RS and Turner NC. Treating cancer with selective CDK4/6 inhibitors. Nat Rev Clin Oncol 2016; 13: 417-430.

[5] Hamilton E and Infante JR. Targeting CDK4/6 in patients with cancer. Cancer Treat Rev 2016; 45: 129-138.

[6] Sanchez-Martinez C, Lallena MJ, Sanfeliciano SG and de Dios A. Cyclin dependent kinase (CDK) inhibitors as anticancer drugs: recent advances (2015-2019). Bioorg Med Chem Lett 2019; 29: 126637.

[7] Poratti M and Marzaro G. Third-generation CDK inhibitors: a review on the synthesis and binding modes of palbociclib, ribociclib and abemaciclib. Eur J Med Chem 2019; 172: 143-153.

[8] Liu M, Liu H and Chen J. Mechanisms of the CDK4/6 inhibitor palbociclib (PD 0332991) and its future application in cancer treatment (review). Oncol Rep 2018; 39: 901-911.

[9] Roskoski R Jr. Cyclin-dependent protein kinase inhibitors including palbociclib as anticancer drugs. Pharmacol Res 2016; 107: 249-275.

[10] Chen L and Pan J. Dual cyclin-dependent kinase 4/6 inhibition by PD-0332991 induces apoptosis and senescence in oesophageal squamous cell carcinoma cells. Br J Pharmacol 2017; 174: 2427-2443.

[11] Qie S, Yoshida A, Parnham S, Oleinik N, Beeson GC, Beeson CC, Ogretmen B, Bass AJ, Wong KK, Rustgi AK and Diehl JA. Targeting glutamine-addiction and overcoming CDK4/6 inhibitor resistance in human esophageal squamous cell carcinoma. Nat Commun 2019; 10: 1296.

[12] Wang J, Li Q, Yuan J, Wang J, Chen Z, Liu Z, Li Z, Lai Y, Gao J and Shen L. CDK4/6 inhibitor-SHR6390 exerts potent antitumor activity in esophageal squamous cell carcinoma by inhibiting phosphorylated Rb and inducing G1 cell cycle arrest. J Transl Med 2017; 15: 127.

[13] Zhou J, Wu Z, Wong G, Pectasides E, Nagaraja A, Stachler M, Zhang H, Chen T, Zhang H, Liu JB, Xu X, Sicinska E, Sanchez-Vega F, Rustgi AK, Diehl JA, Wong KK and Bass AJ. CDK4/6 or MAPK blockade enhances efficacy of EGFR inhibition in oesophageal squamous cell carcinoma. Nat Commun 2017; 8: 13897.

[14] Karasic TB, O'Hara MH, Teitelbaum UR, Damjanov N, Giantonio BJ, d'Entremont TS, Gallagher M, Zhang PJ and O'Dwyer PJ. Phase II trial of palbociclib in patients with advanced esophageal or gastric cancer. Oncologist 2020; 25: e1864-e1868.

[15] Chun SG, Skinner HD and Minsky BD. Radiation therapy for locally advanced esophageal cancer. Surg Oncol Clin N Am 2017; 26: 257-276.

[16] Liao Z, Cox JD and Komaki R. Radiochemotherapy of esophageal cancer. J Thorac Oncol 2007; 2: 553-568.

[17] Yang Y, Luo J, Chen X, Yang Z, Mei X, Ma J, Zhang Z, Guo X and Yu X. CDK4/6 inhibitors: a novel strategy for tumor radiosensitization. J Exp Clin Cancer Res 2020; 39: 188.

[18] Xia D, Zhang XR, Ma YL, Zhao ZJ, Zhao R and Wang YY. Nrf2 promotes esophageal squamous cell carcinoma (ESCC) resistance to radiotherapy through the CaMKIIalpha-associated activation of autophagy. Cell Biosci 2020; 10: 90.

[19] Jorgensen TJ. Enhancing radiosensitivity: targeting the DNA repair pathways. Cancer Biol Ther 2009; 8: 665-670.

[20] Kim YC and Guan KL. mTOR: a pharmacologic target for autophagy regulation. J Clin Invest 2015; 125: 25-32.

[21] Munson MJ and Ganley IG. MTOR, PIK3C3, and autophagy: signaling the beginning from the end. Autophagy 2015; 11: 2375-2376.

[22] Su D, Zhang D, Jin J, Ying L, Han M, Chen K, Li B, Wu J, Xie Z, Zhang F, Lin Y, Cheng G, Li JY, Huang M, Wang J, Wang K, Zhang J, Li F, Xiong L, Futreal A and Mao W. Identification of predictors of drug sensitivity using patient-derived models of esophageal squamous cell carcinoma. Nat Commun 2019; 10: 5076.

[23] Li F, Xu Y, Liu B, Singh PK, Zhao W, Jin J, Han G, Scott AW, Dong X, Huo L, Ma L, Pizzi MP, Wang Y, Li Y, Harada K, Xie M, Skinner HD, Ding S, Wang L, Krishnan S, Johnson RL, Song S and Ajani JA. YAP1-mediated CDK6 activation confers radiation resistance in esophageal cancer-rationale for the combination of YAP1 and CDK4/6 inhibitors in esophageal cancer. Clin Cancer Res 2019; 25: 2264-2277.

[24] Glick D, Barth S and Macleod KF. Autophagy: cellular and molecular mechanisms. J Pathol 2010; 221: 3-12.

[25] Chude CI and Amaravadi RK. Targeting autophagy in cancer: update on clinical trials and novel inhibitors. Int J Mol Sci 2017; 18: 1279.
[26] Yoshida GJ. Therapeutic strategies of drug repositioning targeting autophagy to induce cancer cell death: from pathophysiology to treatment. J Hematol Oncol 2017; 10: 67.
[27] Wu W, Ma J, Shao N, Shi Y, Liu R, Li W, Lin Y and Wang S. Co-targeting IGF-1R and autophagy enhances the effects of cell growth suppression and apoptosis induced by the IGF-1R inhibitor NVP-AEW541 in triple-negative breast cancer cells. PLoS One 2017; 12: e0169229.
[28] Esner M, Graifer D, Lleonart ME and Lyakhovich A. Targeting cancer cells through antibiotics-induced mitochondrial dysfunction requires autophagy inhibition. Cancer Lett 2017; 384: 60-69.
[29] Tam SY, Wu VW and Law HK. Influence of autophagy on the efficacy of radiotherapy. Radiat Oncol 2017; 12: 57.
[30] Iriyama N, Hino H, Moriya S, Hiramoto M, Hatta Y, Takei M and Miyazawa K. The cyclin-dependent kinase 4/6 inhibitor, abemaciclib, exerts dose-dependent cytostatic and cytocidal effects and induces autophagy in multiple myeloma cells. Leuk Lymphoma 2018; 59: 1439-1450.
[31] Valenzuela CA, Vargas L, Martinez V, Bravo S and Brown NE. Palbociclib-induced autophagy and senescence in gastric cancer cells. Exp Cell Res 2017; 360: 390-396.
[32] Vijayaraghavan S, Karakas C, Doostan I, Chen X, Bui T, Yi M, Raghavendra AS, Zhao Y, Bashour SI, Ibrahim NK, Karuturi M, Wang J, Winkler JD, Amaravadi RK, Hunt KK, Tripathy D and Keyomarsi K. CDK4/6 and autophagy inhibitors synergistically induce senescence in Rb positive cytoplasmic cyclin E negative cancers. Nat Commun 2017; 8: 15916.
[33] Okada Y, Kato S, Sakamoto Y, Oishi T and Ishioka C. Synthetic lethal interaction of CDK inhibition and autophagy inhibition in human solid cancer cell lines. Oncol Rep 2017; 38: 31-42.
[34] Su Z, Yang Z, Xu Y, Chen Y and Yu Q. Apoptosis, autophagy, necroptosis, and cancer metastasis. Mol Cancer 2015; 14: 48.
[35] Tilija Pun N, Jang WJ and Jeong CH. Role of autophagy in regulation of cancer cell death/apoptosis during anti-cancer therapy: focus on autophagy flux blockade. Arch Pharm Res 2020; 43: 475-488.