

RAD001 Enhances the Radiosensitivity of SCC4 Oral Cancer Cells by Inducing Cell Cycle Arrest at the G₂/M Checkpoint

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Abstract. *Background:* Inhibition of mammalian target of rapamycin (mTOR) kinase enhances the radiosensitivity of some cancer cells. We investigated the effect of RAD001, an mTOR inhibitor, on irradiated oral cancer cell lines. *Materials and Methods:* Clonogenic assays were performed to determine the radiosensitivity of SCC4 and SCC25 cells after treatment with RAD001. Target protein phosphorylation, apoptosis, and cell-cycle progression were assessed in SCC4 cells treated with RAD001 with and without ionizing radiation. *Results:* RAD001 increased the radiosensitivity of SCC4 cells without affecting cell death; it also inhibited phosphorylation of mTOR, S6, and factor 4E binding protein 1 and reduced the clonogenic survival of irradiated cancer cells. RAD001 combined with radiation increased G₂ arrest by activating CHK1, which phosphorylates CDC25C at Ser216, thereby inhibiting CDC2–cyclin B 1 complex formation. *Conclusion:* RAD001 enhances the radiosensitivity of SCC4 cells by inhibiting mTOR signaling and inducing G₂ cell-cycle arrest through disruption of the G₂ checkpoint.

Oral squamous cell carcinoma (OSCC) is one of the most common malignant neoplasms in Taiwan and is currently

the fourth most common cause of cancer-related mortality in men (1).

Radiation therapy (RT) is an important treatment modality for oral cancer as early-stage and patients with locally advanced disease can often be treated with local radiation alone. However, loss of local control of the disease and the significant toxicity associated with radiation therapy remain problematic. Therefore, increasing the radiosensitivity of oral cancer in order to maintain local control of the disease, reduce the radiation dose and radiation toxicity, and thereby improve efficacy is crucial.

mTOR, a downstream serine/threonine protein kinase in the phosphatidylinositol 3-kinase (PI3K)/AKT pathway, and its downstream effectors, S6 and factor 4E binding protein 1 (4EBP1), play important roles in multiple cellular functions, including cell proliferation and growth (2, 3). The mTOR signaling pathway is abnormally activated in many types of human cancers and is associated with the transformation process and oncogenesis (2, 3). Consequently, the anti-neoplastic effects of mTOR inhibitors combined with chemotherapeutic agents, or other targeted-therapeutic agents for treating various types of cancer have been evaluated in clinical studies (4, 5). In addition, mTOR inhibitors increase the radiosensitivity of cancer cells *in vitro* and *in vivo* (6-8) through different mechanisms, including increased autophagy (9) and apoptosis (6) as well as through anti-angiogenic effects.

RAD001 (everolimus), a derivative of rapamycin, acts as an allosteric inhibitor of mTOR. Its antitumor effects *in vitro* and *in vivo* have been reported in some carcinoma models (11-13). Currently, RAD001 is being tested as an antitumor drug in phase II/III clinical trials (14, 15). However, the molecular mechanism underlying increased OSCC radiosensitivity due

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to RAD001 has not been elucidated. Therefore, we evaluated the antitumor effects of RAD001 alone and in combination with radiation in OSCC-derived cell lines. In addition, we investigated the mechanism by which combined treatment with RAD001 and radiation inhibits tumor growth.

Materials and Methods

Agent. RAD001 (everolimus) was supplied by Novartis Pharmaceuticals Corporation (East Hanover, NJ, USA), dissolved in DMSO at a concentration of 10 mM, and stored at -20°C until further use. The stock solution was diluted to the appropriate concentration in culture medium containing serum just before addition to cell cultures.

Cell lines and cell culture. The human head and neck squamous cell carcinoma cell lines, SCC4 and SCC25, derived from squamous cell carcinoma of the tongue, were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in DMEM/F12 containing 10% fetal bovine serum (FBS), 1% penicillin-streptomycin (10,000 U/ml penicillin and 10 mg/ml streptomycin), and 2 mM glutamine in 10-cm dishes at 37°C under a humidified atmosphere of 5% CO_2 and 95% air.

Ionizing radiation. The cells were irradiated with a 6 MV X-ray medical linear accelerator (LINAC; Elekta, Crawley, Surrey, UK) at different doses (0, 2, 4, 6, and 8 Gy) with 159 MU. RAD001 was added to the cells 1 h before irradiation.

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Cells (5×10^3) were seeded in 96-well plates overnight and then treated with either DMSO (vehicle) or RAD001 (30-300 nM) for 24 and 48 h. MTT solution (0.5 mg/ml in PBS) was then added (10 μl /well), and the cells were incubated for 4 h at 37°C . The formazan crystals formed were dissolved by the addition of 0.04 N HCl in 2-propanol (110 μl /well). Absorbance was measured with a microculture plate reader (Anthos; Anthos Labtec, Wals, Austria) at 540 nm.

Cell survival clonogenic assay. The cells were seeded in 10-cm dishes at a density of 700 cells/dish. After the cells were cultured overnight, they were pretreated with vehicle (DMSO) or RAD001 (30 or 300 nM) for 1 h at 37°C . The cells were then irradiated (0, 2, 4, 6, or 8 Gy) for 6 h after which the culture medium was replaced with fresh medium, and the cells were returned to a 37°C incubator for further growth. After 14 days, the colonies (defined as groups >50 cells) were stained with 0.05% crystal violet and counted. The relative surviving fraction was determined by dividing the plating efficiency of the irradiated cells by the plating efficiency of the control cells (without irradiation) in three independent experiments.

Western blot analysis. The cells were treated with 300 nM RAD001 with/without 6 Gy radiation in individual experiments, after which they were washed twice with ice-cold PBS. Total cell extracts were prepared using the PRO-PREP protein extraction solution (iNtRON Biotechnology, Sungnam, Republic of Korea) containing a cocktail of phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO, USA). Cell suspensions were then centrifuged to collect clear lysates, and the protein concentration was measured using the Bio-Rad protein assay kit (Bio-Rad, Richmond, CA, USA). Proteins (50 μg) were separated

using 10% and 12.5% polyacrylamide gels containing 0.1% SDS and transferred to membranes (Millipore, Billerica, MA, USA). After blocking the membranes with 5% (w/v) non-fat dry milk in TBS containing 0.1% TweenTM 20 (TBS-T) for 1 h at room temperature, they were immunoblotted with the following monoclonal primary antibodies (all rabbit): p-mTOR (Ser2448), p-AKT (S473), p-S6 (Ser235/236), p-4EBP1 (Ser65), eukaryotic translation initiation factor 4E (eIF4E), microtubule-associated protein 1A/1B-light chain 3 (LC3), beclin 1, caspase 3, CDC25C, and p-CDC25C (Ser216), Rictor, H2AX, BAK, BAX (all from Cell Signaling Technology, Beverly, MA, USA). A mouse polyclonal antibody to actin was purchased from Santa Cruz Biotechnology. (Santa Cruz, CA, USA). Mouse monoclonal antibodies to poly(ADP-ribose) polymerase (PARP), p-CHK2 (Thr68), p-CHK1 (Ser345), and cyclin B were purchased from Epitomics (Burlingame, CA, USA). Appropriate horseradish peroxidase-conjugated secondary antibodies, including anti-mouse IgG and anti-rabbit IgG antibodies (Abcam, Cambridge, MA, USA), were incubated for 1 h at room temperature. Specific signals were visualized using a chemiluminescence (ECL) detection kit (Millipore).

Cell-cycle analysis. After treatment with 300 nM RAD001 with/without 6 Gy radiation as described, the cells were trypsinized, washed with PBS, and subjected to cell-cycle analysis using NucleoCounter NC-3000 (Chemometec, Allerød, Denmark), a two-step method that involves cell lysis and staining of the nuclei with DAPI.

Apoptosis assay. The apoptotic effects of RAD001 and radiation on SCC4 cells were assessed with the Annexin V-FITC Apoptosis Detection Kit (Strong Biotech, Taipei, Taiwan). Cells (1×10^5) in 6-cm dishes were treated with RAD001 with/without radiation for 48 and 72 h. After they were harvested, washed with PBS, and collected in a trypsin/EDTA solution, the suspended cells were centrifuged at $270 \times g$ for 5 min. Each sample was then re-suspended in binding buffer containing annexin V-FITC and propidium iodide, incubated for 15 min at room temperature, and analyzed with a flow cytometer (FACSCalibur; Becton Dickinson, San Jose, CA, USA).

Statistical analysis. All experiments were repeated at least three times. All data are presented as the mean \pm SD. Significance levels were calculated using Student's *t*-test, and *p*-values of less than 0.05 were considered statistically significant.

Results

RAD001 induces cytotoxicity in human oral cancer cell lines. We evaluated the antiproliferative effect of the mTOR inhibitor, RAD001 (10-300 nM), on SCC4 and SCC25 oral cancer cells using MTT assays. The addition of 300 nM RAD001 significantly reduced the cell viability of SCC4 and SCC25 cells by 21.03% and 17.94%, respectively, after 24 h ($p < 0.01$, Figure 1A) and by 25%-30% after 48 h ($p < 0.01$, Figure 1B). These data suggest that SCC4 and SCC25 cells are insensitive to the effect of RAD001-alone.

Combining RAD001 with radiation significantly reduces colony formation in SCC4 cells. Next, we assessed the radiosensitizing effects of RAD001 on the SCC4 and SCC25 cell lines. SCC4

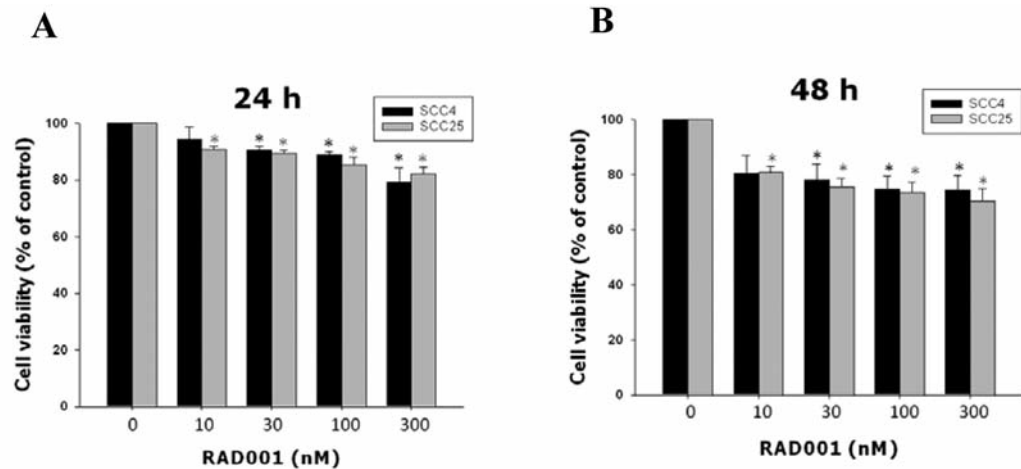


Figure 1. Effect of RAD001 on SCC4 and SCC25 cell proliferation. SCC4 and SCC25 cell proliferation after treatment with RAD001 (0–300 nM) for 24 h (A) and 48 h (B) using the MTT assay. Data are the mean \pm SD of three independent experiments performed in triplicate. * p <0.01 compared to the vehicle control.

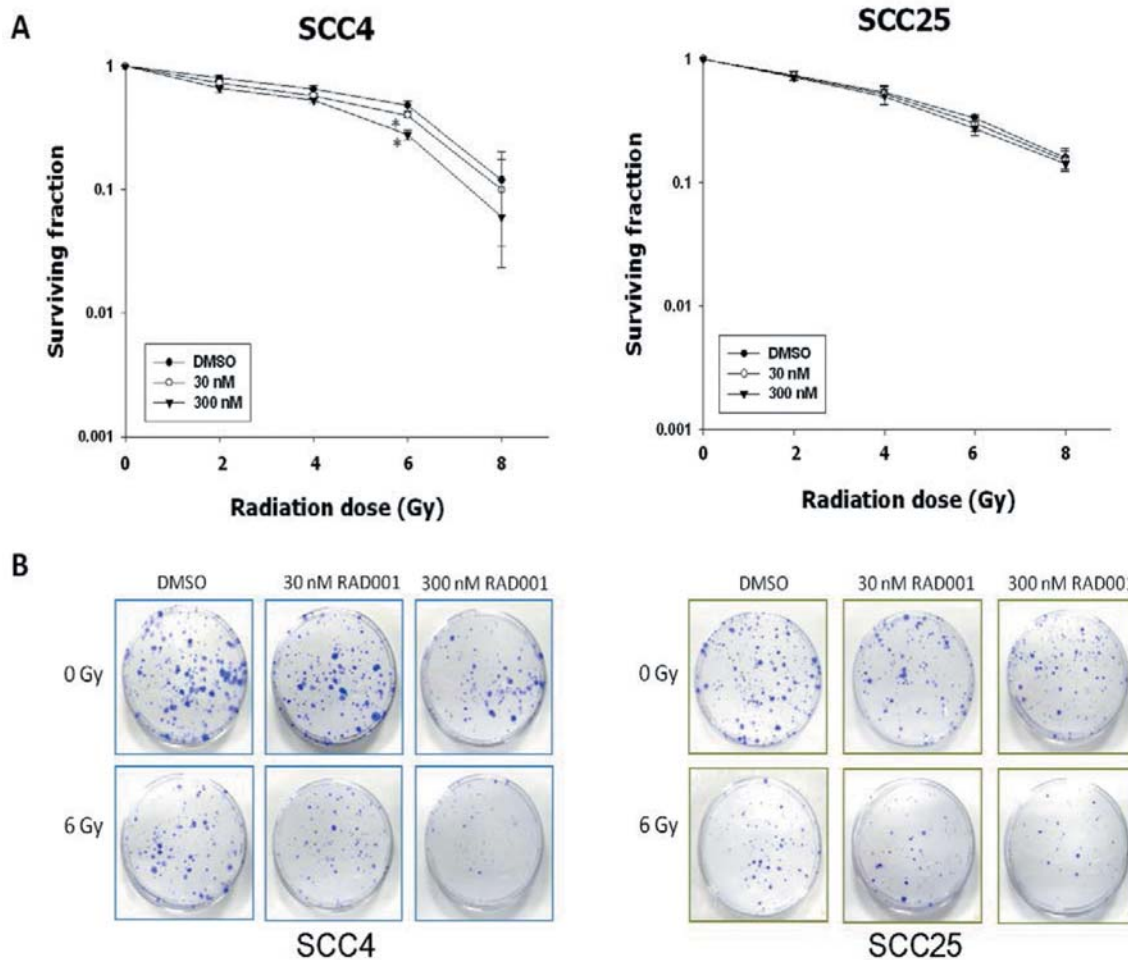


Figure 2. Effect of RAD001 and radiation on SCC4 and SCC25 colony formation. Cells were exposed to radiation (0–8 Gy) with and without RAD001 (30 or 300 nM for 1 h) and cultured for 14 days (A). The colonies were imaged at 14 days (B). Data are the mean \pm SD of three independent experiments performed in triplicate. * p <0.01 compared to the vehicle control.

and SCC25 cells were treated with 30 or 300 nM RAD001 or DMSO (control) for 1 h and then irradiated with 0 to 8 Gy for 6 h. Analysis of colony formation after 14 days revealed that both 30 and 300nM RAD001 significantly increased the radiosensitivity of SCC4 cells treated with 6 Gy radiation ($p<0.05$; Figure 2). In addition, treatment with 300 nM RAD001 and 6 Gy radiation significantly suppressed SCC4 cell growth compared to treatment with 6 Gy-alone ($p=0.004$) or with 30 nM RAD001-plus-6 Gy radiation ($p=0.008$; Figure 2). However, no additional effects of RAD001 on SCC25 cell radiosensitivity were noted (Figure 2). Thus, RAD001 enhances the sensitivity of SCC4 cells to radiation.

RAD001 inhibits mTOR activity and phosphorylation of its downstream effectors, S6 and 4EBP1, but induces AKT phosphorylation. To understand the mechanisms underlying the increased radiosensitivity induced by RAD001, we investigated the phosphorylation status of the downstream targets of mTOR. SCC4 cells were treated with 300 nM RAD001 with/without 6 Gy radiation, and levels of p-mTOR, p-AKT, p-S6, p-4EBP1, and eIF4E were measured with western blot analysis. Treatment of cells with both RAD001 and radiation inhibited phosphorylation of mTOR as well as S6 and 4EBP1, indicating that mTOR activity was attenuated (Figure 3). No changes in eIF4E levels were noted; however, AKT phosphorylation at Ser473 was up-regulated in RAD001-treated SCC4 cells (Figure 3). Inhibition of mTOR induces AKT Ser473 phosphorylation in a subset of cancer cell lines and patient tumors (16, 17), which may attenuate tumor responses to radiation (18, 19).

RAD001-mediated radiosensitization of SCC4 cells does not involve cell death. Previous studies indicated that RAD001 increased radiosensitivity by activating autophagy and enhancing radiation-induced apoptosis (9, 6). To investigate the effects of RAD001 on radiation-induced apoptosis, we first analyzed the expression of the apoptosis-related proteins, including BAX, BAK, caspase 3, and PARP in SCC4 cells after administration of RAD001 (300 nM) and/or radiation (6 Gy). As shown in Figure 4A, the levels of these proteins in irradiated SCC4 cells did not increase in the presence of RAD001. Flow cytometric analysis to examine the apoptotic sub- G_1 cell fraction confirmed that the proportion of sub- G_1 cells among the irradiated cells did not increase with exposure to RAD001 (Figure 4B). We then assessed the expression of the autophagy-related proteins, LC3 and beclin-1. Although LC3 expression increased with RAD001 treatment, it was not further increased in irradiated cells (Figure 5). Beclin-1 protein levels did not differ between the treatment groups (Figure 5). Taken together, these data indicate that neither autophagy nor apoptosis contribute to the molecular mechanism underlying the increase in radiosensitivity of SCC4 cells by RAD001.

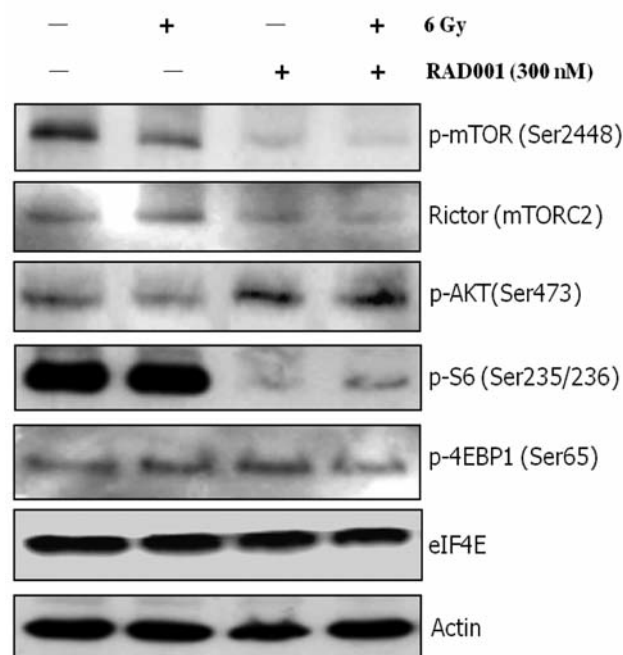


Figure 3. Effects of RAD001 and radiation on mTOR signaling. SCC4 cells were treated with 300 nM RAD001 with and without 6 Gy radiation. mTOR signaling proteins were assessed by western blot analysis.

Treatment with both RAD001 and radiation increases the percentage of cells in the G_2/M phase of the cell cycle. Cell-cycle changes induced by mTOR inhibition have been previously reported (10). To determine whether changes in cell-cycle distribution contributed to increased radiosensitivity of SCC4 cells, cell-cycle analysis was performed 48 h after treatment with RAD001 with/without radiation. As shown in Figure 6, no notable change in cell-cycle distribution was observed in RAD001-treated cells compared to control cells; radiation-alone led to G_2/M arrest. However, in cells treated with RAD001 and 6 Gy radiation, the proportion of cells in the G_2/M phase was 15.01%, compared to 12.56% observed in cells treated with radiation alone (Figure 6), suggesting that RAD001 may enhance the cytostatic effect of radiation by promoting G_2/M phase accumulation and inhibiting cell-cycle progression.

RAD001 and radiation alter the levels of G_2 checkpoint regulators. Given the G_2/M arrest observed in SCC4 cells treated with both RAD001 and radiation, we next assessed the expression and phosphorylation of cell-cycle regulators, particularly those associated with the G_2/M checkpoint, including the checkpoint kinases, CHK1 and CHK2, which induce cell cycle arrest following DNA damage. Activation of the CHK1 and CHK2, subsequently phosphorylates CDC25C, inactivating the CDC2-cyclin B1 complex and

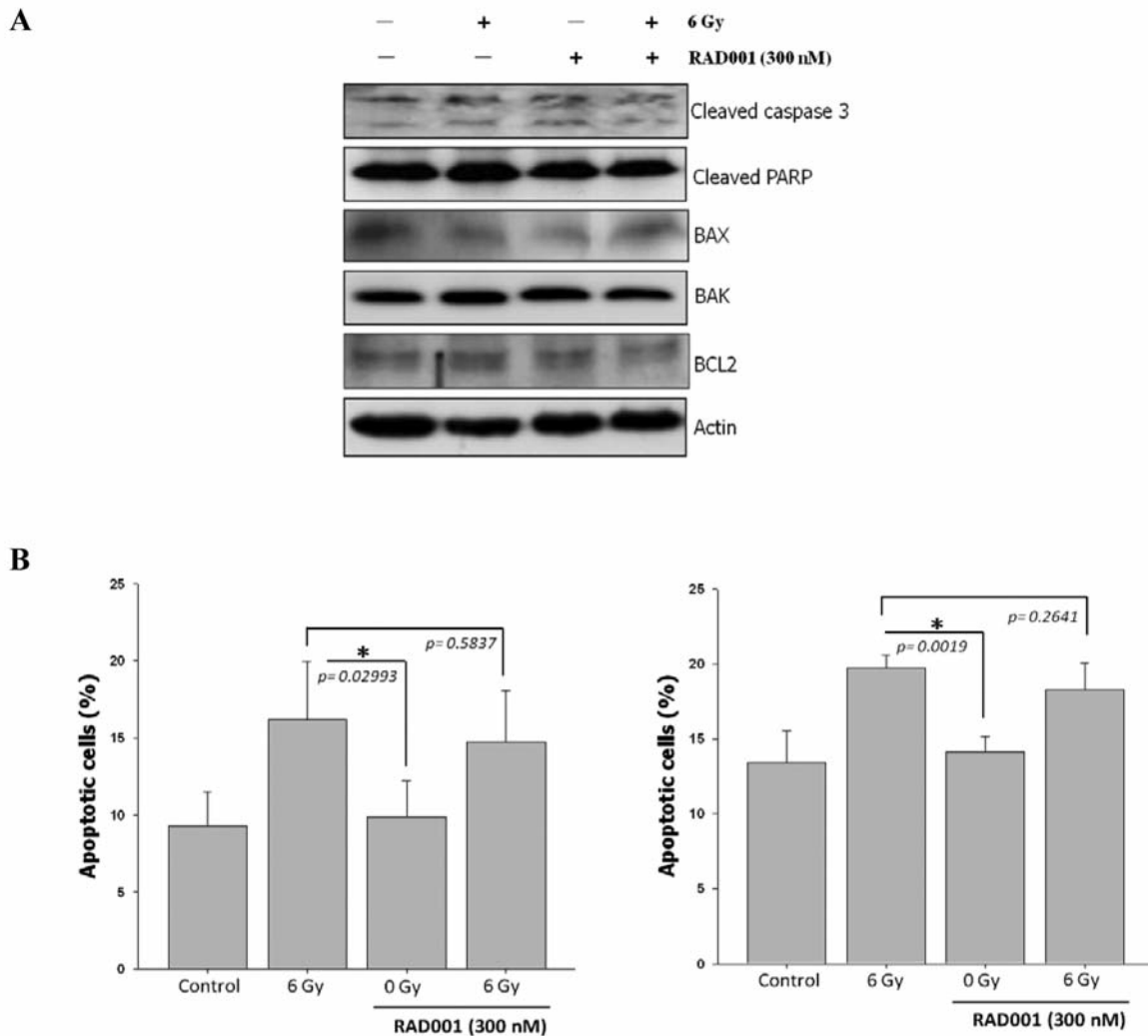


Figure 4. Effect of RAD001 and radiation on apoptosis-mediated cell death. SCC4 cells were treated with DMSO (control), 300 nM RAD001, 6 Gy radiation, or both. A: Protein levels of apoptosis-inducing factors (cleaved PARP and cleaved caspase 3), pro-apoptotic proteins (BAX, BAK), and the anti-apoptotic protein, BCL2, were detected by western blot analysis. B: Annexin V flow cytometric analysis of the effect of RAD001 on radiation-induced apoptosis in SCC4 cells. The cells were cultured with and without 300 nM RAD001 and then exposed to 6 Gy radiation or no radiation for 48 h (left panel) and 72 h (right panel). Data represent the proportion of annexin V-positive cells (mean \pm SEM of three independent experiments). * p <0.05.

thereby inducing G₂/M arrest (20). As shown in Figure 7, although no changes were observed with radiation or RAD001 alone, 300 nM RAD001 and 6 Gy irradiation substantially increased the phosphorylation of CHK1 (Ser345) at 48 h; no changes in phosphorylation of CHK2 (Thr68) in SCC4 cells were noted. Analysis of CDC25C, CDC2 and cyclin B1, which are involved in G₂/M arrest, revealed no changes in the overall expression of CDC25C in response to both 300 nM RAD001 and 6 Gy radiation alone or in combination (Figure 7). However, phospho-CDC25C (Ser216) and phospho-CDC2 (Thr14) levels were increased and cyclin B1 levels were decreased 48 h after the combined treatment. Radiation alone also increased the levels of

phospho-CDC25C (Ser216) and phospho-CDC2 (Thr14), but did not alter cyclin B1 levels. Cyclin B1 is the principal mitotic kinase; therefore, its inhibition would effectively block entry into mitosis.

We also checked the phosphorylation levels of histone H2AX (S139), γ -H2AX, a well-known marker of DNA damage (21). Treatment with radiation alone and in combination with RAD001 increased the level of γ -H2AX in SCC4 cells when compared to control treatment and treatment with RAD001-alone (Figure 7). These data suggest that RAD001 increases cellular sensitivity to radiation by activating CHK1, which inhibits CDC2–cyclin B1 kinase activation, leading to G₂/M arrest.

Discussion

Aberrant activation of mTOR promotes the growth of malignant tumors, including head and neck squamous cell carcinoma (22). The mTOR inhibitor, RAD001, improves the treatment outcomes in several types of cancer (4, 23). Therefore, we wanted to determine the efficacy of RAD001 in the OSCC cell lines, SCC4 and SCC25. In both cell lines, a high dose of RAD001 (300 nM) reduced cell viability by only 25%-30%, suggesting that they were insensitive to RAD001. We hypothesized that RAD001 and radiation would act synergistically and observed that 300 nM RAD001 with 6 Gy radiation significantly suppressed SCC4 cell colony formation. Although we did not attempt to maximize SCC4 growth inhibition by adjusting the duration of RAD001 treatment, RAD001 significantly enhanced the anticancer effects of radiation. However, similar effects were not observed in SCC25 cells. Thus, RAD001 may represent an effective radiosensitizing agent.

Several studies have reported a transient increase in signaling along the AKT/mTOR survival pathway after radiation, thus paradoxically associating radiotherapy with the activation of radioresistance (6, 24). Although radiation-alone did not significantly alter the phosphorylation of mTOR and the ribosomal S6 protein, RAD001, alone or combined with radiation, almost completely abolished mTOR and S6 phosphorylation, with the greatest reduction seen with the combined treatment. However, treatment with RAD001 increased AKT phosphorylation following mTOR inhibition, which is consistent with previous reports that mTOR inhibition inactivates S6, resulting in a feedback loop that activates AKT (25). Inhibition of mTORC1 can induce AKT Ser473 phosphorylation in a subset of cancer cell lines and patient tumors (16, 17), and may attenuate tumor responses (18, 19). Although activation of AKT might limit RAD001-mediated antitumor effects, it appears that the greatest benefit of combined treatment with RAD001 and radiation may derive from inhibition of the pro-survival response in SCC4 cells.

In general, radiation induces apoptotic cell death through activation of caspases in the presence of BAX and BAK (26, 27). Another study showed that that increased radiosensitivity by mTOR inhibition is mediated by induction of the apoptotic cell death pathway (6). However, in SCC4 cells treated for 48 h or 72 h, the proportion of apoptotic cells that were irradiated was similar with and without RAD001.

Autophagy is an alternative mechanism of cell death. Whereas in some cases, it is an adaptive response that promotes survival, in others, it appears to promote cell death and morbidity (28). In addition, autophagy-related stress tolerance can maintain energy production, thereby promoting cell survival, which can lead to tumor growth and resistance to therapy (29). Prior studies have reported that the

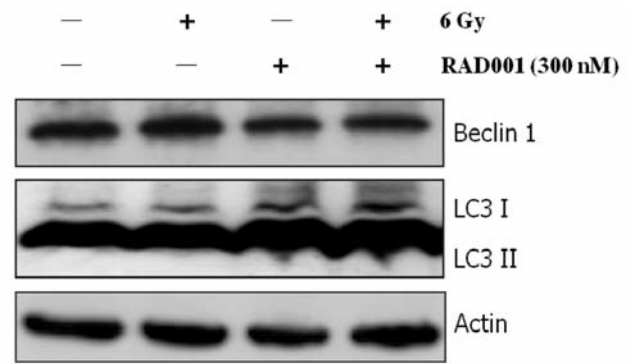


Figure 5. Effect of RAD001 and radiation on autophagy. SCC4 cells were treated with DMSO (control), RAD001 (300 nM), radiation (6 Gy), or their combination for 48 h. The expression of the autophagy-related proteins, LC3I /LC3II and beclin-1, was assessed by western blot analysis.

PI3K/AKT/mTOR pathway, and mTOR in particular, regulates autophagy, as well as cell survival and proliferation pathways (30). Current investigations suggest that mTOR inhibitors, when used in combination with radiation, appear to potentiate the ability of radiation to induce autophagy (9). We found that inhibition of mTOR by RAD001 increased the expression of the autophagy-associated protein, LC3; however, no additional enhancement was observed with the addition of radiation. Thus, the cytotoxic effect of RAD001 enhanced the effect of radiation on SCC4 cells independent of apoptotic and autophagic cell death pathways.

RAD001 combined with radiation induced significant G₂/M arrest in MDA-MB-231 breast cancer cells (6), similar to that observed in the present study after 48 h of treatment. Specifically, the combined treatment increased the proportion of cells in the G₂/M phase as compared to radiation alone, which is consistent with another previous study (31). Therefore, cell-cycle arrest may contribute to increased radiosensitivity observed in this study.

The G₂/M checkpoint is located at the end of G₂/M phase, controlling cell-cycle progression from G₂ to M phase. Because radiation-induced G₂/M phase blocking is a universal event in tumor cells, the G₂/M checkpoint is a target for improving the efficacy of radiation therapy (32). CHK1 and CHK2 are both major effectors of the G₂ checkpoint kinase that are required for the initiation of G₂/M arrest in response to ionizing radiation and thus, are implicated in the DNA damage response pathway (33). Phosphorylation activates CHK1 and CHK2, which phosphorylate CDC25C phosphatase on Ser216, thereby blocking the activation of CDC2/cyclin B1 complex and transition into the M phase (34). In the absence of CDC2 kinase/cyclin B1 activity, cells arrest in the G₂/M phase. Our results suggest that CHK1 rather than CHK2 is the primary checkpoint kinase that

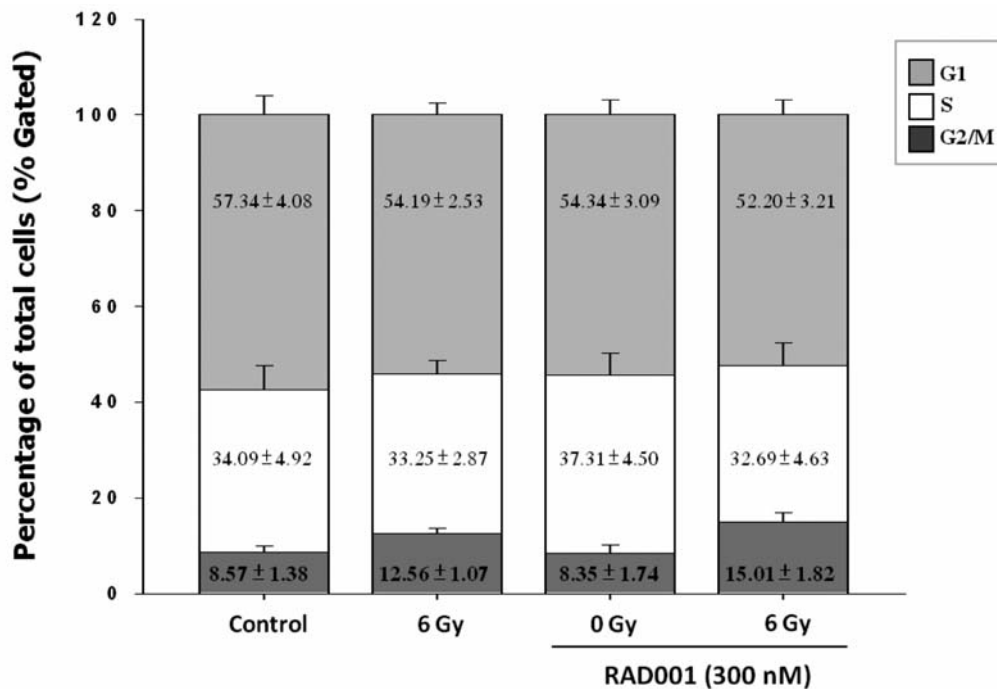


Figure 6. Effect of RAD001 and radiation on cell cycle progression. SCC4 cells were cultured and treated with DMSO, 300 nM RAD001, 6 Gy radiation, or their combination. The proportion of cells in each phase of the cell cycle was calculated as the percentage of the total cell population. Columns show the mean from three independent experiments; bars indicate SD.

responds to the combined treatment with RAD001 and radiation. In addition, treatment with a combination of RAD001 and radiation increased phosphorylation of CDC25C at Ser216 and CDC2 at Thr14, indicating regulation of the G₂ checkpoint. A previous study showed that RAD001 modified cell-cycle checkpoints, enhanced chemosensitivity, and regulated cell-cycle progression at the G₂/M transition (35). Activation of the CDK1–cyclin B1, which triggers a positive feedback loop at the end of the G₂/M phase, is the key event that initiates mitotic entry; expression of cyclin B1 is cyclic and peaks at the G₂ transition (36). Because cyclin B1 has a direct effect on mitosis, its overexpression may lead to uncontrolled cell proliferation. Previous studies have shown that a variety of cancers, such as breast, colorectal, prostate, oral cavity, and head and neck squamous cell cancer, express high levels of cyclin B1 (37–40). In the present study, RAD001 and radiation combination therapy blocked cyclin-B1 expression more effectively than radiation-alone. Thus, RAD001 and radiation inhibited SCC4 growth by inducing G₂/M arrest via the inactivation of CDC25C and the CDC2–cyclin B1 complex.

γ-H2AX formation has normally been associated with the induction of double-strand breaks after exposure to ionizing radiation or other DNA-damaging agents (21). Cells treated with γ-H2AX antagonists exhibit increased radiosensitivity when compared to untreated irradiated cells (41). Thus,

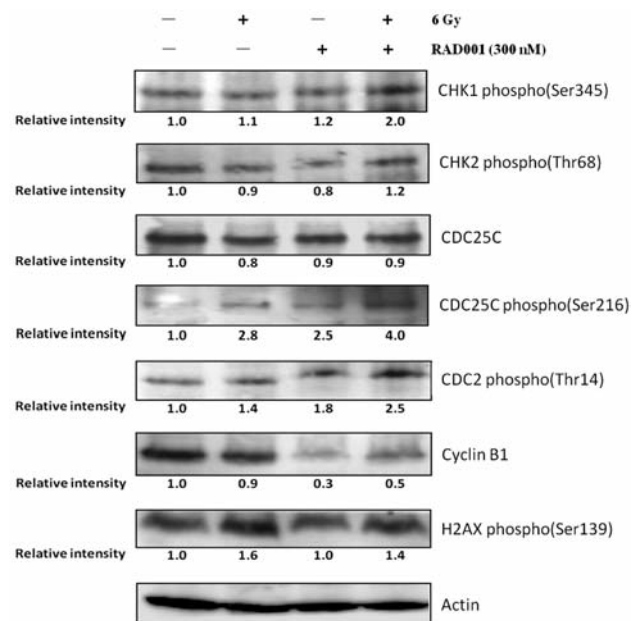


Figure 7. Expression of cell cycle regulatory proteins following RAD001 and radiation treatment. SCC4 cells were treated with 300 nM RAD001 with and without 6 Gy radiation. After 48 h, the expression of the G₂/M checkpoint molecules, p-CHK1, p-CHK2, CDC25C, p-CDC25C, p-CDC2, cyclin B1, and p-H2AX (γ-H2AX), was assessed by western blot analysis. β-Actin was used as an internal control for normalization. The numbers beneath the blots indicate the relative expression of each band when compared to the respective untreated control.

H2AX may be a predictor of radiosensitivity and a target for radiotherapy. In the present study, phosphorylation of H2AX was induced by radiation alone but was not markedly affected by co-treatment with RAD001.

Conclusion

In summary, this study describes a role for RAD001 in OSCC radiosensitization that differs from those previously reported; it attenuates mTOR-S6 and 4EBP1 activity and induces G₂/M phase arrest through the CHK1/CDC25C/CDC2–cyclinB1 pathway, which inhibits SCC4 cell growth.

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References

- Health Do: Executive Yuan, ROC Cancer Registry Annual Report in Taiwan Area Taipei: Department of Health, Executive Yuan, Taipei, Taiwan. 2010.
- Guertin DA and Sabatini DM: Defining the role of mTOR in cancer. *Cancer Cell* 12: 9-22, 2007.
- Zoncu R, Efeyan A and Sabatini DM: mTOR: from growth signal integration to cancer, diabetes and ageing. *Nat Rev Mol Cell Biol* 12: 21-35, 2011.
- Meric-Bernstam F and Gonzalez-Angulo AM: Targeting the mTOR signaling network for cancer therapy. *J Clin Oncol* 27: 2278-2287, 2009.
- Fasolo A and Sessa C: Current and future directions in mammalian target of rapamycin inhibitors development. *Expert opinion on investigational drugs* 20: 381-394, 2011.
- Albert JM, Kim KW, Cao C and Lu B: Targeting the Akt/mammalian target of rapamycin pathway for radiosensitization of breast cancer. *Molecular cancer therapeutics* 5: 1183-1189, 2006.
- Manegold PC, Paringer C, Kulka U, Krimmel K, Eichhorn ME, Wilkowski R, Jauch KW, Guba M and Bruns CJ: Antiangiogenic therapy with mammalian target of rapamycin inhibitor RAD001 (Everolimus) increases radiosensitivity in solid cancer. *Clinical cancer research: an official journal of the American Association for Cancer Research* 14: 892-900, 2008.
- Shinohara ET, Cao C, Niermann K, Mu Y, Zeng F, Hallahan DE and Lu B: Enhanced radiation damage of tumor vasculature by mTOR inhibitors. *Oncogene* 24: 5414-5422, 2005.
- Altmeyer A, Josset E, Denis JM, Gueulette J, Slabbert J, Mutter D, Noel G, and Bischoff P: The mTOR inhibitor RAD001 augments radiation-induced growth inhibition in a hepatocellular carcinoma cell line by increasing autophagy. *International J Oncol* 41: 1381-1386, 2012.
- Murphy JD, Spalding AC, Somnay YR, Markwart S, Ray ME and Hamstra DA: Inhibition of mTOR radiosensitizes soft tissue sarcoma and tumor vasculature. *Clinical cancer research: an official journal of the American Association for Cancer Research* 15: 589-596, 2009.
- Boulay A, Zumstein-Mecker S, Stephan C, Beuvink I, Zilbermann F, Haller R, Tobler S, Heusser C, O'Reilly T, Stolz B, Marti A, Thomas G and Lane HA: Antitumor efficacy of intermittent treatment schedules with the rapamycin derivative RAD001 correlates with prolonged inactivation of ribosomal protein S6 kinase 1 in peripheral blood mononuclear cells. *Cancer Res* 64: 252-261, 2004.
- Jundt F, Raetzl N, Muller C, Calkhoven CF, Kley K, Mathas S, Lietz A, Leutz A and Dorken B: A rapamycin derivative (everolimus) controls proliferation through down-regulation of truncated CCAAT enhancer binding protein {beta} and NF- κ B activity in Hodgkin and anaplastic large cell lymphomas. *Blood* 106: 1801-1807, 2005.
- Zitzmann K, De Toni EN, Brand S, Goke B, Meinecke J, Spottl G, Meyer HH and Auernhammer CJ: The novel mTOR inhibitor RAD001 (everolimus) induces antiproliferative effects in human pancreatic neuroendocrine tumor cells. *Neuroendocrinology* 85: 54-60, 2007.
- Lee SJ, Lee J, Lee J, Park SH, Park JO, Park YS, Lim HY, Kim KM, Do IG, Jung SH, Yim DS and Kang WK: Phase II trial of capecitabine and everolimus (RAD001) combination in refractory gastric cancer patients. *Investigational new drugs* 31: 1580-1586, 2013.
- Huang S and Houghton PJ: Inhibitors of mammalian target of rapamycin as novel antitumor agents: from bench to clinic. *Current opinion in investigational drugs (London, England: 2000)* 3: 295-304, 2002.
- O'Reilly KE, Rojo F, She QB, Solit D, Mills GB, Smith D, Lane H, Hofmann F, Hicklin DJ, Ludwig DL, Baselga J and Rosen N: mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt. *Cancer Res* 66: 1500-1508, 2006.
- Sun SY, Rosenberg LM, Wang X, Zhou Z, Yue P, Fu H and Khuri FR: Activation of Akt and eIF4E survival pathways by rapamycin-mediated mammalian target of rapamycin inhibition. *Cancer Res* 65: 7052-7058, 2005.
- Rosen N and She QB: AKT and cancer—Is it all mTOR? *Cancer cell* 10: 254-256, 2006.
- Shaw RJ and Cantley LC: Ras, PI(3)K and mTOR signalling controls tumour cell growth. *Nature* 441: 424-430, 2006.
- Zhou BB and Elledge SJ: The DNA damage response: putting checkpoints in perspective. *Nature* 408: 433-439, 2000.
- Redon C, Pilch D, Rogakou E, Sedelnikova O, Newrock K and Bonner W: Histone H2A variants H2AX and H2AZ. *Current opinion in genetics & development* 12: 162-169, 2002.
- Gao W, Li JZ, Chan JY, Ho WK and Wong TS: mTOR pathway and mTOR inhibitors in head and neck cancer. *ISRN otolaryngology* 2012: 953089, 2012.
- Don AS and Zheng XF: Recent clinical trials of mTOR-targeted cancer therapies. *Reviews on recent clinical trials* 6: 24-35, 2011.
- Cao C, Subhawong T, Albert JM, Kim KW, Geng L, Sekhar KR, Gi YJ and Lu B: Inhibition of mammalian target of rapamycin or apoptotic pathway induces autophagy and radiosensitizes *PTEN* null prostate cancer cells. *Cancer Res* 66: 10040-10047, 2006.
- Carracedo A, Ma L, Teruya-Feldstein J, Rojo F, Salmena L, Alimonti A, Egia A, Sasaki AT, Thomas G, Kozma SC, Papa A, Nardella C, Cantley LC, Baselga J and Pandolfi PP: Inhibition of mTORC1 leads to MAPK pathway activation through a PI3K-dependent feedback loop in human cancer. *J Clinical Investigation* 118: 3065-3074, 2008.

- 26 Chen Q, Chai YC, Mazumder S, Jiang C, Macklis RM, Chisolm GM and Almasan A: The late increase in intracellular free radical oxygen species during apoptosis is associated with cytochrome c release, caspase activation, and mitochondrial dysfunction. *Cell Death Differentiation* 10: 323-334, 2003.
- 27 Schinzel A, Kaufmann T and Borner C: Bcl-2 family members: integrators of survival and death signals in physiology and pathology [corrected]. *Biochimica et biophysica acta* 1644: 95-105, 2004.
- 28 Patel AS, Lin L, Geyer A, Haspel JA, An CH, Cao J, Rosas IO and Morse D: Autophagy in idiopathic pulmonary fibrosis. *PloS one* 7: e41394, 2012.
- 29 Lomonaco SL, Finniss S, Xiang C, Decarvalho A, Umansky F, Kalkanis SN, Mikkelsen T and Brodie C: The induction of autophagy by gamma-radiation contributes to the radioresistance of glioma stem cells. *International journal of cancer Journal international du cancer* 125: 717-722, 2009.
- 30 Kawamoto H, Koizumi H and Uchikoshi T: Expression of the G₂-M checkpoint regulators cyclin B1 and CDC2 in nonmalignant and malignant human breast lesions: immunocytochemical and quantitative image analyses. *The American journal of pathology* 150: 15-23, 1997.
- 31 Nassim R, Mansure JJ, Chevalier S, Cury F and Kassouf W: Combining mTOR inhibition with radiation improves antitumor activity in bladder cancer cells *in vitro* and *in vivo*: a novel strategy for treatment. *PloS one* 8: e65257, 2013.
- 32 Strunz AM, Peschke P, Waldeck W, Ehemann V, Kissel M and Debus J: Preferential radiosensitization in p53-mutated human tumour cell lines by pentoxifylline-mediated disruption of the G₂/M checkpoint control. *International journal of radiation biology* 78: 721-732, 2002.
- 33 Allen C, Ashley AK, Hromas R and Nickoloff JA: More forks on the road to replication stress recovery. *Journal of molecular cell biology* 3: 4-12, 2011.
- 34 Morgan DO: Principles of CDK regulation. *Nature* 374: 131-134, 1995.
- 35 Saunders PO, Weiss J, Welschinger R, Baraz R, Bradstock KF and Bendall LJ: RAD001 (everolimus) induces dose-dependent changes to cell cycle regulation and modifies the cell cycle response to vincristine. *Oncogene* 32: 4789-4797, 2013.
- 36 Li J, Meyer AN and Donoghue DJ: Nuclear localization of cyclin B1 mediates its biological activity and is regulated by phosphorylation. *Proceedings of the National Academy of Sciences of the United States of America* 94: 502-507, 1997.
- 37 Hassan KA, Ang KK, El-Naggar AK, Story MD, Lee JJ, Liu D, Hong WK and Mao L: Cyclin B1 overexpression and resistance to radiotherapy in head and neck squamous cell carcinoma. *Cancer Res* 62: 6414-6417, 2002.
- 38 Kuwahara Y, Oikawa T, Ochiai Y, Roudkenar MH, Fukumoto M, Shimura T, Ohtake Y, Ohkubo Y, Mori S, Uchiyama Y and Fukumoto M: Enhancement of autophagy is a potential modality for tumors refractory to radiotherapy. *Cell Death & Disease* 2: e177, 2011.
- 39 Mashal RD, Lester S, Corless C, Richie JP, Chandra R, Probert KJ and Dutta A: Expression of cell cycle-regulated proteins in prostate cancer. *Cancer Res* 56: 4159-4163, 1996.
- 40 Wang A, Yoshimi N, Ino N, Tanaka T and Mori H: Overexpression of cyclin B1 in human colorectal cancers. *J Cancer Res Clinical Oncol* 123: 124-127, 1997.
- 41 Kao J, Milano MT, Javaheri A, Garofalo MC, Chmura SJ, Weichselbaum RR and Kron SJ: γ -H2AX as a therapeutic target for improving the efficacy of radiation therapy. *Current Cancer Drug Targets* 6: 197-205, 2006.

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