Everolimus sensitizes Ras-transformed cells to radiation *in vitro* through the autophagy pathway

YU-CHIEH SU[1,6*], CHIH-CHIA YU[1,2*], FEI-TING HSU[3], SHU-LING FU[4], JENG-JONG HWANG[3], LING-CHIEN HUNG[4], MOON-SING LEE[5,6], WEN-YEN CHIOU[5,6], HON-YI LIN[5,6] and SHIH-KAI HUNG[5,6]

[1]Department of Hematological Oncology, Buddhist Dalin Tzu Chi Hospital, Chiayi 62247;
[2]Department of Life Science and Institute of Molecular Biology, National Chung-Cheng University, Chiayi 62102;
[3]Department of Biomedical Imaging and Radiological Sciences, National Yang-Ming University, Taipei 11221;
[4]Institute of Traditional Medicine, National Yang-Ming University, Taipei 11221; [5]Department of Radiation Oncology, Buddhist Dalin Tzu Chi Hospital, Chiayi 62247; [6]School of Medicine, Tzu Chi University, Hualian 97061, Taiwan, R.O.C.

Abstract. Modern radiation therapy strives to minimize injury to organs while increasing the anticancer effects. The present study aimed to investigate the radiosensitizing effects of everolimus and to examine the molecular mechanisms responsible for everolimus-mediated radiosensitization. Radiation in combination with everolimus (30 nM) sensitized Ras-transformed cells to radiation *in vitro*. Radiation induced apoptotic markers (sub-G1 cell accumulation, membrane inversion and DNA fragmentation) and treatment with everolimus did not promote radiation-induced apoptosis. However, LC3-II expression increased following combination treatment with everolimus and radiation, and the radiosensitizing effects of everolimus were reversed following transfection with small interfering RNA (siRNA) targeting Beclin 1. In addition, the protein levels of activated S6 kinase 1 (S6K1) were significantly reduced following treatment with everolimus, and the phosphorylation of factor 4E binding protein 1 (4EBP1) was suppressed following combination treatment. Taken together, our data demonstrate that everolimus sensitizes Ras-transformed cells to radiation *in vitro*. Everolimus-mediated radiosensitization is associated with the autophagy pathway. Thus, everolimus is a novel radiosensitizing agent with potential for use in cancer radiotherapy.

Introduction

Malignant tumors are characterized by uncontrolled growth, invasion and metastasis. The elevated expression and activa-

tion of oncogenes are strongly associated with the initiation and progression of cancer. Ras, one of the well-characterized proto-oncogenes, controls multiple intracellular signaling networks (1). Aberrant Ras activation is highly associated with cancer initiation, as well as progression, and its presence has been shown in 20-30% of all human tumors, particularly in colon and pancreatic cancer (2,3).

Radiation therapy is an important therapeutic strategy in cancer treatment. Minimizing injury to the surrounding normal tissue and organs, while increasing the anticancer effects remains an unresolved issue in the field of radiation therapy. Ras has been studied and linked to radioresistance. A number of studies have reported that the inhibition of Ras activation by anti-Ras antibody or Ras inhibitors can enhance radiation-induced cell death in human tumor cell lines (4,5). The involvement of the phosphoinositide 3-kinase (PI3K)/ protein kinase B (Akt) signaling pathway in Ras-induced radioresistance suggests that the inhibition of PI3K/Akt activity can greatly enhance the radiosensitivity of Ras-transformed cells (6). Everolimus is an inhibitor of mammalian target of rapamycin (mTOR) and a derivative of rapamycin with immunosuppressiveand anticancer properties (7,8). The anticancer effects of everolimus are associated with the inhibition of the PI3K/AKT/mTOR pathway (8,9). Thus, everolimus may act as a radiosensitizer in Ras-transformed cells. The present study aimed to investigate the radiosensitizing effects of everolimus and to elucidate the molecular mechanisms underlying everolimus-mediated radiosensitization.

Materials and methods

Chemicals and antibodies. Everolimus was obtained from Novartis (Basel, Switzerland) and dissolved in dimethyl sulfoxide (DMSO) as a concentrated stock solution. DMSO was used as the vehicle solution. As an internal control, medium only was used. DMSO and anti-actin antibody were from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against Ras, phospho-eukaryotic initiation factor 4E-binding protein 1 (p-4EBP1), 4EBP1, phospho-ribosomal protein S6

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kinase 1 (p-S6K1), S6K1 and phospho-Akt (p-Akt; Ser-473) were from Upstate Biotechnology (Lake Placid, NY, USA). Antibodies against phospho-mTOR (p-mTOR), mTOR, Akt and eukaryotic translation initiation factor 4E (eIF4E) were from BD Biosciences (San Diego, CA, USA). Beclin 1 small interfering RNA (siRNA) was from Thermo Fisher Scientific (Carlsbad, CA, USA).

Cell culture. RK3E/tv-a cells were obtained from the laboratory of Shu-Ling Fu, who established a non-transformed rat kidney epithelial RK3E cell line that constitutively expresses tv-a (receptor for subgroup A avian leukosis virus, ALV) for the delivery of foreign genes via avian retroviral infection. The RK3E/tv-a cells were infected with retrovirus carrying the oncogene, H-ras, or the control gene puromycin N-acetyltransferase (puro) as previously described (10). Ras-expressing cells, but not puro-expressing cells, underwent malignant transformation *in vitro* and tumorigenesis *in vivo*. These cells were maintained as previously described (10).

MTT assays. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) assays were carried out to measure the cytotoxic and radiosensitizing effects of everolimus. Ras-transformed cells ($5x10^3$) were seeded into 96-well plates, incubated overnight, treated with everolimus and/or radiation and cultured for 4 days. The medium was removed and the cells were incubated with MTT solution (0.5 mg/ml, 4 h, overnight, 5% CO_2 incubator, 37°C) and lysed with solubilization solution [12.5% sodium dodecyl sulfate (SDS), 45% dimethylformamide (DMF)]. The absorbance of converted dye was measured at 570 nm using a microplate reader. (Multiskan RC, Labsystems, Stockholm, Sweden). The absorbance of the untreated cells measured at 570 nm was defined as 100%. The relative viability was calculated by dividing the A570 nm of the treated cells by that of the untreated cells.

siRNA-mediated knockdown of Beclin 1. The Ras-transformed cells ($4x10^5$) were seeded into a 6-cm dish, cultured overnight, and transfected at 40% confluency with the indicated specific constructs (Beclin 1 siRNA, or non-targeted control siRNA) for 24 h according to the manufacturer's instructions (Thermo Scientific Dharmacon Inc., Lafayette, CO, USA). These sequences were 5'-GGACAGUUUGGCACAAUCA-3' (951–969). For each transfection, 2 solutions (one of 3 μl of siRNA diluted in 100 μl transfection medium and another of 3 μl of transfection reagents diluted in 100 μl transfection medium) were combined at room temperature for 20 min and added to each well, and the wells were incubated for 24 h. The transfection efficiency was determined at 24 h by western blot analysis. MTT assays were used to measure the effects of everolimus-mediated radiosensitization.

Annexin V staining. An Annexin V-FITC kit was used according to the manufacturer's instructions (R&D Systems Inc., Minneapolis, MN, USA). In brief, the cells (10^5) were grown on 6-well plates, treated with the vehicle (DMSO) or everolimus (10 nM) with or without 6 Gy radiation, cultured for 96 h and stained with Annexin V-FITC and propidium iodide (PI) (5 μg/ml). The stained cells were analyzed using a flow cytometer (FACScalibur; Becton-Dickinson, San Jose, CA, USA).

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay. A (TUNEL) assay was performed to measure cell death according to the manufacturer's instructions (Roche, Mannheim, Germany). The cells (10^5) were seeded into 6-well plates, incubated overnight and subsequently treated with the vehicle, everolimus and/or radiation under various conditions. Following treatment, the floating and adherent cells were collected and fixed with 4% paraformaldehyde in PBS, permeablized with 0.1% Triton X-100, stained with TUNEL reaction mixture for 60 min and analyzed by flow cytometry using a FACScan flow cytometer (Becton-Dickinson).

Cell cycle analysis. Cells (10^5) were grown on 6-well plates, treated under various conditions, incubated for 96 h at 37°C, then trypsinized, washed, fixed in 80% cold ethanol for 30 min, stained with 50 μg/ml propium iodide in PBS containing 50 μg/ml of RNase A for 30 min, and immediately analyzed by flow cytometry using a FACScan flow cytometer.

Western blot analysis. The cells (10^6) were seeded into 100-mm plates, incubated overnight, treated with the vehicle, everolimus or radiation under various conditions, and collected by scraping and centrifugation at 3,000 rpm for 5 min. The cell pellets were then lysed in radioimmune precipitation assay buffer (RIPA) in the presence of general protease inhibitors. Total protein (50 μg of lysate proteins) was analyzed by 10% sodium dodecyl sulfate polyacrylamide-gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes. After blocking with 5% non-fat milk/TBS-Tween, the membranes were incubated with primary antibodies, including anti-p-Akt, anti-Akt, anti-p-mTOR, anti-mTOR, anti-phospho-S6K (p-S6K), anti-S6K, anti-p-4EBP1, anti-4EBP1, anti-eIF4E and anti-LC3 antibodies overnight at 4°C, reacted with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h, treated with Immobilon™ Western Chemiluminescent HRP Substrate (ECL) and exposed to X-ray film to detect the signals.

Statistical analysis. Data are expressed as the means ± SD of 3independent experiments. Statistical analyses were performed using the unpaired two-tailed Student's t-test to compare 2 groups of data. A value of P<0.05 was considered to indicate a statistically signficant difference.

Results

Everolimus-mediated radiosensitization of Ras-transformed cells in vitro. We first evaluated the cytotoxicity of everolimus to Ras-transformed cells by MTT assay. The cytotoxic effects were found to increase with the higher everolimus concentrations. The survival fraction of the cells treated with everolimus was (10, 30 and 50 nM) was 0.91±0.03, 0.84±0.04 and 0.8±0.02, respectively. Thus, everolimus (30 nM; 96 h of incubation) was used in the subsequent radiosensitivity experiments. As shown in Fig. 1, treatment with everolimus (30 nM) sensitized the Ras-transformed cells to radiation (6 Gy). The survival fractions of the irradiated cells in the medium, vehicle and everolimus solution were 0.45±0.04, 0.42±0.04 and 0.12±0.04, respectively.

Everolimus does not promote radiation-induced apoptosis. We wished to determine whether everolimus, with or

Table I. Cell cycle distribution under various treatment conditions.

Phase	M0	M6	D0	D6	R0	R6
Sub-G_1	2.9±0.8	7.1±2.0	3.1±0.3	7.8±4.1	2.2±1.4	5.8±2.9
G_0/G_1	64.9±6.4	47.5±6.1	66.0±4.8	48.1±6.0	62.0±3.7	49.4±6.3
S	14.3±2.3	15.2±1.8	13.3±0.9	14.5±1.2	13.9±0.7	13.9±1.4
G_2/M	18.1±4.6	30.5±3.1	18.1±4.6	30.0±2.6	22.3±2.9	31.3±2.9

M0: medium, 0 Gy; M6: medium, 6 Gy; D0: 0.1% DMSO, 0 Gy; D6: 0.1% DMSO, 6 Gy; R0: everolimus (30 nM), 0 Gy; R6: everolimus (30 nM), 6 Gy.
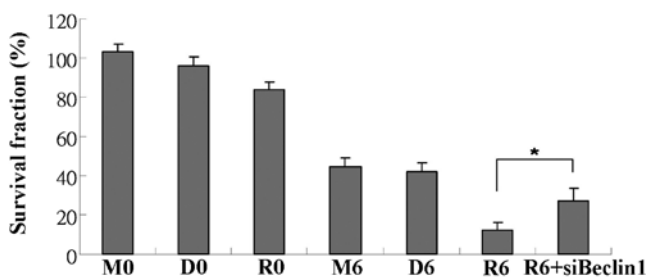

Figure 1. Everolimus sensitizes Ras-transformed cells to radiation *in vitro*. MTT assay was used to determine the radiosensitizing effects of everolimus. The cell survival fractions were calculated, and the calculated cell survival fractions following 6 Gy of radiation and treatment with medium only, vehicle (DMSO) or everolimus solution (stock diluted appropriately in medium) were 0.45±0.04, 0.42±0.04 and 0.12±0.04, respectively. In addition, the radiosensitizating effects of everolimus were reversed following transfection with Beclin 1 siRNA. *P<0.05. M0: medium, 0 Gy; M6: medium, 6 Gy; D0: 0.1% DMSO, 0 Gy; D6: 0.1% DMSO, 6 Gy; R0: everolimus (30 nM), 0 Gy; R6: everolimus (30 nM), 6 Gy radiation.
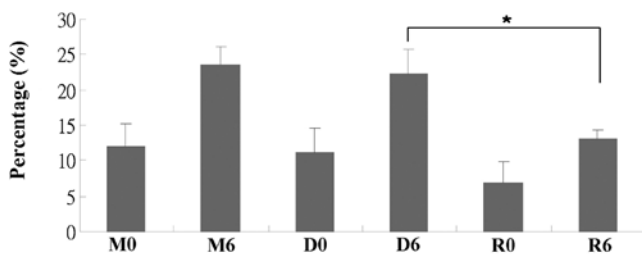

Figure 3. Everolimus does not promote radiation-induced apoptosis. The Ras-transformed cells were treated with everolimus (10 nM) with or without radiation (6 Gy), incubated for 96 h and stained with TUNEL. DNA fragmentation was caused by radiation. *P<0.05. M0: medium, 0 Gy; M6: medium, 6 Gy; D0: 0.1% DMSO, 0 Gy; D6: 0.1% DMSO, 6 Gy; R0: everolimus (30 nM), 0 Gy; R6: everolimus (30 nM), 6 Gy.

Figure 2. Everolimus does not promote radiation-induced apoptosis. The Ras-transformed cells were treated with everolimus (10 nM) with or without radiation (6 Gy), incubated for 96 h, and stained with Annexin V-FITC and propidium iodide. Radiation increased the percentage of Annexin V-stained cells, and everolimus in combination with radiation decreased the percentage of apoptotic cells. *P<0.0. M0: medium, 0 Gy; M6: medium, 6 Gy; D0: 0.1% DMSO, 0 Gy; D6: 0.1% DMSO, 6 Gy; R0: everolimus (30 nM), 0 Gy; R6: everolimus (30 nM), 6 Gy.

without radiation, induces apoptosis. Thus, we performed Annexin V staining and TUNEL assay. The percentage of Annexin V-stained cells was increased following radiation, but not following treatment with everolimus (Fig. 2). Consistent with the above observations, DNA fragmentation (measured by TUNEL assay) was mainly caused by radiation (Fig. 3). By contrast, combination treatment with everolimus and radiation decreased the percentage of dead/apoptotic cells. Taken
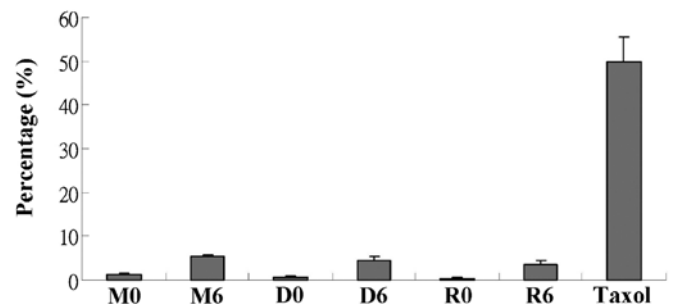
together, these data indicate that apoptosis is mainly induced by radiation and not is promoted by everolimus. The decreased percentage of apoptotic cell death suggests that everolimus-mediated radiosensitization leading to cell death may occur through a mechanism other than apoptosis.

Radiation affects cell cycle distribution. We wished to determine whether everolimus, with or without radiation, affects cell cycle distribution. Thus, we used PI staining followed by flow cytometry using a FACScan flow cytometer. As shown in Table I, radiation significantly increased the size of the sub-G_1 and G_2/M fractions (P<0.05), and radiation in combination with everolimus (compared to radiation alone) only slightly decreased the size of the sub-G_1 fraction.

LC3-II expression increases following treatment with everolimus in combination with radiation and the radiosensitizing effects of everolimus are reversed by transfection with Beclin 1 siRNA. We wished to determine whether everolimus, with or without radiation, induces autophagy. Thus, we performed western blot analysis. Fig. 4 shows the protein expression of LC3-II under various conditions. Everolimus in combination with radiation significantly increased LC3-II expression. We then wished to determine whether the everolimus-mediated radiosensitizing effects were dependent on autophagy. Thus, we transfected the cells with Beclin 1 siRNA. As shown in Fig. 1, the radiosensitization effects of everolimus were partially reversed following transfection with Beclin 1 siRNA.
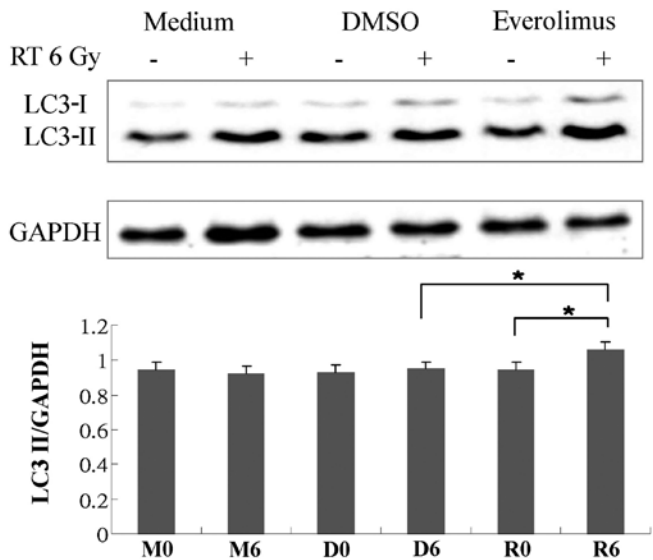
Figure 4. LC3-II expression is increased following combination treatment with everolimus and radiation. (Upper panel) representative western blots showing the protein expression of LC3-I and LC3-II under various treatment conditions. GAPDH was used as the loading control. (Bottom panel) Quantitative analysis of results from western blot analysis. For each treatment, the protein levels of LC3-II were normalized to those of GAPDH. *P<0.05. M0: medium, 0 Gy; M6: medium, 6 Gy; D0: 0.1% DMSO, 0 Gy; D6: 0.1% DMSO, 6 Gy; R0: everolimus (30 nM), 0 Gy; R6: everolimus (30 nM), 6 Gy.
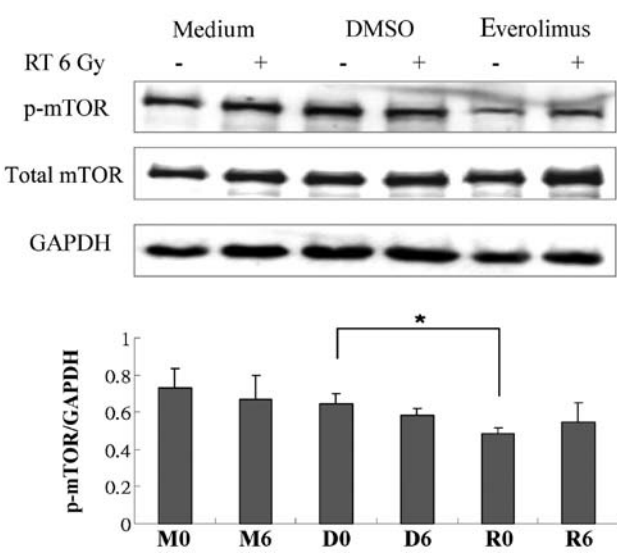

Figure 6. Treatment with everolimus suppresses the phosphorylation of mTOR. (Upper panel) Representative western blots show the protein expression of p-mTOR and mTOR under various treatment conditions. GAPDH was used as the loading control. (Bottom panel) Quantitative analysis of the results from western blot analysis. For each treatment, the protein levels of p-mTOR were normalized to those of GAPDH. *P<0.05. M0: medium, 0 Gy; M6: medium, 6 Gy; D0: 0.1% DMSO, 0 Gy; D6: 0.1% DMSO, 6 Gy; R0: everolimus (30 nM), 0 Gy; R6: everolimus (30 nM), 6 Gy.
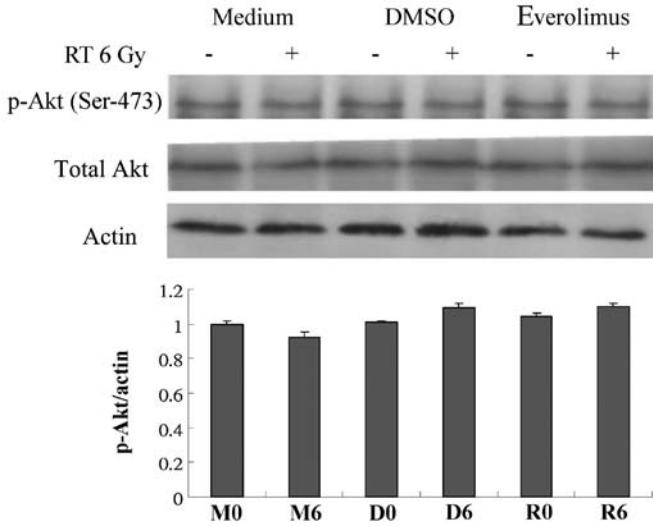

Figure 5. Akt protein expression is not altered by everolimus or radiation. (Upper panel) Representative western blots show the protein expression of p-Akt (Ser473) and Akt under various treatment conditions. Actin was used as the loading control. (Bottom panel) Quantitative analysis of the results from western blot analysis. For each treatment, the protein levels of p-Akt were normalized to those of actin. M0: medium, 0 Gy; M6: medium, 6 Gy; D0: 0.1% DMSO, 0 Gy; D6: 0.1% DMSO, 6 Gy; R0: everolimus (30 nM), 0 Gy; R6: everolimus (30 nM), 6 Gy.
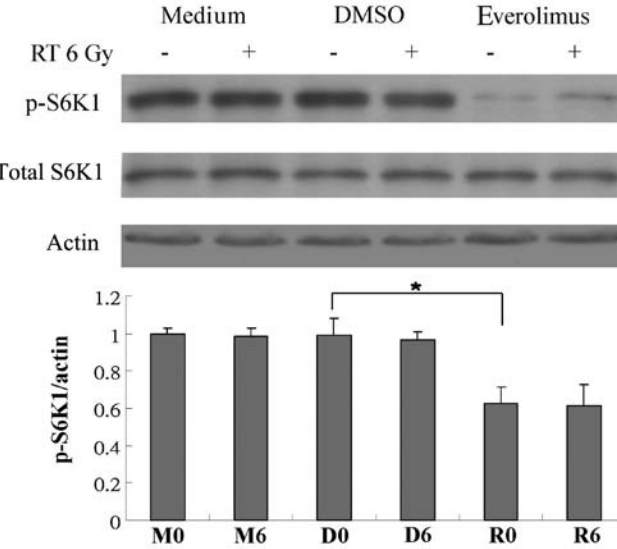

Figure 7. Treatment with everolimus suppresses S6K1 activation. (Upper panel) Representative western blots the protein expression levels of p-S6K1 and S6K1 under various treatment conditions. Actin was used as the loading control. (Bottom panel) Quantitative analysis of the results from western blot analysis. For each treatment, the protein levels of p-S6K1 were normalized to those of actin protein. *P<0.05. M0: medium, 0 Gy; M6: medium, 6 Gy; D0: 0.1% DMSO, 0 Gy; D6: 0.1% DMSO, 6 Gy; R0: everolimus (30 nM), 0 Gy; R6: everolimus (30 nM), 6 Gy.

Akt protein expression is not altered by everolimus or radiation. Since the PI3K/Akt pathway is a target of everolimus, the possible association of the radiosensitizing activity of everolimus with the PI3K/Akt signaling pathway was assessed. Western blot analysis revealed that the expression or activation of Akt protein was not significantly altered by everolimus or radiation (Fig. 5).

Phosphorylation of 4EBP1 is suppressed following treatment with everolimus in combination with radiation. We wished to determine whether everolimus, with or without radiation, regulates the PI3K/AKT/mTOR pathway. Thus, we performed western blot analysis in order to determine the protein expression of p-mTOR, mTOR, p-S6K1, S6K1, p-4EBP1, 4BEP1
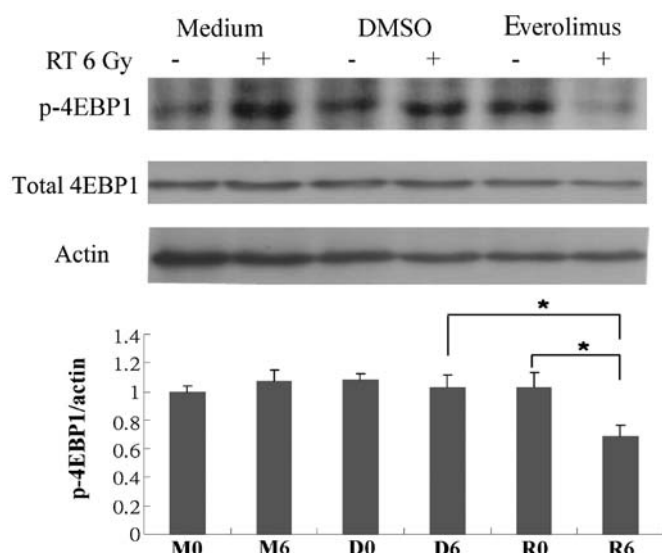
Figure 8. Phosphorylation of 4EBP1 is suppressed following combination treatment with everolimus and radiation. (Upper panel) Representative western blots show the protein expression of p-4EBP1 and 4EBP1 under various treatment conditions. Actin was used as the loading control. (Bottom panel) Quantitative analysis of the results from western blot analysis. For each treatment, the protein levels of p-4EBP1 were normalized to those of actin. $^{*}P<0.05$. M0: medium, 0 Gy; M6: medium, 6 Gy; D0: 0.1% DMSO, 0 Gy; D6: 0.1% DMSO, 6 Gy; R0: everolimus (30 nM), 0 Gy; R6: everolimus (30 nM), 6 Gy.

and eIF4E. There was no significant change observed in eIF4E expression (data not shown). However, treatment with everolimus alone attenuated the phosphorylation of mTOR and S6K1 (Figs. 6 and 7), and everolimus in combination with radiation markedly suppressed the phosphorylation of 4EBP1 (Fig. 8).

Discussion

Activating Ras mutations are frequently observed in a number of human cancers and are strongly correlated with radiation resistance (3,4,11). Colorectal cancer is one of the most frequently diagnosed cancers with aberrant Ras activation and a major cause of cancer-related mortality in Taiwan (2,3,12). In the present study, we demonstrated the radiosensitizing activity of everolimus in Ras-transformed cells, suggesting a potential new application of this compound in cancer therapy.

Different types of cell death with possible associations to everolimus-mediated radiosensitization were examined. Our results revealed that radiation-induced apoptotic changes (sub-G_1 cell accumulation, membrane inversion and DNA fragmentation) were not enhanced by treatment with everolimus (Table I; Figs. 2 and 3). Notably, the percentage of apoptotic cells decreased following treatment with everolimus combined with radiation relative to the percentage following radiation alone, suggesting that other non-apoptotic pathways may be involved in the everolimus-mediated radiosensitization. Indeed, increasing evidence indicates that non-apoptotic mechanisms, such as autophagy, are crucial for treatment-induced tumor-cell death (13,14). Whether the cell death caused by everolimus-mediated radiosensitization is induced by autophagy was examined by assessing the protein expression of LC3. Western blot analysis revealed that treatment

with everolimus plus radiation markedly increased LC3-II expression (Fig. 4). In addition, siRNA targeting Beclin 1 was used to further determine whether the everolimus-mediated radiosensitizing effects are dependent on autophagy. As shown in Fig. 1, the radiosensitizing effects of everolimus were reversed following transfection with Beclin 1 siRNA. These data indicate that the autophagy pathway is involved in the everolimus-mediated radiosensitizing effects.

Multiple signaling pathways, including the PI3K/AKT/mTOR and Raf/MEK/ERK pathways, are associated with the pharmacological activity of everolimus (15,16). The importance of the PI3K/AKT/mTOR pathway in tumor progression has been investigated (17). In addition, it has been shown that mTOR inhibitors target a number of cancers and regulate abnormal signaling (18). mTOR signaling is mediated through two multiprotein complexes, the mTOR complex 1 (mTORC1) and 2 (mTORC2). mTORC1 can activate 4EBP1 and S6K1 and lead to translation, increasing protein synthesis and growth (18). Several studies have demonstrated that mTOR inhibitors modulate autophagy. A previous study suggested that p-4EBP1 binds to and stabilizes mTORC1 *in vitro* (19). Another study demonstrated that the effects of 4EBP1 on autophagy were significant, as evidenced by the increased baseline levels of autophagy, the increased rapamycin-induced autophagy and the elimination of 4EBP1 in the presence of the autophagy inhibitor (20). Furthermore, S6K1 is sensitive to the modulation of autophagy and requires a much less stringent inhibition of mTORC1 to be targeted (21). In the present study, the combination of everolimus and radiation markedly suppressed the phosphorylation of 4EBP1 (Fig. 8). In addition, treatment with everolimus alone attenuated the phosphorylation of S6K1 (Fig. 7). These findings suggest that 4EBP1 and S6K1 interact with specific proteins involved in autophagy and may have as yet unrecognized actions that affect mTOR signaling and/or autophagy.

In conclusion, the present study demonstrates that everolimus may be used as a novel radiosensitizer in Ras-transformed cells, and that the mechanisms responsible for the everolimus-mediated radiosensitization are partially dependent on the autophagy pathway.

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