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CDK4/6 Inhibitor LEE011 Is a Potential Radiation-sensitizer in Head and Neck Squamous Cell Carcinoma: An *In Vitro* Study

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Abstract. *Background: Radiotherapy (RT) combined with a radiosensitizer represents an important treatment for head and neck squamous cell carcinoma (HNSCC). Only few chemotherapy agents are currently approved as radiosensitizers for targeted therapy. In this study, the potent cyclin-dependent kinase 4/6 (CDK4/6) inhibitor LEE011 was tested for potential to act as a radiosensitizer during RT. Materials and Methods: RT enhancement by LEE011 was assessed by in vitro clonogenic assay, flow cytometry, and western blot in a variety of HNSCC cell lines. The HNSCC cell line OML1 and its radiation-resistant clone OML1-R were used. Results: LEE011 induced cell-cycle arrest in SCC4/SCC25 cells during the G_1/M phase through inhibition of retinoblastoma protein phosphorylation. LEE011 enhanced the effects of radiation in OML1 cells and overcame radiation resistance in OML1-R cells. Conclusion: LEE011 is a potential radiosensitizer that can enhance the cytotoxic effects of RT. Clinical trials including LEE011 during RT for HNSCC should be considered.*

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide, with more than 500,000 new cases and 300,000 deaths reported annually (1). The primary treatment for HNSCC is total surgical removal of the primary tumor and lymph nodes, and radiotherapy (RT) can also be considered as a treatment option for some patients. Radiosensitizers are promising agents that enhance injury to tumor tissue by accelerating DNA damage and producing free radicals. Since 1965, several chemotherapy agents have been used in the development of highly effective radiosensitizers (2), and data from the Meta-analysis of Chemotherapy in Head and Neck Cancer group has indicated improved overall survival associated with concurrent chemotherapy combined with RT (3), but more toxic effects were also observed. Cisplatin, carboplatin, and fluorouracil are currently the most common chemotherapy agents used as radiosensitizers during RT, while cetuximab is the only targeted therapy agent approved for use during RT for HNSCC (3).

The primary mechanism for radiation resistance in cancer cells is activation of the phosphoinositide 3-kinase (PI3K)/thymoma viral proto-oncogene 1(AKT)/the mammalian target of rapamycin (mTOR) pathway (4, 5), and blocking this pathway has been observed to increase sensitivity to radiation and improve the effect of RT on tumor cells. In previous studies, we used mTOR-specific and dual-targeted PI3K/AKT inhibitors in oral cancer cell lines and found that blocking PI3K/mTOR increased the radiation sensitivity of oral cancer cells and reversed radiation resistance in a resistant strain (OML1-R) (6-9). Notably, we identified modulation of checkpoint kinase 2 (CHK2) activity and the resulting effects on cyclin-dependent protein

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Key Words: CDK4/6 inhibitor, LEE011, radiosensitization, head and neck cancer, radioresistance.

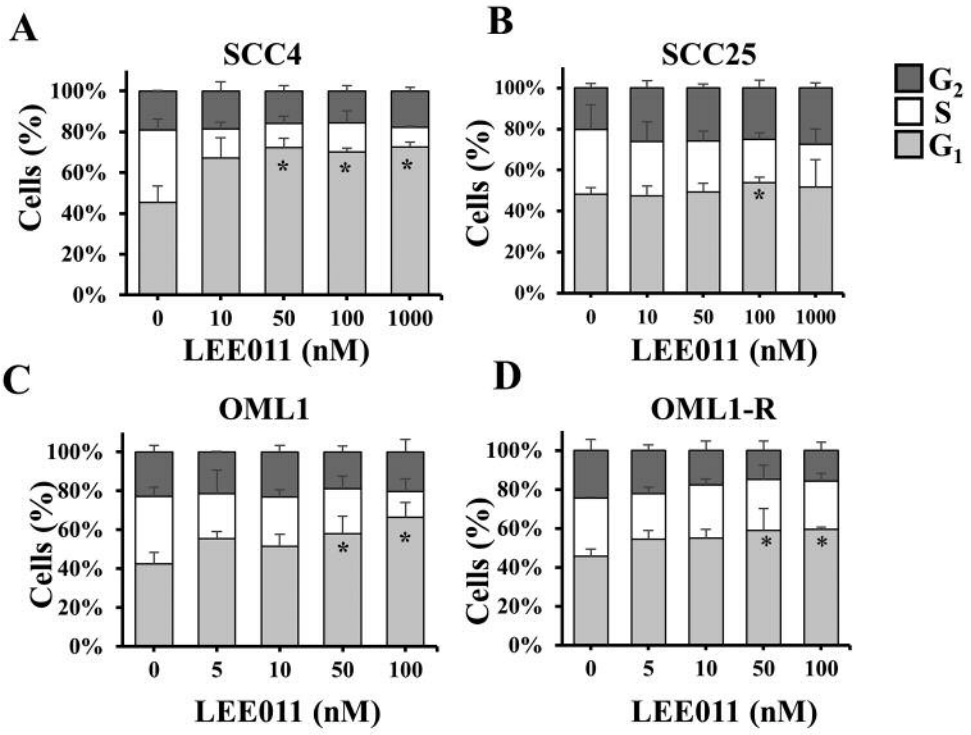

Figure 1. *Treatment with LEE011 blocked cell-cycle progression. SCC4 (A), SCC25 (B), OML1 (C), and OML1-R (D) cells were treated with the indicated dose of LEE011 for 72 h. Cells were harvested and stained with propidium iodide (PI) hypotonic buffer for cell-cycle analysis. The results of a Student's t-test comparing G_1 phase for each treatment and control are shown. Data represent the results of three independent experiments and is presented as the mean ± the SD. *Significantly different at $p<0.05$.*

kinase 1 (CDK1)/cyclin B1 activity as a potential mechanism (7, 8) and we concluded that cell-cycle arrest is the major mechanism of radiation sensitization associated with PI3K/mTOR inhibition. In the current study, we assessed the potential anti-tumor and radiation resistance-reversing effects of the CDK4/6 inhibitor LEE011, and explored the mechanism of cell-cycle arrest in radioresistance in HNSCC. This study provides important clinical information regarding development of radiosensitizer drugs for HNSCC therapy.

Materials and Methods

Reagents and chemicals. LEE011 was provided by the Novartis Pharmaceuticals Corporation (East Hanover, NJ, USA). Stock solutions were prepared in dimethylsulfoxide (DMSO) at 30 mM, stored at −20˚C until further use, and diluted in culture medium for each experiment.

Cell lines and culture. The human head and neck squamous cell carcinoma cell lines SCC4 and SCC25 derived from a squamous cell carcinoma of the tongue were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium/nutrient mixture F-12 (DMEM/F12) containing 10% fetal bovine serum (FBS), penicillin-streptomycin (10,000 U/ml penicillin and 10 mg/ml streptomycin), and 2 mM L-glutamine(10). OML1 and OML1-R cells were established as previously described (10) and maintained in RPMI1640 containing 10% FBS, 1% penicillin-streptomycin, and 2 mM L-glutamine. Cells were cultured in an incubator at 37˚C under a humidified atmosphere of 5% CO_2 and 95% air.

Cell proliferation WST-1 assay. 4-(3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio)-1,3-benzene disulfonate (WST-1; Roche Applied Science, Indianapolis, IN, USA) colorimetric assays were performed according to the manufacturer's instructions. Briefly, 5×10^3 cells were seeded in 96-well plates for overnight culture, and then treated with either DMSO (vehicle) or LEE011 (5-1,000 nM) for 24 and 48 h. Cell viability was measured after treatment by adding 10 μl of WST-1 solution to the culture medium. After incubation for 4 h at 37˚C, the absorbance was measured by a microplate ELISA reader (Sunrise Absorbance Microplate Reader; Tecan, Männedorf, Switzerland) at a wavelength of 450nm with a reference wavelength of 650nm.

Cell survival clonogenic assay. Cells were seeded at 100 cells per 10 cm dish for overnight culture. The cells were pre-treated with LEE011 (100 nM in DMSO) for 72 h before irradiation, then irradiated at 4 Gy. The medium was replaced after 6 h and the cells were placed in an incubator at 37˚C for further culture. After 7 days, colonies, defined as groups of >50 cells, were stained with 0.05% crystal violet and counted. Images for colony counting were acquired with a digital camera.

Cell-cycle profiling. Cells were seeded 1×10^4 cells/10 cm dish for LEE011 (100 nM) treatment for 72 h, then irradiated at 4 Gy.

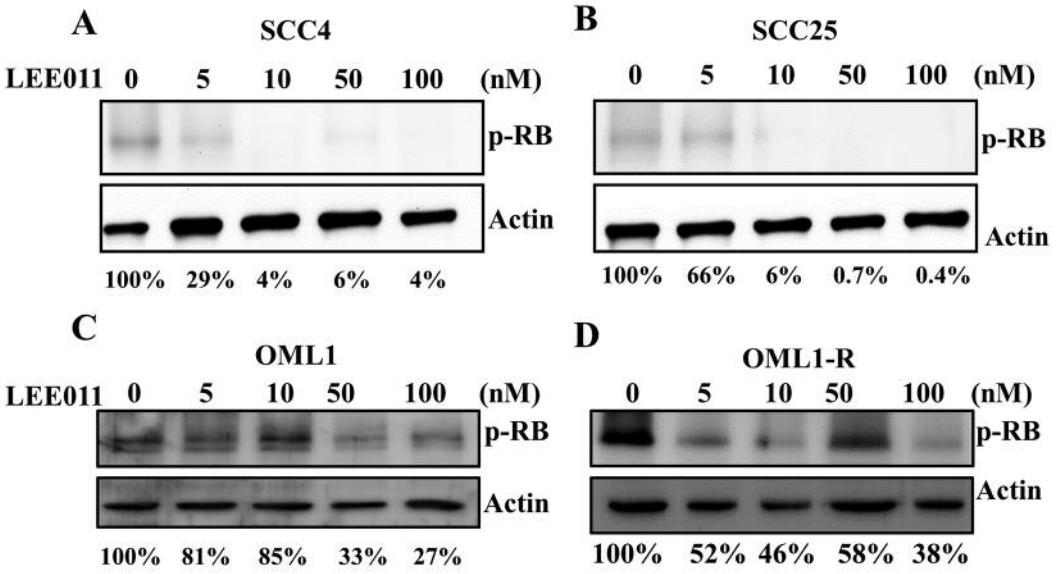

Figure 2. *LEE011 inhibited retinoblastoma protein (RB) phosphorylation in head and neck squamous-cell carcinoma cells. SCC4 (A), SCC25 (B), OML1 (C), and OML1-R (D) cells were treated with the indicated dose of LEE011 for 72 h. Cell lysates were prepared and analyzed by western blot using antibodies to phosphor-RB (p-RB) and actin. The p-RB band was normalized by actin and the percentage relative to that of the untreated control is shown.*

Control cells were not irradiated. Cells were harvested following overnight culture and stained with hypotonic buffer (1.5 μM propidium iodide, P4170; Sigma-Aldrich, St. Louis, MO, USA) for at least 30 min at 4˚C. Cells were collected and analyzed by Accuri C6 (BD Bioscience, San Jose, CA, USA).

Western blotting. Cells were harvested and lysed with cell lysis buffer (50 mM Tris·HCl pH 7.4, 150 mM NaCl, 2 mM EDTA pH 8, 0.5% Nonidet P-40, and 1% Triton-X 100) after treatment. Protein concentrations were measured using the Bio-Rad protein assay kit (Bio-Rad, Richmond, CA, USA). Forty micrograms of protein from each sample were separated by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). Membranes were incubated with the corresponding primary antibodies and secondary antibodies after blocking with non-fat dry milk for 1 h. The following antibodies were used: anti-RB, anti-phospho-RB(S780), anti-phospho-RB(S795) (all at 1:2,000; Cell Signaling, Beverly, MA, USA); and anti-β-actin (1:2,000; Santa Cruz Biotechnology, Dallas, Tx, USA). Proteins were visualized using a chemiluminescence (ECL) detection kit (Millipore, Billerica, MA, USA) and all blots were quantified with ImageJ (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. All data are presented as the mean±standard deviation. Significance levels were calculated using Student's *t*-test and *p*-values of less than 0.05 were considered statistically significant.

Results

LEE011 arrested the cell cycle in human oral cancer cells. We investigated whether LEE011 can increase radiosensitivity of oral cancer cell lines by blocking cell-cycle progression. Four different HNSCC cell lines were treated with LEE011 and subjected to cell-cycle analysis. The G1 percentage increased from 40% to 70% following 10 nM LEE011 treatment in SCC4 cells (Figure 1A). G_1 arrest became statistically significant after 50 nM LEE011 treatment in OML1 and OML1-R cells (Figure 1C and D). While SCC25 cells were more resistant to LEE011 inhibition, G1 arrest became statistically significant after 100 nM LEE011 treatment in this cell line (Figure 1B). Together, these observations suggest that LEE011 can arrest the cell cycle in HNSCC cells.

LEE011 inhibited RB phosphorylation. We examined the phosphorylation status of RB following LEE011 treatment to confirm the inhibition of CDK4/6 activity by LEE011. LEE011 inhibited RB phosphorylation at 5 nM in the four cell lines tested (Figure 2). RB phosphorylation was completely inhibited in SCC4 and SCC25 cells following treatment with 10-50 nM LEE011 (Figure 2A and B) and RB phosphorylation was inhibited up to 70% in OML1 and OML1-R cells following treatment with 100 nM LEE011 (Figure 2C and D). LEE011 treatment led to a significant difference in RB phosphorylation, especially in the SCC4 line. These data indicate that LEE011 blocks cell-cycle progression through RB inhibition of phosphorylation.

LEE011 did not induce cytotoxicity in human oral cancer cells. We used the WST-1 method to examine the survival

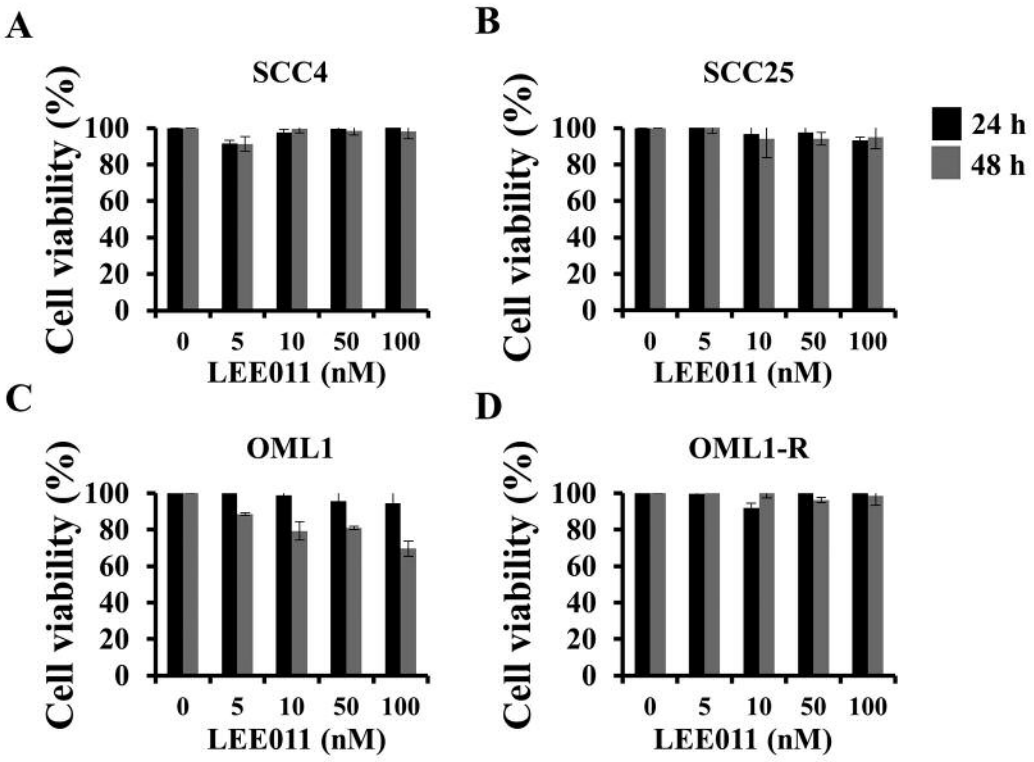

Figure 3. *LEE011 induced cell cytotoxicity in head and neck squamous-cell carcinoma cells. SCC4 (A), SCC25 (B), OML1 (C), and OML1-R (D) cells were treated with the indicated dose of LEE011 for 24 and 48 h. Cell viability was examined by the WST-1 assay.*

rate of head and neck cancer cells treated with LEE011 at different concentrations. LEE011 did not induce cell toxicity at concentrations up to 100 nM in any of the four cell lines at 24 h and there was no apparent inhibition of cell survival after treatment for 48 h (Figure 3). A slight decrease in cell viability was seen in OML1 cells after 100 nM LEE011 treatment, but this did not reach statistical significance (Figure 3C). These data indicate that LEE011 did not induce cell cytotoxicity in human oral cancer cell lines.

The effects of LEE011 combined with RT on OML1 and OML1-R cell lines. While LEE011 blocks cell-cycle progression, it does not induce cell cytotoxicity. Our previous studies suggested that cell-cycle arrest by PI3K/mTOR inhibitors increases radiosensitivity in human oral cancer cell lines (7, 8). Therefore, we examined the effects of LEE011 on radiosensitization in OML1, with the radioresistant cell line OML1-R as a control. OML1 and OML1-R cells were irradiated with 4 Gy alone or in combination with LEE011 and colony formation and cell proliferation effects were evaluated by clonogenic and WST-1 proliferation assays, respectively (Figure 4). Radiation alone did not affect colony formation in the radioresistant cell line OML1-R; however, radiation combined with LEE011 reduced colony formation in these cells (Figure 4A and B). The WST-1 proliferation assay showed there to be a significant decrease in proliferation of OML1-R cells when treated with RT combined with LEE011 (Figure 4C). These data suggest that LEE011 might supplement RT to achieve radiosensitization.

RT with LEE011 blocked cell-cycle progression. The decrease of proliferation and clonogenic formation after LEE011 combined with RT suggests that the combined treatment blocks cell-cycle progression. We found that the percentage of OML1 cells in the G_1 phase increased from 50% to 70% after treatment with LEE011 and RT (Figure 5A). A similar effect was seen in radioresistant OML1-R cells, in which the G1 percentage increased 54% to 65% following treatment (Figure 5B).

RT with LEE011 blocked RB phosphorylation. The effect of combined LEE011 and RT treatment on the cell cycle may be due to a loss of CDK4/6 activity. We measured RB phosphorylation following LEE011 and RT treatment to examine CDK4/6 activity. Compared to the untreated control, combined treatment with LEE011 and RT reduced phosphorylation of RB in OML1 cells by up to 60% (Figure

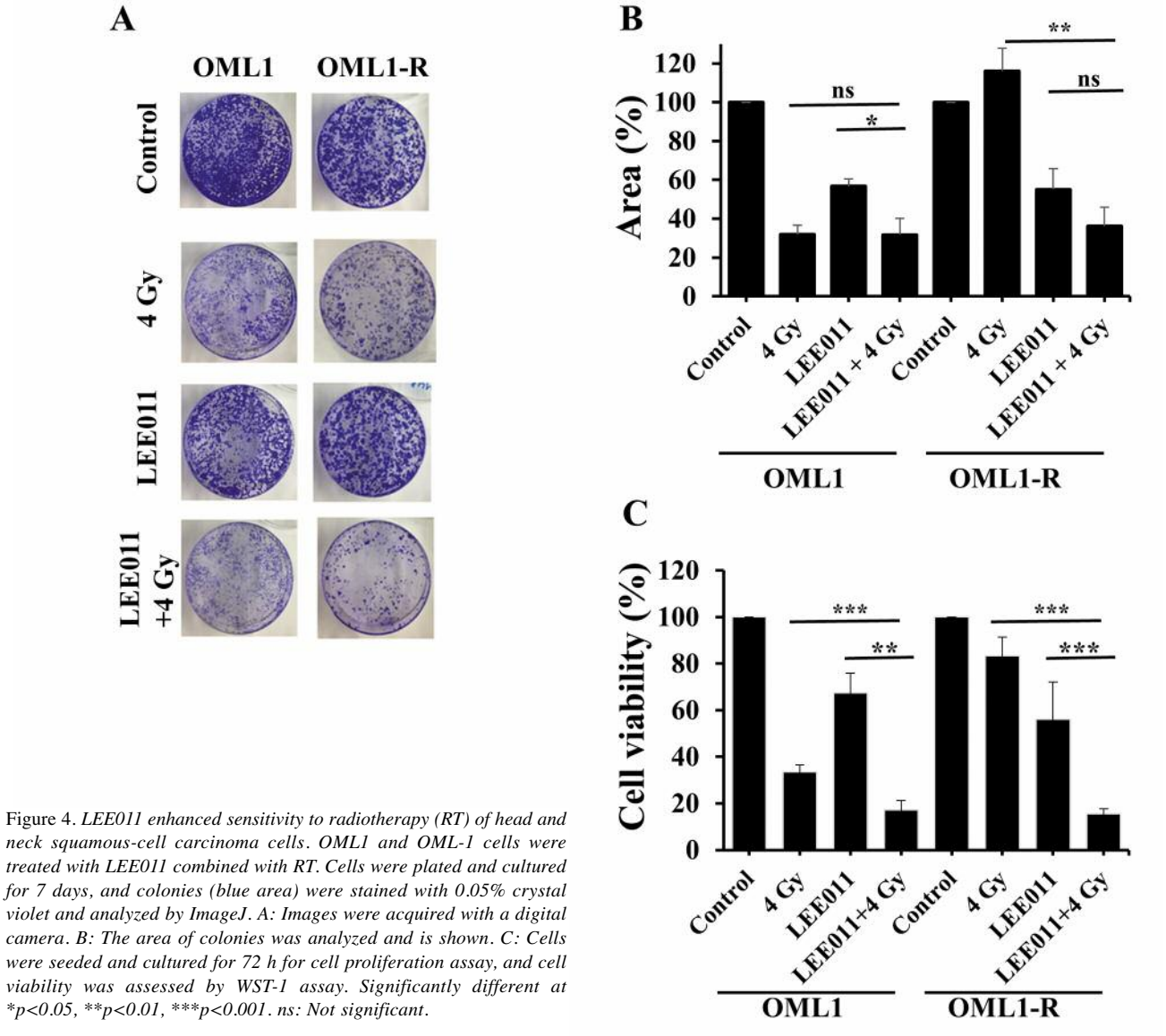

Figure 4. *LEE011 enhanced sensitivity to radiotherapy (RT) of head and neck squamous-cell carcinoma cells. OML1 and OML-1 cells were treated with LEE011 combined with RT. Cells were plated and cultured for 7 days, and colonies (blue area) were stained with 0.05% crystal violet and analyzed by ImageJ. A: Images were acquired with a digital camera. B: The area of colonies was analyzed and is shown. C: Cells were seeded and cultured for 72 h for cell proliferation assay, and cell viability was assessed by WST-1 assay. Significantly different at *p<0.05, **p<0.01, ***p<0.001. ns: Not significant.*

6A), and by 80% in radioresistant OML1-R cells (Figure 6B). These observations suggest that the inhibition of cell-cycle progression seen in human oral cancer cell lines was caused by decrease of RB phosphorylation, which may be due to a loss of CDK4/6 activity. Blocking the cell cycle at the G_1 phase leads to growth inhibition in oral cancer cells.

Discussion

RT is a still mainstay treatment modality for HNSCC, but radiation resistance remains a major cause of treatment failure. There are many challenges associated with enhancing radiation damage to tumor tissue, also known as radiation sensitization. Radiosensitizers such as cisplatin and hydroxyurea are typical agents used to enhance injury to tumor tissue by accelerating DNA damage and producing free radicals. Multiple signaling pathways related to apoptosis, metastasis, DNA repair, protein degradation, and others were shown to influence the efficacy of RT [reviewed in (11)]. Recent studies investigating the mechanisms of radioresistance have reported PI3K/AKT/mTOR pathways associated with radiosensitivity (12). Our previous studies identified RAD001 (8), AZD2014 (7), and BEZ235 (6) as potential small-molecule drugs for radiosensitivity, and these three compounds all influence radiation sensitization by the same mechanism, cell-cycle arrest.

The CDK4/6 inhibitor LEE011 is among a new generation of therapeutics. Building upon the striking success of the

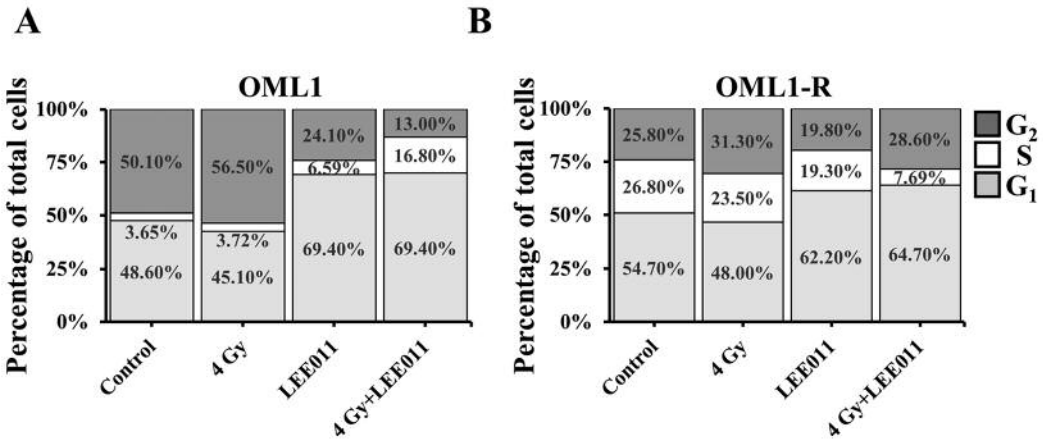

Figure 5. *Effect of LEE011 combined with radiotherapy (RT) on cell-cycle progression in head and neck squamous-cell carcinoma cells. OML1 and OML-1 cells were left untreated, treated with LEE011 or with RT alone, or treated with LEE011 combined with RT for 72 h. Cells were harvested and stained with propidium iodide (PI) hypotonic buffer for cell-cycle analysis, and the number indicates the percentage of cells in the cell-cycle phase.*

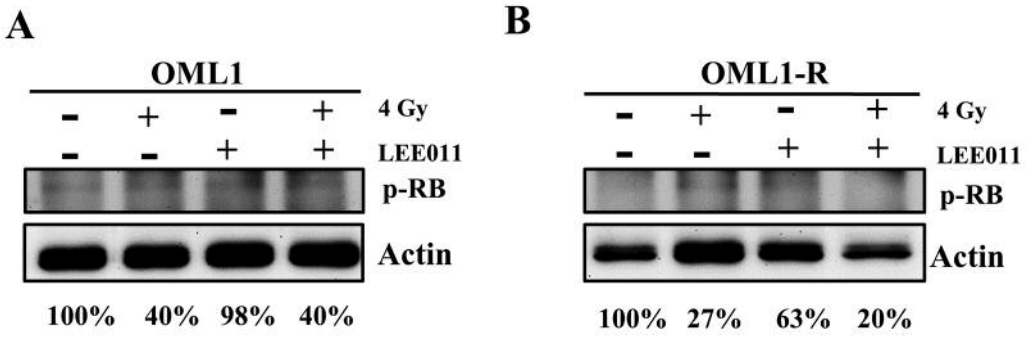

Figure 6. *Effect of LEE011 combined with radiotherapy (RT) on retinoblastoma protein (RB phosphorylation in head and neck squamous-cell carcinoma cells. OML1 (A) and RT-resistant cell line OML1-R (B) cells were treated with 100 nM of LEE011 for 72 h with and without 4 Gy RT. Cell lysates were prepared and analyzed by western blot using antibodies to phosphor-RB (p-RB) and actin. The p-RB band was normalized by actin and the percentage relative to that of the untreated control is shown.*

combination of CDK4/6 inhibitors and the hormone receptor antagonist letrozole in breast cancer (13), many other combination therapies have recently entered clinical trials in multiple diseases [reviewed in (14)]. Naz *et al.* recently reported that the CDK4/6 inhibitor abemaciclib enhanced radiation effects in lung cancer and was a radiosensitizer for lung cancer in preclinical models (15). They found a unique role for CDK4/6 inhibition in radiation-induced vasculogenesis contributing to the enhanced RT response *in vivo*. Here we report that the radiation sensitization effect is due to cell-cycle arrest in the G_1 phase.

The cell cycle is a key factor influencing radiosensitivity, therefore the inhibition of checkpoint kinases in combination with DNA-damaging chemotherapy or RT was proposed as a promising cancer treatment strategy (16). The cell-cycle phase determines the relative radiosensitivity of a cell, and cells are most radiosensitive in the G_2-M phase, less sensitive in the G_1 phase, and least sensitive during the latter part of the S phase. Previous studies have shown that metallic nanoparticles can alter cell-cycle distribution and improve radiosensitivity (17-19), but the clinical application of metallic nanoparticles remains untested. We found that SCC4/SCC25 cell lines were arrested in G_1 phase when treated with LEE011, and LEE011 treatment inhibited cell survival of radiation-resistant OML1-R head and neck squamous cells (10). These results confirm that CDK4/6 inhibition enhances the effects of radiation and can overcome radioresistance in head and neck cell lines. These findings confirm the result of a previous study on non-small cell lung cancer (15), and suggest that the combination of CDK4/6

inhibitor and RT represents a reasonable way to manage patients with HNSCC in advanced disease settings.

By using OML1-R and OML1 cell lines, we created a model to test the radiosensitization effects of various drugs in HNSCC (6-8, 10). We had previously explored the radiosensitization effects of PI3K/mTOR/CDK inhibition and demonstrated that the most important mechanism is cell-cycle arrest. CDK4/6 appears to be the most efficient and least toxic target, but its clinical application still needs further investigation in clinical trials. Currently, chemotherapeutic agents such as cetuximab, cisplatin, and fluoruracil are the most popular drugs approved for clinical use in concurrent chemotherapy combined with RT for head and neck cancer (3). These drugs have been well-investigated for their safety and pharmacokinetic properties, and although it may be more convenient to introduce them into clinical application, more toxic effects were reported in the groups treated with concurrent chemotherapy combined with RT (20). There are also reports investigating other small-molecule agents, such as hypoxic radiosensitizers (21) tirapazamine (22) and nimorazole (23), but the trials failed to identify evidence of an overall survival benefit. Our study suggested the possibility of PI3K/mTOR/CDK inhibitor usage in concurrent chemotherapy combined with RT settings, leading to a new method of treatment for advanced/recurrent HNSCC.

Conflicts of Interests

The Author(s) declared no potential conflicts of interest with respect to the research, authorship, or publication of this article.

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