LCL161, a SMAC-mimetic, Preferentially Radiosensitizes Human Papillomavirus-negative Head and Neck Squamous Cell Carcinoma

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Abstract

Targeting inhibitor of apoptosis proteins (IAP) with second mitochondria-derived activator of caspase (SMAC) mimetics may promote cancer cell death. We tested whether cIAP1 predicts poor prognosis in head and neck squamous cell carcinoma (HNSCC) and whether a novel Smac-mimetic, LCL161, could radiosensitize human papillomavirus–positive (HPV+) and -negative (HPV−) HNSCC. The association of BIRC2 (encoding cIAP1) mRNA level with HPV status in HNSCC was analyzed using The Cancer Genome Atlas (TCGA) database. cIAP1 was assessed by IHC on an HNSCC tissue microarray (TMA, n = 84) followed by correlation analysis with HPV status and patient outcomes. Human cell culture and animal models of HNSCC were used to analyze the outcome and molecular characteristics following radiotherapy in combination with LCL161. cIAP1 expression is increased in HPV− compared with HPV+ HNSCC tumors in the TCGA database. In our TMA, cIAP1 was overexpressed in HNSCC compared with normal tissues (P = 0.0003) and associated with a poor overall survival (P = 0.0402). cIAP1 levels were higher in HPV− than that in HPV+ HNSCC tumors (P = 0.004) and patients with cIAP1+/HPV+ HNSCC had the worst survival. LCL161 effectively radiosensitized HPV− HNSCC cells, which was accompanied with enhanced apoptosis, but not HPV+ HNSCC cells. Importantly, LCL161 in combination with radiotherapy led to dramatic tumor regression of HPV− HNSCC tumor xenografts, accompanied by cIAP1 degradation and apoptosis activation. These results reveal that cIAP1 is a prognostic and a potential therapeutic biomarker for HNSCC, and targeting cIAP1 with LCL161 preferentially radiosensitizes HPV− HNSCC, providing justification for clinical testing of LCL161 in combination with radiation for patients with HPV− HNSCC.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer and accounts for 600,000 new cancer cases per year in the world (1, 2). Common treatments include surgery, radiotherapy, chemotherapy, targeted therapy, and more recently immunotherapy. Although improvements in these treatments have greatly benefited patients with HNSCC, the 5-year survival rate of patients with HNSCC remains low at about 50% (1). HNSCC is clinically classified as human papillomavirus–positive (HPV+) and -negative (HPV−) subgroups, with different genetic alteration patterns and clinical outcomes associated with them (3). HPV− HNSCC represents a distinct clinicopathologic entity and typically has a worse prognosis than patients with HPV+ HNSCC (4−7). In comparison with HPV+ HNSCC, patients with HPV− HNSCC are more resistant to radiotherapy. It has been proposed that p53 dysfunction resulting from TP53 and CDKN2A mutations and uncontrolled activity of EGFR/PI3K/AKT signaling may contribute to the radioresistance of HPV− HNSCC (8−10). Indeed, targeting EGFR with cetuximab significantly improved the outcome of HNSCC when compared with radiotherapy alone in a large randomized phase III trial; however, HPV status was not determined for patients on this trial (11). However, the most recent randomized phase III clinical trial has shown that cetuximab does not improve outcomes when used in combination with cisplatin and radiotherapy (12, 13). Because radioresistance is a significant challenge for patients with HNSCC, particularly HPV− patients (14), it is of high importance to elucidate the precise mechanism of radioresistance, which will engender novel strategies to overcome radioresistance of HPV− patients.

Apoptosis is a tightly regulated multi-step cell suicide program that is critical for the development and homeostasis of multicellular organisms (15). Evasion of apoptosis is a characteristic feature of human cancer cells and represents an important basis of resistance to current treatment approaches, including radiation (16, 17). It has been widely accepted that reversal of cancer cell apoptosis evasion is a pivotal strategy for cancer therapy (18, 19). Inhibitor of apoptosis proteins (IAP) originally discovered in Baculoviral genomes by Miller and colleagues in 1993, comprise a family of antiapoptotic proteins that promote prosurvival signaling pathways and prevent activation of apoptosis by interfering with the activation of caspases (20, 21). Overexpression of IAPs frequently occurs in various human cancers, including esophageal...
carcinoma (22), cervical cancer (23), and pancreatic cancer (24), and correlates with tumor progression, treatment failure, and poor prognosis (25–27), making IAPs important targets for therapeutic intervention. Endogenously, the role of IAPs in preventing apoptosis is inhibited by the second mitochondria-derived activator of caspase (SMAC), a mitochondria protein that is released to the cytoplasm upon induction of apoptosis (28, 29). SMAC (also called DIABLO) physically interacts with the conserved Baculovirus IAP repeat (BIR) domains thereby preventing the apoptosis inhibition functions of IAPs. Accordingly, several SMAC mimetics have been designed to prevent IAPs inhibitory action on caspases to promote apoptosis. The SMAC-mimetic, LCL161, is a monovalent SMAC-mimetic, which bind IAPs with high affinity and initiates the destruction of cIAP1 and cIAP2 (encoded by BIRC2 and BIRC3, respectively) and prevention of caspase inhibition by XIAP (30, 31). LCL161 has shown safety and efficacy in a phase 1 study of advanced solid tumors (32). Furthermore, a preclinical study has shown good antitumor activity of LCL161 in human hepatocellular carcinoma (33). LCL161 has also been shown to sensitize esophageal cancer to radiotherapy (34). In addition, SMAC mimetics SM-164 and birinapant were reported to sensitize HNSCC to radiotherapy (35, 36). Dual inhibition of p53 and Rb by E6 and E7, respectively, in HPV+ HNSCC cells may lead to loss of G1 checkpoint, but the presence of wild-type p53 has been proposed to contribute to the sensitivity of HPV+ HNSCC cells to radiotherapy (37). In contrast, TP53 and CDKN2A mutations that are commonly found in HPV+ HNSCC cells may not only result in loss of G1 phase checkpoint, but also apoptosis evasion in response to DNA damage. We hypothesize that HPV+ HNSCC cells might rely on attenuated apoptosis for survival and be more susceptible to radiotherapy following reactivation of apoptosis by a potent SMAC-mimetic, LCL161.

In this study, we compared the expression of cIAP1 between HPV+ HNSCC and HPV− HNSCC in The Cancer Genome Atlas (TCGA) database, cell lines, and tissue microarray (TMA), and evaluated the radiosensitizing potential of LCL161 in in vitro and in vivo models of HPV+ and HPV− HNSCC. We revealed that cIAP1 is a prognostic and therapeutic biomarker for HPV+ HNSCC and targeting cIAP1 with LCL161 preferentially radiosensitizes HPV+ HNSCC. Our findings may provide a novel strategy for the management of patients with HPV+ HNSCC.

Materials and Methods

Cell culture, chemicals, antibodies, and ELISA

HNSCC cell line U-M-SCC-2 was a gift from Henning Bier (University of Düsseldorf, Düsseldorf, Germany, 2009); UM-SCC-47, UM-SCC-1, UM-SCC-11B, and UM-SCC-74A were gifts from Thomas Carey (University of Michigan, Ann Arbor, MI, 2009); 93VU117T was gift from Jim Rocco (Ohio State University, Columbus, OH, 2015); UPCI:SCC090 was gift from Susanne M. Gollin (University of Pittsburgh, Pittsburgh, PA, 2009); Normal Oral Epithelial cells (NOE) was gift from Quintin Pan (University Hospitals Cleveland Medical Center, Cleveland, OH, 2009); and Cal27 and FaDu were purchased from ATCC. Except for UPCI:SCC090 and NOE cells, cell lines were maintained in DMEM (Life Technologies Inc.) containing 10% v/v heat-inactivated FBS (Life Technologies Inc.) and 1% v/v non-essential amino acid (NEAA; Life Technologies Inc.). UPCI:SCC090 cells were cultured in MEM (Life Technologies Inc.) with 10% FBS (v/v) and 1% NEAA (v/v). NOE cells were maintained in a serum-free keratin MEM (Life Technologies Inc.) with 10% FBS (v/v) and 1% (NEAA; Life Technologies Inc.). UPCI:SCC090 cells were cultured (Life Technologies Inc.) and 1% (v/v) non-essential amino acid

Gollin (University of Pittsburgh, Pittsburg, PA, 2009); Normal 2009); 93VU147T was gift from Jim Rocco (Ohio State University, atorium’s instructions (Roche). Briefly, cells were seeded in 96- well plates in 5–6 replicates at a density of 2,000 cells per well in 100 µl medium. The next day, the cells were treated with LCL161.
at various concentrations. After 72 hours, WST-1 cell proliferation reagent was added and incubated at 37°C for 4 hours, and absorbance was measured at 490 nm. IC50 was determined using the nonlinear four parameter regression function in GraphPad Prism.

**Radiation clonogenic assay**

Exponentially growing cells were harvested to generate single-cell suspensions and seeded into 60- or 100-mm tissue culture dishes in triplicate at predetermined densities. Twenty-four hours later, cells were irradiated using a RS-2000 Biological Irradiator (Rad Source Technologies) for different doses, followed by incubation at 37°C for 10–14 days. Colonies were then fixed with methanol/acetic and stained with 1% crystal violet. The number of colony containing at least 50 cells was counted using a dissecting microscope (Leica Microsystems, Inc.). The surviving fractions and dose-enhancement ratios (DER) at 2 Gy were calculated. For LCL161 or Z-VAD-FMK treatment, cells were pretreated with either drug or vehicle (DMSO) for 2 hours prior to radiation, and incubated for additional 24 hours post radiation. The cells were then washed and cultured in fresh media for the remainder of the experiments. Experiments were repeated at multiples for independent times.

**Detection of cell apoptosis**

Cells were seeded into 6-well plates at a density of 3 × 10^5 cells per well in 2 mL medium for 16 hours. The cells were treated with the indicated drugs and ionizing radiation, and cultured for 48 hours. Cell apoptosis was assessed by Annexin V-FITC (Invitrogen) and propidium iodide (PI; Sigma-Aldrich) staining coupled with flow cytometry analysis. For cell-cycle distribution analysis, cells were fixed in 70% ethanol at −20°C and stained with DNA staining solution containing PI and RNaseA (Sigma-Aldrich) overnight. All data were acquired on LSRII Cytometry (BD Biosciences) and each sample was assessed using a collection of 10,000 events, followed by analysis using FlowJo Software (FlowJo).

**Animal experiments**

All animal studies were conducted under our protocol approved by The Ohio State University Institutional Animal Care and Usage Committee. Six- to 8-week-old athymic nude mice (Taconic Farms Inc.) were housed in a pathogen-free facility. A total of 5 × 10^6 Cal27 or FaDu cells were injected subcutaneously into the left flank of each mouse. Mice were randomized to treatment regimens when the tumor size reached 150 mm^3 (−10 days after inoculation). As reported previously (41), LCL161 was formulated for oral gavage by dissolving in 0.1 N HCl and diluting with sodium acetate buffer (100 mmol/L, pH 4.6) to produce a solution with pH 4.3 to 4.6. LCL161 (50 mg/kg, oral gavage) and radiation (6 Gy) were given once a day for 5 continuous days over 1 week. Radiation was delivered directly to the tumor with the rest of the animal body shielded. For combination treatment, LCL161 was given to the mouse 2–3 hours before radiation exposure. The tumor growth was monitored three times a week. The average tumor volumes of at least 10 tumors in each group were calculated according to the formula [(Length × Width^2)/2].

**Statistical analysis**

Data are presented as the mean ± SEM for clonogenic survival and tumor growth experiments. Statistical comparisons were made between the control and experimental groups using the unpaired two-tailed Student t test. Cox proportional hazards models were used to assess univariate associations of cIAP1 expression as predictors for death. Unadjusted HR and 95% confidence intervals (CI) were reported. cIAP1 expression was grouped into two categories: positive (cIAP1 H-score ≥ 100) and negative (cIAP1 H-score < 100). Kaplan–Meier survival curves were plotted by cIAP1 status or by cIAP1 and HPV dual markers. Log-rank test was used to compare survival curves with the Sidák multiple-comparison adjustment. A multivariate Cox proportional hazards model including tumor stage, node stage, gender, age, and cIAP1 expression was built to estimate adjusted HRs. cIAP1 expression was compared between HPV− and HPV+ using Wilcoxon two-sample test. A P < 0.05 was considered as statistically significant. All analyses were conducted in SAS, version 9.3 (SAS Institute).

**Results**

**cIAP1 expression is upregulated in HPV− HNSCC**

Overexpression of IAPs has been detected in numerous cancers and found to be associated with the resistance of cancer cells to radiotherapy (42). To explore the association between the expression of IAPs and HPV status in HNSCC, we compared the mRNA expression of BIRC2 (cIAP1), BIRC3 (cIAP2), XIAP, and SURVIVIN, well-characterized members of the IAP family, and other apoptosis related genes, between the 36 HPV− (13%) and 243 HPV+ (87%) HNSCC tumors with mRNA expression available in TCGA database. These tumor samples are derived from primary, previously untreated tumors. Between HPV− and HPV+ HNSCC tumors in this cohort, BIRC2 and SURVIVIN mRNA levels were significantly upregulated in HPV− HNSCC, whereas there were no apparent differences in XIAP mRNA level; BIRC3 mRNA levels were elevated in HPV+ tumors but not statistically significant (Fig. 1A). We also noted significantly higher expression of FADD and RELA/p65 in HPV− HNSCC. We next determined the protein expression of these IAPs by immunoblotting in the well-characterized HPV− HNSCC cell lines UD-SCC-2, UM-SCC-47, and UPCI:SCC090, as well as the HPV− HNSCC cell lines Cal27, UM-SCC-1, and U-M-SCC-7A. Consistent with the significant upregulation of BIRC2 and SURVIVIN mRNA in HPV− HNSCC, from the TCGA database, we found cIAP1 and Survivin protein levels were also elevated in HPV− HNSCC cell lines when compared with that in HPV+ HNSCC cell lines (Fig. 1B). We also noted higher levels of XIAP, suggesting that multiple IAP family members are upregulated in HPV− HNSCC. Finally, we noted several other proteins were also elevated in HPV− HNSCC cells, such as RELA/p65, TNFα, FADD, and BCL-xL, which paralleled the trends or significant differences observed with mRNA expression in TCGA data.

**cIAP1 overexpression is associated with poor overall survival in HNSCC and is associated with HPV-negative status**

To confirm the upregulation of cIAP1 in HPV− HNSCC, we evaluated the protein expression pattern of cIAP1 by IHC in an institutional TMA, which was constructed from 84 surgically treated HNSCC samples and 72 paired normal tissues (39). The 84 HNSCC cases were composed of 46 HPV− and 38 HPV+ HNSCC (Supplementary Table S1). Representative IHC images of low and high cIAP1 protein staining are shown in Fig. 2A, and validation of the antibody in Supplementary Fig. S1. We graded
the cores for intensity and percent of cells staining positive and derived an "H-score" for each tumor sample. Wilcoxon signed rank sum test was used to assess the difference between cIAP1 tumor H-score versus normal H-score. We found cIAP1 protein levels in tumor cells were significantly higher than that in normal cells (P < 0.0003; Fig. 2B). When cIAP1 H-score increased by 10 units, the HR increased by 3.7% (HR, 1.037; 95% CI, 1.001–1.074; P = 0.0422). Kaplan–Meier survival plot showed that patients having cIAP1-positive expression had worse overall survival than patients having negative cIAP1 expression (log-rank P = 0.0322; Fig. 2C). Similar to TCGA data analysis, HPV− tumors had significantly higher expression of cIAP1 protein as compared with HPV+ tumors (Wilcoxon two-sample test, P = 0.004; Fig. 2D). In multiple comparisons, cIAP1-positive/HPV-negative (cIAP1+/HPV−) group had the worst survival rate compared with cIAP1+/HPV+ (P = 0.0004) or cIAP1−/HPV− (log-rank P = 0.0046; Fig. 2E). Finally, we performed multivariate analysis with common clinical covariates, including gender, T stage, N stage, and age. After adjusting for other covariates, cIAP1 expression remained a significant predictor of overall survival. Notably, when the cIAP1 H-score increased by 10 units, the HR for death increased by 3.9% (HR, 1.039; 95% CI, 1.002–1.074; P = 0.0422; Table 1).

**HPV+ HNSCC cells are preferentially radiosensitized by LCL161**

The higher expression of cIAP1 in HPV+ HNSCCs and the poor survival of cIAP1+/HPV+ HNSCC subgroup implies that HPV+ HNSCC cells may depend on cIAP1 for survival. LCL161 is a small molecule Smac-mimetic that binds to cIAP1 and cIAP2 with high affinity, initiating their destruction, and also binds to XIAP and inhibits XIAP activity. Previous studies have shown LCL161 potently induces the degradation of cIAP1 (34, 41). This led us to hypothesize that LCL161 may be, in general, more effective on HPV− tumors than HPV+ tumors (representative UD-SCC-2 cells shown in Fig. 3A). To explore the potential of LCL161 as a radiosensitizer, cells were treated with different doses of radiation in the presence or absence of 100 nmol/L LCL161. We compared the radiosensitivity of HPV+ cells (representative Cal27 cells shown in Fig. 3D) with that of HPV− cells (UD-SCC-2 cells) using standard radiation clonogenic assay (treatment schema shown in Fig. 3C). With 2 hours of treatment prior to radiation, LCL161 significantly induced radiosensitization of HPV+ cells (representative Cal27 cells shown in Fig. 3D) with an average DER of 1.31 (Fig. 3F), but not HPV− cells (representative UD-SCC-2 cells shown in Fig. 3E) with an average DER of 1.01 (Fig. 3F). To determine whether pretreatment of cells...
with LCL161 is critical for radiosensitization, we also compared treatment with an alternate treatment schedule of radiation first, followed by LCL161 treatment 2 hours later. The results showed that LCL161 also radiosensitized Cal27 cells in both treatment schedules (Supplementary Fig. S4). For the remaining in vitro experiments, LCL161 was administered prior to radiation. To determine whether the radiosensitizing effect of LCL161 is tumor type–specific, we treated many additional types of cancer cells with radiation in combination with 100 nmol/L LCL161 followed by clonogenic assays, and found all other cancer cell lines except for HPV−/C0 HNSCC cells, were essentially resistant to LCL161 radiosensitization with a mean DER of 1.1 or less (Fig. 3F).

LCL161 enhances radiation-induced apoptosis of HPV−/C0 HNSCC cells
Impaired apoptosis is one of the mechanisms for radiation resistance in cancer cells. To assess whether LCL161-mediated inhibition of IAPs enhances radiation-induced apoptosis, 2 HPV− and 2 HPV+ HNSCC cell lines, as well as NOE were treated with 4 Gy radiation alone or in combination with 100 nmol/L LCL161 for 24 hours (including pretreatment for 2 hours), and subjected to analysis of cell death by PI flow cytometry and apoptosis by Annexin V staining. In all cell lines, LCL161 treatment alone did not lead to an increase of cells in the sub-G1 fraction, indicating that LCL161 monotherapy was not cytotoxic to both tumor and normal cells (Fig. 4A). On the other hand, radiation monotherapy

![Figure 2](image)

**Figure 2.**
cIAP protein expression is associated with poor overall survival and elevated in HPV−/C0 HNSCC. **A,** Representative IHC images of HNSCC tumor cores negative (left) and positive (right) for cIAP1 expression in the TMA. **B,** cIAP1 protein expression in tumor versus paired normal tissues. **C,** Kaplan-Meier survival plot by cIAP status. **D,** cIAP1 expression in HPV− versus HPV+ patients. **E,** Kaplan-Meier survival plot by cIAP status and HPV status (*, P < 0.05; **, P < 0.001).

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<th>Parameter</th>
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<td>Tumor stage T3/T4</td>
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<td>Node stage N2/N3</td>
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<tr>
<td>Gender Male</td>
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<td>Age (continuous)</td>
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did induce a mild increase in sub-G1 fraction in the four tumor cell lines. However, when compared with radiation alone, the combined treatment of radiation with LCL161 significantly increased the percentage of sub-G1 cells from 2.2% and 2.24%, to 7.98% and 6.5% in the 2 HPV− cell lines Cal27 and UM-SCC-1, respectively. In sharp contrast, LCL161 did not significantly increase the radiation-induced sub-G1 fraction in either HPV+ cell lines or NOE cells (Fig. 4A).

Furthermore, Annexin V/PI staining coupled with flow cytometry analysis demonstrated that the percentage of Annexin V-positive cells was significantly enhanced by the combination treatment (7.54%) compared with radiation alone (4.39%) in HPV− Cal27 cells; however, there was no difference of Annexin V-positive cells in HPV+ UM-SCC-47 and normal NOE cells treated with radiation alone or radiation plus LCL161 (Fig. 4B).

Our above results imply that LCL161 may preferentially enhance radiation-induced apoptosis and cell death in HPV− HNSCC to a greater extent than HPV+ HNSCC and normal NOE cells. To further support this conclusion, caspase activation was assessed by immunoblotting. Treatment with 100 nmol/L LCL161 for 24 hours led to marked degradation of cIAP1 in all the four HNSCC cell lines and NOE cells, whereas radiation alone had minimal effect on cIAP1 protein expression (Fig. 4C). Consistent with the cell death and apoptosis assays (Fig. 4A and B), LCL161 monotherapy caused marginal cleavage of caspase-3, 7, 8, 9, and PARP in all five cell lines, whereas radiation alone had moderate caspase cleavage in HPV+ and HPV− cells but not NOE cells. Compared with radiation alone, the combined treatment of LCL161 and radiation resulted in increased cleavage of caspase-3, 7, 8, 9, and PARP only in HPV+ cells, but not HPV− and NOE cells, suggesting potentiation of apoptotic pathway activation with the combination (Fig. 4C). Also noted was persistence of the DNA damage marker phosphorylated-H2A.X (Ser139) in the combination treated HPV− cells, suggestive of heightened DNA damage at 24 hours after the combination, which was not observed in the HPV+ or NOE cells.

To confirm that caspase activation contributes to LCL161-mediated radiosensitization of HPV− HNSCC cells in vitro, Cal27 cells were treated for 24 hours with 100 nmol/L LCL161 + 4 Gy irradiation in the presence or absence of 10 μmol/L z-VAD-fmk, a pan-caspase inhibitor, followed by detection of cleavage of...
LCL161 Preferentially Radiosensitizes HPV− HNSCC

caspase-3, 8, and PARP by immunoblotting, and apoptosis by Annexin V staining, z-VAD-fmk suppressed the cleavage of caspase-3, 8, and PARP (Fig. 4D), and abolished the increase of Annexin V−positive cells by LCL161 in combination with radiation (Fig. 4E). Consistent with these findings, radiation clonogenic assay showed that LCL161-mediated radiosensitization of Cal27 cells was blocked by z-VAD-fmk (Fig. 4F). Collectively, these data indicate that LCL161 enhances radiosensitivity of HPV− HNSCC cells through caspase-mediated apoptosis.

Because of the important role of TNFα in the tumor microenvironment, as well as in the recent study with birinapant and radiation (36), we assessed TNFα levels in our cell lines and indeed found upregulation of TNFα in our HPV− cells compared with HPV+ cells (Fig. 1B). Next, we assessed whether the various treatments could result in further activation of TNFα expression in HPV+ cell lines and xenograft tumors 24–48 hours after treatment, but did not find significant upregulation of TNFα expression after treatment with LCL161, radiation, or the combination (Supplementary Fig. S5A and S5B). Interestingly, we did detect heightened levels of TNFα in the medium of tumor cells by ELISA after 48 hours of treatment with LCL161 + radiation in Cal27 cells, but not UD-SCC-2 cells (Supplementary Fig. S5C). Because TNFα (and other death receptor ligands like TRIAL or Fas ligand) could contribute to heightened apoptotic-mediated tumor cell death during this process, we investigated whether supplementing TNFα could increase rates of apoptosis in HPV+ tumor cells. Indeed, treatment with TNFα led to significantly increased rates of radiation-induced apoptosis in Cal27 cells, but only slightly increased rates of apoptosis further when LCL161 was added to this combination (Supplementary Fig. S5D).
LCL161 radiosensitizes HPV^+ HNSCC cells in mouse tumor xenografts

To translate our findings to preclinical investigation, we evaluated LCL161 radiosensitization of HPV^+ HNSCC in nude mice xenografts. LCL161 was delivered by oral gavage with a dose of 50 mg/kg as described previously (43). When tumors derived from HNSCC HPV^+ Cal27 and FaDu cell lines reached approximately 100–150 mm^3, the mice were randomized to treatment with vehicle, LCL161 alone, 6 Gy radiation alone, or the combination of LCL161 + 6 Gy, all for 5 consecutive days (Fig. 5A). Consistent with in vitro data, LCL161 monotherapy did not alter tumor growth, whereas radiation treatment resulted in partial tumor growth delay. However, LCL161 in combination with radiation treatment led to remarkable and sustained regression of both Cal27 (Fig. 5B) and FaDu (Fig. 5C) xenografts, with the majority of Cal27 tumors showing no evidence of tumor recurrence (survival curves shown in Supplementary Fig. S6). The treatment was tolerated well, although mice in the combination treatment cohort lost weight during the first week (no more than 10% or less of starting body weight), but fully recovered within 1–2 weeks after the last treatment (Supplementary Fig. S7). Immunoblotting of tumor lysates several hours after day 3 of treatment in each of the groups demonstrated degradation of cIAP1 by LCL161 alone and in combination with radiation, confirming pharmacodynamic inhibition by LCL161. Consistent with the cell culture results, radiation alone did not induce degradation of cIAP1 and cleavage of caspase-3, 7, 8, 9, and PARP. In sharp contrast, combination treatment of LCL161 with radiation resulted in potent cleavage of caspase-3, 7, 8, 9, and PARP, implicating activation of apoptosis as a major mechanism for tumor growth regression and sustained response (Fig. 5D).

Discussion

In this study, we found that cIAP1 (BIRC2) expression is significantly higher in HNSCC tumor cells than normal tissue cells, and increased cIAP1 is significantly associated with a poor overall survival of patients with HNSCC. For the first time, to our
knowledge, we demonstrated that HPV+ HNSCC has significantly higher expression of cIAP1 as compared with HPV− HNSCC and that patients with cIAP1+/HPV− HNSCC have reduced survival. Most importantly, we showed that targeting IAPs with LCL161 preferentially sensitizes HPV+ HNSCC cells to radiotherapy both in vitro and mouse xenograft models, which is accompanied by degradation of cIAP1 and enhanced apoptosis. The effects of the combination of LCL161 and radiation appear to demonstrate tumor selectivity, as minimal induction of apoptotic response was observed in the normal oral epithelial (nontransformed) cell line.

The HPV status of HNSCC has an important impact on the prognosis and response of patients to treatments. It has been well documented that patients with HPV+ HNSCC have a better prognosis than patients with HPV− HNSCC (4–7); moreover, patients with HPV+ HNSCC are resistant to radiotherapy (37). However, the exact underlying molecular mechanisms are largely unknown. The TCGA study show that HPV+ HNSCC has a characteristic coamplification of 11q22 (containing BIRC2 and YAP1) and 11q13 (containing CCND1, FADD, and CTTN), suggesting an important role for cIAP1 in HPV+ HNSCC. Indeed, data from TCGA suggests approximately 30% of HNSCC harbors chromosome 11q13/22 amplifications and overexpression of FADD, with or without BIRC2/3 genes that encode cIAP1/2 (36). In addition, 86% of HPV+ HNSCC harbor TP53 mutations (3). In this study, using a TMA, we found that HPV+ HNSCC has significantly higher cIAP1 protein expression as compared with HPV− HNSCC, and patients with cIAP1+/HPV− HNSCC have the lowest survival. cIAP1 is a potent inhibitor of apoptosis and p53 proapoptosis pathways by stimulating the expression of proapoptosis genes including PUMA and BAX, while suppressing the expression of antiapoptosis genes such as BCL-2 and BCL-XL (44). It is therefore possible that simultaneous BIRC2 overexpression and TP53 mutation may contribute in part to the resistance of HPV+ HNSCC to therapies which rely on activation of apoptosis.

Radiation is commonly used to treat patients with HNSCC, both in the curative and palliative settings. However, radioresistance, which contributes to local recurrence, is currently a challenge for the management of patients with HNSCC, particularly with HPV− HNSCC. The only proven radiosensitizer in HNSCC that was shown to improve overall survival in combination with radiation was cetuximab, targeting EGFR, in the RTOG 0522 randomized trial (45). Current ongoing trials are exploring various other radiosensitizers, including cytotoxic/tetrahydrodouridine (NCT00077051), olaparib (PARP inhibitor, NCT02229656), PS-341/bortezomib (proteasome inhibitor, NCT00077051), olaparib (PARP inhibitor, NCT011778), and nimorazole (hypoxia modifying drug, NCT00077051), SM164 in HNSCC (35) and breast cancer (48), Debio 1143 in HNSCC (49), ASTX660 in HNSCC (50), and ip-1201 in colorectal cancer (51). Likewise, a more recent study showed that SMAC-mimetic compound, birinapant, sensitizes HNSCC with FADD and BIRC2 amplification (36). It is interesting to note that tumors with FADD and BIRC2 amplification are generally HPV− tumors.

In our study, we demonstrated that LCL161 enhances the radiosensitization of HNSCC cell lines with the greatest effect in HPV− cells. In preclinical modeling, we demonstrated that oral administration of LCL161 synergizes with radiation to induce remarkable and virtually complete regression of HPV+ HNSCC tumour xenografts. Such dramatic findings of SMAC-mimetic in combination with radiation using xenografts were likewise noted in the study with birinapant and radiation (36). Consistently, LCL161 has previously been tested in combination with paclitaxel chemotherapy for triple-negative breast cancer and found to result in higher pathologic response rates compared with chemotherapy alone for patients with a TNFRα gene expression signature (52). Similar to radiotherapy, one of the main mechanisms by which many chemotherapy drugs act is to induce DNA damage. Thus, it will be interesting to investigate whether LCL161 sensitizes HPV+ HNSCC to DNA-damaging based chemotherapy. In addition, the pharmacology of SMAC-mimetic sensitization of cancer cells to radiotherapy and chemotherapy is complex. It has been reported that IAP antagonists can initiate NF-kB activation, as well as autocrine and/or paracrine TNF signalling to induce cell apoptosis (53). Of note, IAPs are emerging as modulators of both innate and adaptive immunity during pathogen recognition and inflammation activation (54). A weakness of our study is that the mouse modeling was performed in an immunocompromised mouse model, with lack of proper T-cell-mediated cell killing. Because checkpoint inhibitors such as anti-PD-1 antibodies (which activate T-cell infiltration and T-cell-mediated
immunogenic tumor cell death) are increasingly being used in the clinic, it will be important in the future to explore the modulation of immune response by LCL161 in response to radiotherapy, chemotherapy, and immune checkpoint blockade based therapy. Certainly, there is increasing data that radiation may help to activate an immune response by enhanced tumor neoantigen release and cross-presentation of tumor antigens, leading to activation of tumor-specific T cells (55). As proof of principle, a recent study found that another IAP antagonist (ASTX660) enhances antitumor immunity when combined with PD-1 inhibition and radiation in an immunocompetent model of HNSCC (50).

In conclusion, higher cIAP1 expression is correlated with HPV status and is associated with a poor prognosis of HNSCC. Targeting IAPs with LCL161 preferentially sensitizes HPV+ HNSCC to radiotherapy over HPV− HNSCC and normal epithelial cells. Our study has important implications for the use of cIAP1 expression as a surrogate biomarker in future clinical trials combining LCL161 and radiation. Finally, our preclinical data demonstrate the susceptibility of HPV+ HNSCC tumors to SMAC-mimetics in combination with radiotherapy, and supports initiation of clinical trials with LCL161 in combination with radiation, particularly in patients with HPV+ HNSCC tumors.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References


LCL161, a SMAC-mimetic, Preferentially Radiosensitizes Human Papillomavirus-negative Head and Neck Squamous Cell Carcinoma

Linlin Yang, Bhavna Kumar, Changxian Shen, et al.