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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 (IAPs) 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 analysed using TCGA database. cIAP1 was assessed by immunohistochemistry 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 to HPV[+] HNSCC tumors in the TCGA database. In our TMA, cIAP1 was over-expressed in HNSCC compared to 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 cIAP1-positive/HPV[−] HNSCC patients 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 HPV[−] HNSCC patients.

Keywords

SMAC mimetic; LCL161; human papillomavirus (HPV); head and neck squamous cell carcinoma (HNSCC); inhibitor of apoptosis protein (IAP)

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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. While improvements in these treatments have greatly benefited HNSCC patients, the 5-year survival rate of HNSCC patients 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 clinicopathological entity and typically has a worse prognosis than patients with HPV[+] HNSCC (4–7). In comparison to 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 signalling 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). Since radioresistance is a significant challenge for HNSCC patients, 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 (IAPs) originally discovered in Baculoviral genomes by Lois Miller and colleagues in 1993, comprise a family of anti-apoptotic proteins that promote pro-survival signalling 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 binds 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 been shown safety and efficacy in a phase I study of advanced solid tumours (32). Furthermore, a preclinical study

has shown good anti-tumor activity of LCL161 in human hepatocellular carcinoma (33). LCL161 has also been shown to sensitize esophageal carcinoma to radiotherapy (34). In addition, SMAC-mimetics SM-164 and birinipant 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 TCGA database, cell lines and tissue microarray, 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 HPV[-] HNSCC patients.

MATERIALS AND METHODS

Cell culture, chemicals, antibodies, and ELISA

HNSCC cell line UD-SCC-2 was a gift from Henning Bier (University of Dusseldorf, 2009); UM-SCC-47, UM-SCC-1, UM-SCC-11B, UM-SCC-74A were gifts from Thomas Carey (University of Michigan, 2009); 93VU147T was gift from Jim Rocco (Ohio State University, 2015); UPCI:SCC090 was gift from Susanne M. Gollin (University of Pittsburgh, 2009); Normal Oral Epithelial cells (NOE) was gift from Quintin Pan (University Hospitals Cleveland Medical Center, 2009); Cal27, FaDu were purchased from ATCC. Except for UPCI:SCC090 and NOE cells, cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Life Technologies Inc.) containing 10% (v/v) heat inactivated fetal bovine serum (FBS; Life Technologies Inc.) and 1% (v/v) none essential amino acid (NEAA; Life Technologies Inc.). UPCI:SCC090 cells were cultured in MEM medium (Life Technologies Inc.) with 10% FBS (v/v) and 1% NEAA (v/v). NOE cells were maintained in a serum-free keratinocyte growth medium (KGM; Life Technologies Inc.). All cell lines were routinely cultured at humidified atmosphere in an incubator with 5% CO₂ at 37°C. Typically cells were kept in culture for minimum two passages prior and maximum 20 passages when the experiments were performed. The identity of all cell lines was confirmed by STR genotyping (Identifier Kit, Applied Biosystems, Carlsband, CA). For the detection of Mycoplasma in cell culture, the Universal Mycoplasma Detection Kit (ATCC) was used. LCL161 was synthesized and provided by Novartis Pharma AG (Basel, Switzerland). Pan-caspase inhibitor Z-VAD-FMK was purchased from Selleck Chemicals LLC (Huston, TX). Anti- c-IAP1, c-IAP2, XIAP, Survivin, Smac, Bcl-xL, Bcl-2, caspase3, cleaved caspase3, caspase 7, cleaved caspase 7, caspase 8, cleaved caspase 8, caspase 9, cleaved caspase 9, PARP, FADD, c-FLIP, p65/RELA, phospho-p65, TNF- α , gamma-H2A.X, and GAPDH antibodies were purchased from Cell Signalling Technology (Danvers, MA). TNF- α enzyme

linked immunosorbent assay (ELISA) kit (#ELH-TNF α -1) was purchased from Raybiotech Life (Norcross, GA) and experiments performed per the manufacturer's instructions. Recombinant human TNF α (cat# 210-TA-005) was purchased from R&D Biosystems (Minneapolis, MN).

Immunoblotting

Immunoblotting was performed as previously described (38). Briefly, total proteins were extracted from cells using RIPA buffer (Thermo Fisher, Waltham, MA) supplemented with 1x phosphatase inhibitors (PhosSTOP, Roche, Indianapolis, IN) and protease inhibitors (cOmplete, Roche) followed by protein quantification with Dc protein assay kit (Bio-Rad, Hercules, CA). Equal amounts of protein were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes, followed by incubation with primary antibodies (dilution of 1:500–1,000) at 4°C overnight. After washing with TBS-Tween, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (dilution of 1:2,500–5,000) at room temperature for 1 hour, followed by signal detection with enhanced chemiluminescence reagent (Immobilon, Millipore, Billerica, MA).

Study population, tissue microarray (TMA), immunohistochemistry (IHC) and scoring

A tissue microarray (TMA) was constructed from human oropharyngeal squamous cell carcinoma tissue specimens, which were obtained from the surgical resection of the patients at The Ohio State University James Cancer Hospital and Solove Research Institute from 2002 to 2009 (39). All patients underwent surgical resection as a first line of therapy with a curative intent. This was followed by no additional treatment or adjuvant chemotherapy and/or radiotherapy as needed. The Ohio State University Institutional Review Board approved a retrospective analysis study of these specimens and a waiver of HIPAA authorization was obtained. Patient characteristics including HPV status are shown in Supplementary Table S1. TMA slides were stained to assess the tumour expression of cIAP1 using immunohistochemistry as previously described (40). Stained slides were interpreted by a pathologist who was blinded to treatment outcome at the time of review. Tumour cells were scored for staining proportion (0–100%) and intensity (0: none, 1: low, 2: moderate, 3: high). An H-Score was generated by multiplying the stain proportion scores with stain intensity to obtain values between 0–300.

WST-1 assay and IC₅₀ determination

WST-1 cytotoxicity assay was performed according to manufacturer's instructions (Roche, Basel, Switzerland). Briefly, cells were seeded in 96-well plates in 5–6 replicates at a density of 2,000 cells per well in 100 μ L 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. Half maximal inhibitory concentration (IC₅₀) was determined using the nonlinear four parameter regression function in GraphPad Prism (La Jolla, CA).

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 (RadSource, GA) for different doses, followed by incubation at 37°C for 10 to 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. Buffalo Grove, IL). The surviving fractions and dose enhancement ratios (DER) at 2 Gy were calculated. For LCL161 or Z-VAD-FMK treatment, cells were pre-treated 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 300,000 cells per well in 2 mL medium for 16 hours. The cells were treated with the indicated drugs and ionizing radiation (IR), 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 10000 events, followed by analysis using FlowJo software (FlowJo, Ashland, Oregon).

Animal experiments

All animal studies were conducted under our protocol approved by The Ohio State University Institutional Animal Care and Usage Committee (IACUC). Six to eight-week-old athymic nude mice (Taconic Farms Inc.) were housed in a pathogen-free facility. Five million of Cal27 or FaDu cells were injected subcutaneously (s.c.) into the left flank of each mouse. Mice were randomized to treatment regimens when the tumour size reached 150 mm³ (~10 days after inoculation). As previously reported (41), LCL161 was formulated for oral gavage by dissolving in 0.1N HCL and diluting with sodium acetate buffer (100 mM, pH 4.63) to produce a solution with pH 4.3–4.6. LCL161 (50mg/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 tumour with the rest of the animal body shielded. For combination treatment, LCL161 was given to the mouse 2 to 3 hours before radiation exposure. The tumor growth was monitored 3 times a week. The average tumor volumes of at least 10 tumors in each group were calculated according to the formula $[(\text{Length} \times \text{Width}^2)/2]$.

Statistical analysis

Data are presented as the mean $\pm$ standard error of the mean (SEM) for clonogenic survival and tumor growth experiments. Statistical comparisons were made between the control and experimental groups using the unpaired 2-tailed Student's t-test. Cox proportional hazards models were used to assess univariate associations of cIAP1 expression

as predictors for death. Unadjusted hazard ratios (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 Šídák multiple-comparison adjustment. A multivariable 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-value <0.05 was considered as statistically significant. All analyses were conducted in SAS, version 9.3 (SAS Institute, Cary, North Carolina).

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). In order 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 the 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, while 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 UM-SCC-74A. 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 to that in HPV[+] HNSCC cell lines (Fig. 1B). We also noted higher levels of XIAP, suggesting that multiple IAP family members are up-regulated 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 tumour H-score versus normal H-score. We found cIAP1 protein levels in tumor cells were significantly higher than that in normal cells (Fig. 2B, $p=0.0003$). When cIAP1 H-Score increased by 10 units, the hazard ratio (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 to HPV[+] tumors (Fig. 2D, Wilcoxon two-sample test, $p=0.004$). In multiple comparisons, cIAP1-positive/HPV-negative (cIAP1+/HPV[–]) group had the worst survival rate compared to cIAP1-/HPV+ ($p=0.0004$) or cIAP1+/HPV+ (log rank $p=0.0046$, Fig. 2E). Finally, we performed multivariable analysis with common clinical co-variables, 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 hazard ratio for death increased by 3.9% (Table 1, $HR=1.039$, 95% CI: 1.002–1.078, $p=0.0402$).

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[–] than HPV[+] cells with regard to radiosensitization. To this end, we first compared the radiosensitivity of HPV[–] cell lines (Cal27, and UM-SCC-1) with that of HPV[+] cell lines (UD-SCC-2, and UM-SCC-47) using standard radiation clonogenic assay. Consistent with the previous observations that HPV[–] HNSCC cells generally display higher resistance to radiation than HPV[+] cells (37), we found that HPV[–] cell lines were relatively resistant to radiation while HPV[+] cell lines were relatively sensitive (Supplementary Fig. S2A).

We next investigated the cytotoxic effect of LCL161 as a single agent on HNSCC cell lines through WST1 assay. We observed that all HNSCC cell lines were highly resistant to LCL161 as a single agent with the IC₅₀ values ranging from 32 to 95 μ M (Supplementary Fig. S2B), suggesting LCL161 as monotherapy may not be effective in HNSCC. However, HPV[–] cells showed significantly lower IC₅₀ than HPV[+] cells, suggesting increased sensitivity to cIAP1 inhibition ($p=0.006$, Supplementary Fig. S2C). We further determined the doses and timing of LCL161 to reduce IAPs in HNSCC cells. LCL161 treatment for 2 hours led to a dose-dependent decrease of both cIAP1 and cIAP2 expression, but did not decrease XIAP expression in HPV[–] Cal27 and UM-SCC-1 cells, consistent with its known effects on XIAP enzymatic activity (not expression) (Fig. 3A). LCL161 at 100 nM induced potent downregulation of cIAP1 and cIAP2 in all tested HNSCC cell lines and was chosen for subsequent experiments. We next treated HPV[–] UM-SCC-1 and Cal27 cells with 100 nM LCL161 for different time periods and found LCL161 treatment resulted in rapid

decrease of cIAP1 and cIAP2 expression as early as 30 minutes and sustained after 24 hours (Fig. 3B). We determined whether LCL161 treatment for 2 hours could alter the expression and activity of important tumor cell proliferation and survival pathways. We did not detect any significant acute changes in the phosphorylation levels of STAT3, AKT and ERK in HNSCC cells (Supplementary Fig. S3), suggesting that LCL161-mediated inhibition of IAPs does not alter the acute activation of the JAK/STAT, PI3K/AKT and MEK/MAPK signaling pathways in HNSCC cells.

To explore the potential of LCL161 as a radiosensitizer, cells were treated with different doses of radiation in the presence or absence of 100 nM LCL161, followed by 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 pre-treatment of cells with LCL161 is critical for radiosensitization, we also compared treatment of 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. In order 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 nM LCL161 followed by clonogenic assays, and found all other cancer cell lines except for HPV[−] 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[−] 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 (a normal oral epithelial cell line) were treated with 4 Gy radiation alone or in combination with 100 nM LCL161 for 24 hours (including pre-treatment for 2 hrs), and subjected to analysis of cell death by propidium iodide 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 did induce a mild increase in sub-G1 fraction in the 4 tumor cell lines. However, when compared to 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 cells, 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). Further, 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 to 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 nM LCL161 for 24 hours led to marked degradation of cIAP1 in all the 4 HNSCC cell lines and NOE cells, while radiation alone had minimal effect on cIAP1 protein expression (Fig. 4C). Consistent with the cell death and apoptosis assays (Figs. 4A and B), LCL161 monotherapy caused marginal cleavage of caspase-3, –7, –8, –9, and PARP in all 5 cell lines, while radiation alone had moderate caspase cleavage in HPV[+] and HPV[–] cells but not NOE cells. Compared to 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 hrs 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 nM LCL161+4 Gy irradiation in the presence or absence of 10 μ M z-VAD-fmk, a pan-caspase inhibitor, followed by detection of cleavage of 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 TNF α in the tumor microenvironment as well as in the recent study with birinipant and radiation(36), we assessed TNF α levels in our cell lines and indeed found up-regulation of TNF α in our HPV[–] cells compared to HPV[+] cells (Figure 1B). Next, we assessed whether the various treatments could result in further activation of TNF α expression in HPV[–] cell lines and xenograft tumors 24–48 hrs after treatment, but did not find significant up-regulation of TNF α expression after treatment with LCL161, radiation, or the combination (Supplementary Fig. S5A–B). Interestingly, we did detect heightened levels of TNF α in the medium of tumor cells by ELISA after 48 hrs of treatment with LCL161 + radiation in Cal27 cells, but not UD-SCC-2 cells (Supplementary Fig. S5C). Since TNF α (and other death receptor ligands like TRAIL or Fas ligand) could contribute to heightened apoptotic-mediated tumor cell death during this process, we investigated whether supplementing TNF α could increase rates of apoptosis in HPV[–] tumor cells. Indeed, treatment with TNF α 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 previously described (43). When tumors derived from HNSCC HPV[−] Cal27 and FaDu cell lines reached ~100–150 mm³, 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, while 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 HNSCC patients. For the first time, to our knowledge, we demonstrated that HPV[−] HNSCC has significantly higher expression of cIAP1 as compared to HPV[+] HNSCC and that cIAP1-positive/HPV[−] HNSCC patients have reduced survival. Most importantly, we showed that targeting IAPs with LCL161 preferentially sensitizes HPV[−] HNSCC cells to radiotherapy both in *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 (non-transformed) 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 co-amplification 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 ~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 harbour *TP53* mutations (3). In the present study, using a TMA, we found that HPV[−] HNSCC has significantly higher cIAP1 protein expression as compared to HPV[+] HNSCC, and cIAP1-positive/HPV[−] HNSCC patients have the lowest survival. cIAP1 is a potent inhibitor of apoptosis and p53 promotes apoptosis by stimulating the expression of pro-apoptosis genes including *PUMA* and *BAX*, while suppressing the expression of anti-apoptosis genes such as *BCL-2*, *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 HNSCC patients, particularly HPV[−] HNSCC patients. 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 cytochlor/tetrahydrouridine (NCT00077051), olaparib (PARP inhibitor, NCT02229656), PS-341/bortezomib (proteasome inhibitor, NCT00011778), and nimorazole (hypoxia modifying drug, NCT01880359), but results have yet to be reported. Our findings support that an IAP antagonist may be an effective radiosensitizer for HNSCC. Our finding that overexpression and negative correlation of cIAP1 with the survival of HPV[−] HNSCC patients led us to explore the potential of targeting cIAP1 with SMAC-mimetic in the sensitization of HPV[−] HNSCC to radiotherapy. LCL161 is an orally bioavailable, cell permeable small molecule mimetic of SMAC developed by Novartis Pharmaceuticals. LCL161 promotes the degradation of certain IAP family members (i.e. cIAP1, cIAP2) and disengages IAPs from caspases (e.g. XIAP), thereby sensitizing cells to apoptosis-inducing treatments (46,47). Certainly, while we found that LCL161 promotes a rapid and potent degradation of cIAP1 in HNSCC cells at nanomolar concentrations, we can not exclude the possibility that the effects we observed are not all dependent on cIAP1 (or cIAP2), since LCL161 potentially inhibits XIAP activity by forcing disengagement of XIAP from caspases. Further evaluation into the role of XIAP in LCL161 response is warranted, but cIAP1 may be a more effective pharmacodynamic biomarker since degradation may be more readily measurable than caspase disengagement.

LCL161 has been shown to exert antitumor activity against multiple cancers including myeloma, glioblastoma and sarcoma. In agreement with the finding from a preclinical study in pediatric tumors (41), the IC50 values of LCL161 on HNSCC cells are at range of 32 to 95 μ M, indicating LCL161 may not have therapeutic potential as a single treatment for HNSCC. With regards to a role for LCL161 in combination therapy, several SMAC-mimetics have been reported to sensitize cancer cells to radiation, including SM164 in HNSCC (35) and breast cancer (48), Debio 1143 in HNSCC (49), ASTX660 in HNSCC (50), and JP-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 modelling, 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 birinipant and radiation (36). Consistent with previous reports showing that SMAC mimetics radiosensitize cancer cells through activation of caspases and enhanced induction of apoptosis (35,48,49), we revealed that the combination treatment of LCL161 and radiation activates caspases and induces apoptosis of HPV[–] HNSCC cells both in *in vitro* and in tumour xenografts. Our findings suggest that cIAP1+/HPV[–] might serve as a predictive biomarker for HNSCC patients who would benefit most from the combination of SMAC mimetic and radiotherapy.

Aside from radiotherapy, IAP antagonists have also been shown to sensitize various cancers including pancreatic, lung, colon and acute leukaemia to chemotherapy such as gemcitabine, doxorubicin, cisplatin, and irinotecan (19). Clinically, LCL161 has been tested in combination with paclitaxel chemotherapy for triple-negative breast cancer and found to result in higher pathologic response rates compared to chemotherapy alone for patients with a TNF α 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- κ B 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 inflammasome 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. Since 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 anti-tumor 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 could have implications for the use of cIAP1 expression as a surrogate biomarker in future clinical trials combining LCL161 and radiation. Lastly, 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 tumours.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Differential expression of IAPs between HPV[–] and HPV[+] HNSCC. **A**, The mRNA expression of BIRC2 (cIAP1), BIRC3 (cIAP2), XIAP and SURVIVIN between HPV[–] and HPV[+] HNSCC of HNSCC cohort in TCGA database was compared using *t*-test. The mRNA expression data were derived from RNA-Seq and converted to Z scores.

p*<0.05. *p*<0.01 **B**, Exponentially growing HPV[–] and HPV[+] HNSCC cells of the indicated cell lines were subjected to immunoblotting analyses of cIAP1, cIAP2, XIAP, Survivin, SMAC/DIABLO and other NF-κB and apoptosis pathway components. GAPDH shown as loading control.

Figure 2.

cIAP1 protein expression is associated with poor overall survival and elevated in HPV[–] HNSCC. **A**, Representative IHC images of HNSCC tumor cores negative (left panel) and positive (right panel) for cIAP1 expression in the TMA. **B**, cIAP1 protein expression in tumor versus paired normal tissues. **C**, Kaplan–Meier Survival plot by cIAP1 status. **D**, cIAP1 expression in HPV[–] versus HPV[+] patients. **E**, Kaplan–Meier Survival plot by cIAP1 status and HPV status. * $p < 0.05$, ** $p < 0.001$.

Figure 3.

LCL161 sensitizes HPV[-] HNSCC cells to radiation. **A**, The indicated HNSCC cell lines were treated with increasing concentrations of LCL161 for 2 hours. Total proteins were extracted for immunoblotting of cIAP1, cIAP2, XIAP and Survivin with GAPDH as loading control. **B**, UM-SSC-1 and Cal27 cells were treated with 100 nM LCL161 for different time points and subjected to immunoblotting for the indicated proteins. **C**, Schedule of combined treatment in clonogenic assay. Cells were seeded in 6-well plates for 24 hours, followed by exposure to irradiation of increasing dose. LCL161 (100 nM) was added to the culture media 2 hours prior to irradiation, and removed 24 hours after radiation. The cells were cultured for 10–14 days to determine colony formation. Representative radiation clonogenic assay results of HPV[-] Cal27 (**D**) and HPV[+] UD-SSC-2 (**E**) are shown. **F**, Dose enhancement ratios (DER) at 2 Gy were compared between various types of cancer using radiation clonogenic assay. Multiple independent experiments were performed. ** $p < 0.001$.

Figure 4.

LCL161 enhances radiation-induced apoptosis in HPV[-] but not HPV[+] HNSCC and normal oral epithelial cell lines. **A** and **B**, Cells were treated with 4 Gy radiation, 100 nM LCL161 alone, or in combination for 24 hours, followed by flow cytometry analysis of sub-G1 populations (**A**) and Annexin V positive cells (**B**). **C**, Cells were treated as in **A** and subjected to immunoblotting for the indicated proteins. **D**, Cal27 cells were treated with 4 Gy radiation and 100 nM LCL161 in the presence or absence of 10 μ M pan-caspase inhibitor-z-VAD-fmk for 24 hours. Total proteins were extracted for immunoblotting of the indicated proteins. **E**, Cal27 cells were treated with 4 Gy radiation, 100 nM LCL161, 4 Gy radiation + 100 nM CL161 in the presence or absence of 10 μ M z-VAD-fmk for 24 hours. Cell apoptosis was assessed by Annexin V staining coupled with flow cytometry analysis. **F**, Effect of z-VAD-fmk on LCL161 radiosensitization was assessed by clonogenic assay. Cal27 cells were treated with LCL161 alone (2 hours prior to radiation) or in combination with z-VAD-fmk (10mM). For all assays, multiple independent experiments were performed. ** $p < 0.001$.

Figure 5.

LCL161 radiosensitization of HPV[−] HNSCC in *in vivo* xenograft tumor models. **A**, schematic schedule of combined *in vivo* treatment. Five million Cal27 or FaDu cells were inoculated subcutaneously in the left flank of nude mice. Indicated treatments were started on 21 days after inoculation when tumor size reached 100–150 mm³, followed by tumor growth measurement (mean ± SEM, n=10). **B–C** The change of the average tumor volume of each group was shown for Cal27 (**B**) and FaDu (**C**) xenografts models. **D**, Cal27 tumor lysates were made from 2 representative tumors per group 3 hrs after the indicated treatment on Day 3. LCL161 eliminated cIAP1 and enhanced radiation-induced apoptosis as assessed by immunoblotting of cleaved caspase-3, 7, 8, 9, and PARP. IR, ionizing radiation.

**p<0.001.

Table 1.

Overall Survival Multivariate Analysis

Parameter		Hazard Ratio	95% Hazard Ratio Confidence Limits	P-value
cIAP1 H-Score (per 10 unit increase)		1.039	1.002 1.078	0.0402
Tumor Stage	T3/T4	1.968	1.009 3.838	0.0469
Node Stage	N2/N3	1.057	0.533 2.095	0.874
Gender	Male	2.162	0.748 6.253	0.1546
Age (continuous)		0.999	0.964 1.036	0.9573