p16 Expression Represses DNA Damage Repair via a Novel Ubiquitin-Dependent Signaling Cascade.

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Abstract

Human papillomavirus (HPV) drives the development of squamous cell carcinoma at several sites, including the oropharynx. Generally, the presence of HPV renders a tumor more sensitive to DNA-damaging therapies such as radiation; however, the mechanism behind this phenomenon is elusive. Previous studies have shown that p16, the clinically utilized surrogate for HPV tumor positivity, can render cells more sensitive to radiation. In the current manuscript, using a combination of immunoprecipitation mass spectrometry (IP/MS), *in vivo* and *in vitro* modulation and clinical tumor profiling, we identify a novel ubiquitin-dependent signaling pathway linking p16 to increased activity of the transcription factor SP1 leading to increased HUWE1 transcription and degradation of ubiquitin-specific protease 7 (USP7). This pathway is activated in HPV-positive tumor cells, leading to an absence of TRIP12, decreased DNA damage repair and increased mitotic death following radiation. As USP7 inhibitors are currently in clinical trials, this pathway provides a novel means by which radioresistant tumors may be targeted to increase response and improve outcome.

Introduction

HPV infection is associated with several malignancies, including head and neck squamous cell carcinoma (HNSCC)¹. Analysis of patient outcomes following treatment of HNSCC reveals that patients with HPV(+) tumors fare significantly better following standard of care radiation and platinum chemotherapy^{2,3} and further suggests that p16INK4 (hereafter referred to as p16) is a surrogate biomarker for HPV status⁴. *In vitro*, HPV(+) tumor cell lines are more sensitive to ionizing radiation⁵⁻⁹. These findings suggest that there is an inherent molecular mechanism by which HPV infection confers sensitivity of tumor cells to genotoxic therapy through the DNA damage response (DDR) pathway¹⁰. As noted above, it is now recognized that the hallmark of HPV infection is the expression of p16 protein^{2,4,11,12}, a consequence of E7-dependent pRb inhibition and degradation. P16 regulates the cell cycle¹³, cell response to DNA damage¹⁴⁻¹⁶ and cellular senescence following genotoxic exposure¹⁷. Overexpression of p16 can simulate HPV-dependent radiation sensitivity, suggesting that this phenomenon is at least partially dependent on the function of p16^{16,8}. Yet how p16 achieves this effect remains unclear. Understanding the basis of p16's ability to modulate the radiosensitivity of HPV(+) HNSCC cells could conceivably lead to strategies to enhance the response of HPV(-) tumors as well as perform rational treatment deintensification, both of which are sorely needed.

Recently, we established that one mechanism by which p16 modulates the cell response to DNA damage relies on its control of TRIP12⁸. TRIP12 is a HECT domain ubiquitin E3 ligase that binds to and inhibits RNF168 – an E3 ligase RING finger protein – and thus prevents excessive spreading of 53BP1-specific DNA repair foci by controlling the extent of chromatin ubiquitination at the sites of DNA damage¹⁸. Cells expressing p16 have significantly downregulated protein levels of TRIP12⁸ and enlarged 53BP1 foci in response to radiation therapy¹⁸. Thus, it appears that p16 leads to a decrease in TRIP12 protein levels that compromises DNA repair at sites of radiation-induced DNA damage, specifically repair of DNA

double strand breaks (DSBs) by homologous recombination (HRR). In light of these findings, we proposed that an inverse relationship between p16 and TRIP12 may at least partially explain the increased positive response of HPV(+) patients to radiotherapy. Although we previously demonstrated p16 posttranslational control over TRIP12, it is currently unclear how p16 regulates TRIP12 ubiquitination and degradation. Moreover, it is unknown whether this pathway is important to the clinical responses of patients with HNSCC. In this manuscript, we delineate a p16-HUWE1-USP7-TRIP12 pathway regulating the DNA damage response in HNSCC that is both targetable and clinically relevant.

Materials And Methods

Cell Lines and Culture Conditions

Head and neck squamous cell carcinomas HN5, HN30, HN31, UM-SCC-1, UM-SCC-25 and UM-SCC-47 were obtained from Dr. Jeffrey Myers (UT MD Anderson). HEK-293T, FaDu, UPCI:SCC-152, UPCI:SCC-154, NCI-H460, NCI-H1299 and Detroit562 cells were purchased from ATCC. At every new frozen batch generation, DNA fingerprinting and mycoplasma testing were performed by the Cancer Center Support Grant-funded Characterized Cell Line core at MD Anderson (CA016672). HN5, HN30, HN31 and UM-SCC-1 cells were cultured in DMEM/F-12 (Mediatech) supplemented with 10% heat-inactivated (56°C, 30 min) FBS (Sigma) and 1% Pen-Strep (Gibco). NCI-H460 and NCI-H1299 cells were cultured in RPMI 1640 (Gibco) supplemented with 10% heat-inactivated FBS and 1% Pen-Strep. UM-SCC-47 cells were cultured in DMEM (Gibco) supplemented with 10% heat-inactivated FBS, 1% Pen-Strep, 2% MEM vitamins (Gibco), 1% sodium pyruvate (Lonza), and 1% nonessential amino acids (Gibco). FaDu, Detroit562, UPCI:SCC-152 and UPCI:SCC-154 were grown in MEM (Gibco) with 10% heat-inactivated FBS, 1% Pen-Strep and 1% sodium pyruvate. All cells were incubated at 37°C, 5% carbon dioxide.

Clonogenic Survival Assays

Clonogenicity was tested following radiation using an X-RAD 320 biological irradiator (Precision X-Ray) as previously described⁸. Briefly, single cells were plated into 6-well dishes and incubated overnight. The next day, the cells were irradiated and then returned to the incubator for 10-21 days until colonies formed. Colonies with more than 50 cells were counted. Survival curves were generated by extrapolation from radiation surviving fractions using alpha/beta analysis with GraphPad Prism. Each experiment was plated in triplicate and repeated at least three independent times. Error bars represent standard error.

Antibodies and reagents

USP7, RNF168, ARF-BP1 (HUWE1) and TRIP12 antibodies were purchased from Abcam, p16 antibody from BD Biosciences, BRCA1, alpha tubulin and HA from Santa Cruz, K48 and K63-linked ubiquitin and Aurora Kinase A from Cell Signaling Technology, and Actin from Millipore. MG132 was purchased from Cell Signaling Technology, and cells were treated with doses ranging from 5-10 μ M for 5-12 h. Cycloheximide was purchased from Sigma-Aldrich, and cells were treated with 300 μ g/ml for the times indicated. GNE-6640 was purchased from Sigma-Aldrich, and cells were treated with doses ranging from

0.1 to 10 μ M for 6 to 48 h prior to irradiation and left on until collection or staining of cells. P5091 was purchased from Sigma-Aldrich, and cells were treated with doses ranging from 1-5 μ M for 1 h prior to and 18 h post irradiation.

Western blot analysis

Following treatment, cells were lysed with extraction buffer containing 20 mM HEPES, pH 7.9, 0.4 mM NaCl, 1 mM EDTA pH 8.0, and 1 mM EGTA pH 7.0, and XPert protease and phosphatase inhibitors were added at a 1:100 dilution (GenDepot) and then sonicated. Equal amounts of protein were loaded into 4-15% gradient polyacrylamide gels (Bio-Rad) and then transferred to PVDF membranes for 10 min at 25 V using a Trans-Blot Turbo (Bio-Rad). Membranes were incubated in 5% dry milk for 1 h and then incubated with primary antibody overnight at 4°C. Immunoblots were detected using horseradish peroxidase-conjugated secondary antibodies (GE) and ECL2 chemiluminescent substrate (Pierce). Densitometry was measured using ImageJ. Western blots for TRIP12 were always run on the same day the cells were collected due to the instability of TRIP12 protein.

Over-expression and shRNA lentiviral infection

pLenti puro HA-Ubiquitin was a gift from Melina Fan (Addgene plasmid # 74218). Stable overexpression of USP7, p16 or rfp pLOC Turbo lentiviral vector (Precision LentiORF, Dharmacon) or stable shRNA knockdown of USP7 or p16 GIPZ lentiviral shRNA (Dharmacon) or GIPZ nonsilencing lentiviral shRNA control were cotransfected with lentiviral particles DR.8 and VSVG in HEK-293T cells for 48 h using Fugene (Promega) transfection reagent. Media plus lentivirus was then filtered through a 0.45 micron PES syringe filter and added to cells. Polybrene (5 μ g/ml) was added, and the cells were transduced for 6 h. The transduction procedure was repeated for 2 consecutive days. Three days after initial transduction, stably expressing cells were selected with either 20 μ g/ml blasticidin (overexpression) or 2 μ g/ml puromycin (shRNA).

siRNA transfection

siRNA was transfected using Nucleofector II technology (Amaxa). Briefly, 1 million cells were resuspended in 100 μ l Reagent T (Lonza) and 200 nM siRNA (Dharmacon). Cells were electroporated with program T-001, plated in 6-well dishes containing complete media, and collected at the times indicated.

p16 CRISPR

A single colony of the LentiCRISPRv2 plasmid (Addgene) was expanded in LB broth containing 100 μ g/ml ampicillin, and plasmid DNA was isolated using the QIAfilter Plasmid Midi Kit (Qiagen). The plasmid was then linearized and dephosphorylated by BsmBI digestion and purified with a QIAquick Gel Extraction Kit (Qiagen). p16 guide RNA, sgCDKN2A CACCGTTCGGCTGACTGGCTGGCCA, and reverse complement, AAAGTGGCCAGCCAGTCAGCCGAAC (Sigma), were annealed by PCR. gRNA was ligated into the purified LentiCRISPRv2 plasmid and transformed into One shot Stbl3 Chemically Competent *E. coli* (Invitrogen). A single clone was then selected, propagated, and Sanger sequenced to

confirm the insert. The sg-p16 CRISPR plasmid was then cotransfected with DR.8 lentiviral particles and VSVG in HEK-293T cells for 48 h using Eugene (Promega) transfection reagent. Media containing lentivirus was then filtered through a 0.45 micron PES syringe filter and added to cells. Polybrene (5 µg/ml) was added, and the cells were transduced for 6 h. The transduction procedure was repeated for 2 consecutive days. Three days after initial transduction, stably expressing cells were selected with 2 µg/ml puromycin.

RT-PCR

Cells were collected and then lysed using a QIAshredder Kit (Qiagen). RNA extraction was performed using an RNeasy Kit (Qiagen), and RNA was quantified on a Take3 plate (BioTek) and read on an Epoch spectrophotometer (BioTek). Reverse transcription was performed using iScript Reverse Transcription Supermix (Bio-Rad) with 1 µg of total RNA/reaction. Fifty nanograms of cDNA template was mixed with primers and SsoAdvanced Universal SYBR Green Supermix (BioRad). PrimePCR primer sets for GAPDH, HUWE1, TRIP12 or USP7 were purchased from BioRad. Real-time PCR was run on a CFX Connect Real-Time PCR system (BioRad). Data was normalized to GAPDH.

Immunocytochemistry

Cells were plated directly on a coverslip and allowed to adhere with overnight incubation. The following day, the cells were irradiated and fixed with 4% paraformaldehyde for 10 minutes at room temperature. Cells were then washed with PBS and permeabilized with 70% ethanol overnight at 4°C and for 20 minutes with 0.1% Igepal at room temperature. Cells were washed, blocked with 2% bovine serum albumin for 1 h and incubated overnight with 1:1000 Aurora Kinase A antibody (Cell Signaling Technology). Centrosomes were visualized by 1 h incubation with a 1:500 AlexaFluor 594 fluorochrome (Invitrogen). Cells were then incubated with 1:500 alpha tubulin (Santa Cruz) for 1 h at room temperature. Mitotic spindles were visualized by 1 h incubation with 1:600 FITC (Jackson Immuno), and pictures were captured with a Leica microscope. DNA was stained with 1 µg/ml DAPI (Sigma).

BRCA1 foci were visualized by incubating 1:500 BRCA1 antibody overnight (Santa Cruz) and 1:600 FITC (Jackson Immuno) for 45 minutes at room temperature.

Micronuclei quantification

HN5 cells stably expressing control or shUSP7 constructs were irradiated with 6 Gy and then incubated in medium containing 664 nM nocodazole (Sigma-Aldrich) for 4 h. At the end of nocodazole treatment, mitotic cells were harvested by gentle shaking and replated on coverslips in media without nocodazole for 24 h. Cells were then fixed and stained as described above.

Immunoprecipitation

Following treatment, cells were lysed with extraction buffer containing 20 mM Tris-HCl, pH 7.8, 150 mM NaCl, 1 mM EDTA pH 8.0, and 1 mM EGTA pH 7.0, and XPert protease and phosphatase inhibitors

were added at a 1:100 dilution (GenDepot) and then sonicated. One milligram of cell lysate per sample was incubated with 5 µg of antibody of interest with rotation at 4°C overnight. Then, 30 µL of 100 mg/ml Protein-A Sepharose beads (GE Healthcare) were added to each sample and rotated at 4°C for 2 h. The beads were sedimented by centrifugation at 400 rcf, and the bead-bound samples were washed three times with 1 ml lysis buffer. The sample was eluted by heating the bead-bound sample with 25 µL 2X SDS Laemmli Sample Buffer (Bio-Rad) at 100°C for 7 min. After centrifugation, each sample was loaded into a 4-15% gradient polyacrylamide precast gel (Bio-Rad) and transferred to a PVDF membrane. The resulting sample was analyzed by immunoblot. Immunoprecipitations for TRIP12 were always run on the same day the cells were collected due to the instability of TRIP12 protein.

IP Mass Spectrometry

Following treatment, cells were lysed with extraction buffer containing 20 mM Tris-HCl, pH 7.8, 150 mM NaCl, 1 mM EDTA pH 8.0, and 1 mM EGTA pH 7.0, and XPert protease and phosphatase inhibitors were added at a 1:100 dilution (GenDepot) and then sonicated. Twenty-five milligrams of cell lysate per sample was incubated with 25 µg of antibody of interest with rotation at 4°C overnight. Then, 100 µL of 100 mg/ml Protein-A Sepharose beads (GE Healthcare) were added to each sample and rotated at 4°C for 2 h. The samples were sedimented by centrifugation, and the bead-bound samples were washed three times in lysis buffer. Beads were then sent to the MD Anderson Proteomics core for mass spectrometry analysis.

Xenograft Tumors

Mouse experiments were carried out in the specific pathogen-free mouse colony of the Department of Experimental Radiation Oncology at MD Anderson Cancer Center and were approved by the American Association for Accreditation of Laboratory Animal Care, in accordance with current regulations and standards of the U.S. Department of Agriculture and the Department of Health and Human Services. HN5 cells were transfected with USP7 shRNA using lentiviral vectors as described above. Two million cells suspended in 20 µL PBS were injected intramuscularly into the right hind leg of male Swiss Nu/Nu mice. When tumors reached 8 mm in diameter (range 7.7-8.3 mm), the animals were randomized into groups and treated with 4 Gy for 5 consecutive days using a 137 Cesium irradiator (dose rate 4 Gy/min). Mice were immobilized in a jig, and tumors were centered in a 3 cm diameter circular field for irradiation. The tumors were then measured every other day until they reached 14 mm in diameter. Animals were euthanized via CO₂ inhalation followed by cervical dislocation. Following euthanasia, the tumors were excised, and a portion of each was snap frozen and formalin fixed.

The time for tumors to reach 12 mm in diameter was used to determine the dose enhancement factor (DEF). The growth curves for the four conditions shown are approximately linear (coefficient of t^2 not significant for any of the curves), so the enhancement ratio for radiosensitization by USP7 was estimated as the ratio of growth delays between shUSP7 and controls. The calculations were carried out for three diameters (11, 12, and 13 mm) and either all times or times > 6 days (to assess the effect of small

nonlinearities at the start). A complication arises from the fact that the observations are not independent (the same tumors are measured at different times), so we applied so-called mixture models with random and fixed effects. Linear models where intercept and slope were considered random effects were used in a bootstrapping procedure where data were sampled randomly 100 times and estimates with 95% CIs were obtained from the 2.5- and 97.5-centile distributions. DEF at 11 mm shown in the figure, all calculated DEFs had a lower limit of the 95% CI greater than 1 by at least 0.5 arbitrary units.

Clinical data analysis

For expression analysis, HUWE1 mRNA expression was examined for all available patients from The Cancer Genome Atlas (TCGA) Head and Neck cohort for which HPV status was available (n=519). For outcome analysis, data for which HUWE1 mutation status and/or mRNA expression, HPV status and disease-free status were available (n=392). Clinical characteristics (see Supplemental Table 1), outcomes and biologic data were accessed via cBioPortal. Disease-free survival (DFS) was analyzed using Cox regression analysis. Kaplan-Meier survival curves are shown with log rank statistics used to compare groups for statistical significance.

Results

Radiosensitivity in HNSCCs correlates with TRIP12 and USP7 expression and is HPV status dependent

To examine the mechanism of HPV-mediated radiosensitization, we utilized a panel of HNSCC HPV(+) and HPV(-) cell lines. To confirm previously published results, we examined the surviving fraction after 2 Gy of irradiation (SF2) for our panel and found that the HPV(+) cell lines (UMSCC-47, UPCI:SCC152, UPCI:SCC154) were significantly more sensitive to radiation than the HPV(-) (Detroit562, UMSCC-1, HN5, FaDu, HN30, HN31) lines (Fig. 1A). As expected, the HPV(-) cell lines expressed negligible levels of p16, the surrogate marker of HPV infection (Fig. 1B). In agreement with our recently published report, p16 protein levels were inversely correlated with TRIP12 protein levels (Fig. 1B). Because we previously demonstrated that p16 regulates TRIP12 in a posttranslational fashion⁸, we examined the deubiquitinase ubiquitin-specific protease 7 (USP7), which has been shown to bind to TRIP12¹⁹. The immunoblot of USP7 from our cell line panel demonstrated a USP7 protein expression pattern proportional to that of TRIP12 (Fig. 1B). Additionally, densitometric analysis showed that both USP7 and TRIP12 protein expression was highly correlated with radioresistance (Fig. 1C).

USP7 is downregulated by p16 through ubiquitination

To understand whether the protein level correlation was a direct consequence of p16 induction, we examined the impact of direct p16 modulation on USP7 levels. The protein levels of USP7 decreased following forced expression of p16 in HPV(-) HN5 and HN31 cells (Fig. 2A). Moreover, forced expression of p16 in a panel including both HNSCC and non-small cell lung carcinoma (NSCLC) cell lines led to an approximately 50% reduction in USP7 protein expression (Fig. 2B). This panel included p53 wild-type H460 and HN30 cells, which is suggestive of a p53-independent mechanism across multiple cell types.

The converse was observed when p16 expression was inhibited via CRISPR in HPV(+) UM-SCC-47 cells, with USP7 levels increasing following p16 KO (Fig. 2C).

Next, we sought to determine the mechanism by which p16 regulates USP7. We found that forced p16 expression in HPV(-) HN5, HN30 and HN31 cells had no effect on USP7 mRNA (Supplemental Fig. 1A) despite the reduction seen at the protein level (Fig. 2A & B); this led us to suspect that the USP7 decrease may occur through posttranslational modification. To test this hypothesis, we performed cycloheximide chase assays to determine the effect of p16 expression on the stability of USP7. The presence of p16 significantly destabilized USP7 protein in p53 mutant HN5 (Fig. 2D) and p53 wild-type HN30 (Supplemental Fig. 1B). To determine whether this destabilization occurred through ubiquitination of USP7, we tested whether the addition of the proteasome inhibitor MG132 could rescue USP7 expression following forced expression of p16. We found that MG132 was able to at least partially rescue the p16-induced reduction in USP7 protein expression in all three lines tested (Fig. 2E & Supplemental Fig. 1C), which indicated that the mechanism depended on ubiquitination of USP7. To confirm the role of ubiquitin in the destabilization of USP7 by p16, HN5 cells were cotransfected with control or p16 and lenti-HA-ubiquitin expression vectors. Cells were then either immunoprecipitated (IP) with HA-tagged ubiquitin and immunoblotted for USP7 or the reverse (Fig. 2F), both of which showed an increase in ubiquitination of USP7 in the presence of p16 expression. Furthermore, we showed by IP that the ubiquitination of USP7 was K48-linked and not K63-linked (Fig. 2G). Given that K-48-linked ubiquitination is generally associated with degradation, this suggests that the p16-dependent ubiquitination indeed marks the USP7 protein for degradation²⁰.

USP7 stabilizes TRIP12 through deubiquitination

Prior work suggested that USP7 binds to TRIP12; however, data are conflicting as to whether this interaction serves to regulate TRIP12 or USP7²¹. Therefore, we directly asked whether USP7 was responsible for the observed impact of p16 on TRIP12. We reasoned that if USP7 was the link between p16 and TRIP12, modulating USP7 could abrogate the effect of p16 on TRIP12 expression. First, we confirmed that USP7 indeed bound to TRIP12 in HN5 cells by immunoprecipitating TRIP12 and immunoblotting for USP7 as well as the reverse (Fig. 3A). In addition, direct targeting of USP7 via shRNA resulted in significant depression of TRIP12 protein levels in HPV(-) HN5, HN30 and HN31 cells (Fig. 3B). Inhibition of TRIP12 had no effect on USP7 expression (Supplemental Fig. 2A), indicating that USP7 likely regulates TRIP12 in this model and not the converse. The regulation of TRIP12 by USP7 was confirmed in HPV(+) UM-SCC-47 and SCC-154 cells, where forced expression of USP7 led to significant upregulation of TRIP12 (Fig. 3C), despite the presence of p16, suggesting that p16 regulates TRIP12 indirectly through USP7. While inhibition of USP7 reduced TRIP12 protein expression, it did not reduce TRIP12 gene expression (Supplemental Fig. 2B), providing further evidence that TRIP12 regulation occurs through posttranslational modification. Cycloheximide chase assays in p53 mutant HN5 and p53 wild-type HN30 cells both showed a reduced half-life of TRIP12 after USP7 knockdown (Fig. 3D-E), which supports a p53-independent mechanism of TRIP12 stabilization by USP7. Treatment with MG132 at least partially reversed the reduction in TRIP12 protein expression induced by USP7 knockout in HN5, HN30 and HN31

cells (Fig. 3F and Supplemental Fig. 2C), which indicated that USP7 stabilized TRIP12 by deubiquitination. This mechanism of TRIP12 stabilization by USP7 was confirmed by co-IP with HA-tagged ubiquitin. This experiment showed that forced expression of p16 caused increased ubiquitination of TRIP12, which was nearly abolished upon coexpression with USP7 (Fig. 3G), confirming that the mechanism was indeed through deubiquitination by USP7.

USP7 plays an integral role in p16-induced radiosensitivity

As the p16-USP7-TRIP12 axis has not been reported thus far, especially in the context of the radiation response, we tested the effects of modulation of USP7 in combination with radiation both *in vitro* and *in vivo*. Knockdown of USP7 with shRNA sensitized HN5 cells to radiation (Fig. 4A) and reduced BRCA1 expression (Fig. 4B). Immunocytochemical (ICC) analysis of HN5 cells showed that targeting USP7 decreased the formation of BRCA1 foci following radiation exposure, suggesting that in the absence of USP7, the repair of radiation-induced DNA damage was compromised (Fig. 4C). This reduction in BRCA1 foci seen after inhibition of USP7 causes the cells to progress into mitosis with unrepaired DNA damage leading to aberrant mitosis, including increased centrosomes and micronuclei per cell, markers of mitotic death (Fig 4D-F).

To further characterize USP7 in the context of radiation, we overexpressed p16 in HN5 cells, which resulted in significantly enhanced radiosensitivity (Fig. 4G) comparable to our previous results⁸, as well as marked downregulation of USP7, TRIP12, and BRCA1 (Fig. 4H). Forced expression of both p16 and USP7, on the other hand, partially reversed p16-induced radiosensitization (Fig. 4G) and p16-induced downregulation of both TRIP12 and BRCA1 (Fig. 4H), demonstrating that loss of USP7 is a factor in conferring radiosensitivity in HPV(+) tumors.

To examine the role USP7 plays in the HPV(-) tumor response to radiation, we performed a tumor growth delay assay with HN5 xenografts in nude mice. Prior to inoculation, the cells were either infected with control shRNA or USP7 shRNA to mimic USP7 deficiency in HPV(+) tumors. The animals were treated with 4 Gy for 5 consecutive days, and tumor diameters were measured every two days. At the end of the study, tumors were excised and analyzed by western blot, which confirmed shRNA knockdown of USP7 and showed reduced BRCA1 expression consistent with prior *in vitro* results (Supplemental Fig. 3). Moreover, we found that inhibition of USP7 led to radiosensitization of HN5 tumors (Fig. 4I), further supporting that targeting USP7 could be a viable radiosensitization strategy for HPV(-) head and neck tumors.

USP7 is a druggable target for increasing the radiosensitivity of HPV(-) HNSCC

It is necessary to find druggable targets that can be utilized for sensitization of HPV(-) radioresistant tumors. Several USP7 inhibitors have been developed and are available for research purposes, with one, P5091, currently in clinical trials. Here, we tested three USP7 inhibitors: P22077, P5091 and GNE-6640. P22077 and P5091 inhibit USP7 but also USP10 and USP47^{22,23}. GNE-6640 is a more selective inhibitor

of USP7 that inhibits the deubiquitinase activity of USP7 with selectivity over a highly structurally similar deubiquitinase (USP47) and a highly active deubiquitinase (USP5)²⁴.

Modulating USP7 activity via the chemical inhibitor GNE-6640 resulted in decreased TRIP12 expression (Fig. 5A-B) and increased radiosensitivity in HPV(-) HN5 cells (Fig. 5C). Furthermore, radiosensitization by GNE-6640 was only achieved when dose and duration were sufficient to reduce TRIP12 expression and BRCA1 foci formation. TRIP12 expression was only reduced with greater than 6 hours of treatment (Fig. 5A) and at doses greater than 1 μ M (Fig. 5B), which explains the lack of radiosensitization seen at 1 or 10 μ M with 6 hours of treatment prior to irradiation (Supplemental Fig. 4A) or at 1 μ M with 48 hours of pretreatment (Fig. 5C). Similarly, BRCA1 foci were only reduced after treatment with 10 μ M GNE-6640 for 48 hours prior to radiation (Fig. 5D) and not at any of the other doses or schedules tested (Supplemental Fig. 4B). Additionally, similar radiosensitization was achieved using P5091 in HN5 and FaDu cells (Supplemental Fig. 4C-D) as well as with P22077 in UMSCC25 and FaDu cells at various doses and schedules (data not shown). These data suggest that targeting USP7 could partially recapitulate favorable HPV-induced radiosensitivity in head and neck cancer.

HUWE1 is identified as an E3 ligase for USP7 and is transcriptionally regulated by p16, potentially via SP1

While we determined that USP7 linked p16 and TRIP12 and that USP7 appeared to be regulated via ubiquitination, it remained unclear how p16 modulated this cellular cascade. We performed IP mass spectrometry (IP/MS) in 3 HPV(+)/p16(+) and 3 HPV(-)/p16(-) cell lines to discover binding partners of USP7. From this analysis (schema in Fig. 6A), we identified three E3 ubiquitin ligases (HUWE1, TRIM21 and RNF168) binding USP7 in all HPV(-) cell lines tested (Fig. 6B). For two of the HPV(+) cell lines, SCC-152 and SCC-154, USP7 had to be overexpressed prior to IP/MS due to insufficient levels of endogenous USP7. The HPV(+) cell lines also had a similar set of binding partners with HUWE1 and TRIM21 binding USP7 in all 3 cell lines and RNF168 in one of the three (Fig. 6B). IP/MS evaluating proteins bound to HUWE1 confirmed its binding with USP7 in all cell types examined (Fig. 6C).

To further explore the relationship of these USP7 binding partners, we performed western blots for HUWE1, TRIM21, USP7, TRIP12 and p16 in all six cell lines. Basal expression of HUWE1 showed an inverse relationship to USP7 and TRIP12 and correlated with p16/HPV positivity, suggesting that it could be an E3 ubiquitin ligase limiting USP7 expression, while TRIM21 levels trended towards a proportional correlation to USP7, though this effect was not consistent across all cell lines (Fig. 7A).

When examining the relative gene expression of HUWE1 and TRIM21 in the 6 cell lines, we found that HUWE1 gene expression also correlated with p16/HPV positivity, indicating that HUWE1 is likely transcriptionally regulated by p16 (Fig. 7B). On the other hand, the gene expression of TRIM21 was comparable to its basal protein expression levels and did not show the predicted inverse correlation (Fig. 7C). To investigate the implied transcriptional regulation of HUWE1 by p16, we expressed p16 in HN5 cells, which led to an increase in both HUWE1 protein and mRNA levels (Fig. 7D-E), confirming that p16 regulates HUWE1 at the transcriptional level. Conversely, inhibition of p16 in HPV(+)/p16(+) SCC154 cells

led to a decrease in HUWE1 gene expression (Fig. 7F), with a similar pattern observed in UMSCC47 cells (data not shown).

To further characterize this newly discovered p16-USP7-TRIP12 pathway, HN5 cells with forced expression of p16 were cotransfected with HUWE1, TRIM21, TRIP12 or USP7 siRNA. Western blots showed that HUWE1 expression increased with forced p16 expression (Fig. 7G), which suggested that p16 may be responsible for its transcriptional upregulation. TRIM21 did not appear to be affected by p16 expression (Fig. 7G). Most importantly, p16-induced downregulation of USP7 was rescued by cotransfection of HUWE1 siRNA, confirming HUWE1 as an E3 ligase for USP7 (Fig. 7G). In addition, TRIP12 levels correlated with those of USP7, further confirming USP7 as a deubiquitinase for TRIP12 (Fig. 7G). Stable expression of shRNA to HUWE1 reversed the repression of USP7 and TRIP12 in response to p16 (Fig. 7H). Moreover, in SCC-154 HPV(+)/p16(+) cells, inhibition of HUWE1 led to an increase in USP7 protein levels (Fig. 7I). Additionally, forced expression of p16 in HN5 cells led to ubiquitination of USP7, which was rescued by cotransfection with HUWE1 shRNA, thus confirming that p16 regulates USP7 through its ubiquitination by HUWE1 (Fig. 7J).

To understand how p16 controls HUWE1 transcription, we examined the HUWE1 promoter region, which contains binding sites for multiple enhancers and promoters, including specificity protein 1 (SP1). SP1 is a transcription factor and has been found to bind to p16, leading to increased transcriptional activity of the target gene but not total expression level²⁵. To determine whether SP1 is the mediator of p16-driven upregulation of HUWE1, we inhibited SP1 expression in either HPV(-) cells forced to express p16 (Fig. 7K) or HPV(+)/p16(+) cells (Fig. 7L). In HN5 p16+ cells, HUWE1 levels increased as expected upon p16 expression, but this increase was completely reversed by SP1 inhibition (Fig. 7K). We recorded a similar reduction in HUWE1 expression in HPV(+) cells in which SP1 was inhibited (Fig. 7L). Thus, our results suggest that SP1 transcriptional activity is responsible for p16-driven HUWE1 upregulation.

HUWE1 expression is associated with disease-free survival in HPV(-) HNSCC

HUWE1 is mutated in approximately 10% of HNSCC. Based on this observation as well as the pathway we identified in our investigation, we examined the association between HUWE1 and outcome in the TCGA HNSCC patient cohort. We initially examined HUWE1 expression and found it to be elevated in HPV(+) tumors and markedly reduced in the few tumors with truncating HUWE1 mutations (Supplemental Figure 5). In HPV(-) patients, higher HUWE1 expression as a continuous variable was associated with improved DFS in univariate analysis ($p=0.018$) and remained significant in multivariate analysis, including tumor site and clinical stage ($p=0.016$). When divided into groups by HUWE1 expression (upper tertile vs. others), higher HUWE1 expression was associated with improved disease-free survival (DFS) ($p=0.048$) (Fig. 8A). Additionally, truncating mutations in HUWE1 led to a median survival of 9.4 months compared to 67.7 months in the remaining patients ($p=0.008$) (Fig. 8C). Interestingly, in HPV(+) patients, neither HUWE1 mutation nor its gene expression was associated with survival (Fig. 8B & D).

Discussion

The significantly increased radiosensitivity and better overall outcomes for patients with HPV-positive HNSCC suggest that the molecular mechanism by which HPV transforms the cells also confers inherent sensitivity to DNA damage. Deciphering this molecular mechanism allows us an opportunity to mimic this pathway in HPV-negative tumors and potentially achieve radiosensitization for patients with resistant cancers with pharmacological intervention. The first step in this mechanism was recently published and revealed a central role for the E3 ubiquitin ligase TRIP12⁸. TRIP12 binds to RNF168 and in turn prevents excessive spreading of 53BP1 foci¹⁸. Expression of p16, either as a consequence of HPV infection or forced expression, downregulated TRIP12 protein by promoting its ubiquitin-dependent proteasomal degradation. It became clear that p16 regulates TRIP12 through either activation of a ubiquitin ligase or inhibition of a deubiquitinase. In this work, we present clear evidence that p16 regulates TRIP12 and thus the repair of DNA damage through the regulation of USP7, a deubiquitinase that binds to the TRIP12-RNF168 complex and a number of other DNA repair proteins^{21,26}. This is the first report of a direct signaling relationship between p16 and USP7, further elucidating the mechanism by which p16 inhibits DNA repair and the tumor cell response to DNA damage.

The main goal of these studies is to identify druggable targets in the p16-mediated radiosensitization pathway that can pharmacologically imitate the HPV(+) tumor response to DNA-damaging treatments. TRIP12, while a crucial part of this mechanism, is not yet targetable; thus, to advance this goal, we needed to identify other upstream targets. Here, we present data that show that p16 regulates TRIP12 through USP7, and by using pharmacological inhibitors of USP7, we can emulate the p16-mediated radiation response in cell culture. Our initial USP7 inhibitor, P5091, is not specific to USP7 but additionally inhibits USP47 (ubiquitin-specific peptidase 47), which, among several functions, controls tumor cell proliferation^{27,28}. While P5091 is currently in clinical trials for multiple myeloma, it is unclear whether USP7 or a USP47-based mechanism is responsible for its antineoplastic activity. Thus, for clinical translation of our findings, it will be necessary to develop more specific inhibitors of USP7. To this end, we tested GNE-6640, a more specific inhibitor of USP7 deubiquitinase activity²⁴. Although not yet in clinical trials, our data support further exploration of GNE-6640 as a means of targeting USP7 to radiosensitize resistant head and neck tumors.

Our studies also identified HUWE1 as a direct link between p16 and USP7. HUWE1 is a HECT, UBA and WWE domain containing E3 ubiquitin protein ligase 1 that is involved in the stress response, proliferation, differentiation, apoptosis, and DNA repair. As an E3 ligase, HUWE1 marks proteins, including the DNA repair proteins BRCA1 and H2AX, with ubiquitin for degradation through the proteasome, thus decreasing the DNA repair capacity of both homologous recombination and nonhomologous end joining. In addition, HUWE1 has already been demonstrated to polyubiquitinate USP7 under hypoxic conditions²⁹. Because HUWE1 and USP7 were also discovered to bind in our cell lines in the IP/MS analysis, the E3 ubiquitin ligase HUWE1 may be antagonistic to USP7 and play an intermediary role in the p16/TRIP12 pathway and the inhibition of BRCA1 function. As we demonstrated in this report, knockdown of HUWE1 rescued p16-dependent downregulation of USP7 at the protein and gene levels, validating this prediction.

Finally, the E3 ligase HUWE1 was found to be possibly transcriptionally controlled by SP1/p16 binding, connecting p16 to USP7 protein expression (pathway in Figure 9). Previously, it has been shown that p16 can form a complex with SP1, facilitating the transcriptional activity of the latter^{25,30}. SP1 is one of several predicted transcription factors for HUWE1; however, our data are the first to our knowledge to show this regulation in any context, particularly in a p16-driven fashion.

Although our data do not rule out regulation of HUWE1 by p16 via other means, they do provide a direct link between p16 and HUWE1, rounding out a potentially complete pathway. Additionally, while we uncovered the roles of USP7 and HUWE1 in the p16-driven pathway responsible for regulating DNA damage through TRIP12, it is also possible that additional deubiquitinases control TRIP12 cellular levels and could act as intermediaries between p16 and TRIP12, leaving open possibilities for inherent or acquired therapeutic resistance.

In conclusion, we identified a signaling pathway directly connecting HPV positivity and p16 expression to ubiquitin-mediated DNA damage repair via transcriptional upregulation of HUWE1 followed by ubiquitin-dependent signaling to USP7 and TRIP12 (Figure 9). This pathway is both clinically important – as evidenced by the relationship between HUWE1 and clinical outcome – and targetable via inhibitors of USP7.

Declarations

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Figures

Figure 1

Figure 1

USP7 and TRIP12 expression are associated with radiation resistance and repressed in HPV(+)/p16(+) cells. A & B) Surviving fraction at 2 Gy (SF2) (A) and immunoblot (B) of representative HPV(-) and HPV(+) cells. C) Correlation between SF2, USP7 and TRIP12 protein expression.

Figure 2

Figure 2

p16 inhibits USP7 levels by increasing its ubiquitination. A & B) Forced expression of p16 represses USP7 expression in two HPV(-) HNSCCs following radiation (A) and at baseline (A & B) in multiple HNSCC and NSCLC cell types. C) CRISPR KO of p16 in an HPV(+) cell line (UM-SCC-47) leads to increased USP7 expression. D) Cyclohexamide chase assay in HN-5 (HPV(-)/p16(-)) cells expressing exogenous control vector or p16. E) MG132 rescue experiment in several HPV(-)/p16(-) cell types (HN5, HN30 and HN31)

following forced expression of p16. Data are densitometry from immunoblots shown in Supplemental Fig. 1C. F) HN5 cells expressing HA-tagged ubiquitin (Ub) and either control vector or p16 were analyzed via immunoprecipitation (IP) of HA and immunoblot of USP7 (left) or IP of USP7 and immunoblot of HA (right). G) HN5 cells expressing either control vector or p16 were analyzed via IP of USP7 followed by immunoblot for either K48- (left) or K63- (right) linked ubiquitin. * - $p < 0.05$ versus control; + - $p < 0.05$ vs. p16 forced expression.

Figure 3

Figure 3

USP7 stabilizes TRIP12 in HNSCC via deubiquitination. A) IP for the indicated proteins in HPV(-) HN5 cells followed by immunoblot for either USP7 (top) or TRIP12 (bottom). B & C) Immunoblot of USP7 and TRIP12 in representative HPV(-)/p16(-) cells stably expressing control or multiple shRNAs to USP7 (B) or representative HPV(+)/p16(+) cells expressing control vector or USP7 (C). D & E) Cyclohexamide chase experiments in HN5 (D) and HN30 (E) HPV(-)/p16(-) cells expressing control or shRNA for USP7 (shUSP7). F) MG132 rescue experiment in several HPV(-)/p16(-) cell types (HN5, HN30 and HN31) expressing control or shUSP7. Data are densitometry from immunoblots shown in Supplemental Fig. 2C. G) IP for TRIP12 and immunoblot for HA or TRIP12 in HN5 cells expressing Ub-tagged HA and either: control vector, p16 alone or USP7 and p16. * - $p < 0.05$ versus control; + - $p < 0.05$ vs. p16 forced expression.

Figure 4

Figure 4

USP7 modulates p16-induced radiosensitivity. A-C) Clonogenic survival assay (A), immunoblot (B) and BRCA1 foci (representative images below the compiled averages) (C) in HN5 cells stably expressing control or shUSP7 constructs. D) Representative images in the indicated groups (green=α-Tubulin, red=Aurora A kinase, blue=DAPI). E & F) Centrosomes/mitotic cells (E) and micronuclei/cells (F) in HN5 cells stably expressing control or shUSP7 constructs. G & H) Clonogenic survival assay (G) and

immunoblot (H) in HN5 cells stably expressing control vector, p16 or p16 and USP7. I) In vivo tumor growth delay following intramuscular injection of HN5 cells expressing control or shUSP7 and treatment with 4 Gy x 5 days (20 Gy total). * - $p < 0.05$ versus control; + - $p < 0.05$ vs. p16 forced expression.

Figure 5

Figure 5

USP7 is a druggable target for radiosensitizing HPV(-)/p16(-) HNSCC. A-D) Immunoblot (A & B), clonogenic survival (C) and BRCA1 foci formation (D) in HN5 cells treated with the USP7 inhibitor GNE-

6640. * - $p < 0.05$ versus control.

Figure 6

Figure 6

IP/MS identifies TRIM21 and HUWE1 as common E3 ubiquitin ligases for USP7. A) Basic schema for the experiment, with lysate incubated with either USP7 or HUWE1 antibody, followed by incubation with Protein-A Sepharose beads. The beads were then washed, centrifuged and analyzed via mass spectrometry. B & C) E3 ubiquitin ligases (dark blue) and deubiquitinases identified by IP/MS following IP

for either USP7 (B) or HUWE1 (C) cells are shown. Targets common to all cell lines examined are underlined in black.

Figure 7

Figure 7

p16 represses USP7 via transcriptional activation of HUWE1. A-C) Immunoblot (A) and RT-PCR for HUWE1 (B) and TRIM21 (C) in HNSCC cell lines with varying HPV/p16 statuses. D & E) Immunoblot (D) and RT-PCR for the indicated genes (E) in HN5 cells expressing control vector or p16. F) RT-PCR for the indicated

genes in UM-SCC-47 cells transfected with siRNA targeting p16. G & H) Immunoblot in HN5 cells expressing control vector or p16 and siRNA for the indicated genes (G) or stably expressed control or HUWE1 shRNA (shHUWE1) (H). I) Immunoblot in SCC-154 cells stably expressing control or shRNA to HUWE1. J) IP for USP7 and immunoblot for HA in HN5 cells expressing HA-tagged Ub and either: control vector, p16 or p16 and shHUWE1. K) Immunoblot in HN5 cells expressing either control vector, p16 or p16 and siRNA to SP1 (siSP1). L) Immunoblot in UM-SCC-47 cells expressing control or siSP1. * - $p < 0.05$ versus control for indicated gene.

Figure 8

Figure 8

HUWE1 is associated with disease-free survival in HPV(-) HNSCC. Patients from the Head and Neck TCGA cohort were divided into groups based upon HPV status, HUWE1 mRNA expression (uppermost tertile vs. lower two tertiles) and mutation status (truncating vs. other/wild type), and DFS was analyzed using log rank statistics.

Figure 9

Figure 9

Potential pathway linking p16 to DNA damage repair via ubiquitin signaling.

Supplementary Files

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