Enhanced Radiation Sensitivity in HPV-Positive Head and Neck Cancer

Randall J. Kimple1,3, Molly A. Smith1, Grace C. Blitzer1, Alexandra D. Torres1,2, Joshua A. Martin1,2, Robert Z. Yang1,2, Chimera R. Peet1, Laurel D. Lorenz2, Kwangok P. Nickel1, Aloysius J. Klingelhutz4, Paul F. Lambert2,3, and Paul M. Harari1,3

Abstract

Patients with human papillomavirus (HPV+)–associated head and neck cancer (HNC) show significantly improved survival outcome compared with those with HPV-negative (HPV−) tumors. Published data examining this difference offers conflicting results to date. We systematically investigated the radiation sensitivity of all available validated HPV+ HNC cell lines and a series of HPV− HNC cell lines using in vitro and in vivo techniques. HPV+ HNCs exhibited greater intrinsic radiation sensitivity (average SF2 HPV+: 0.59 vs. HPV−: 0.22; P < 0.0001), corresponding with a prolonged G2–M cell-cycle arrest and increased apoptosis following radiation exposure (percent change 0% vs. 83%; P = 0.002). A genome-wide microarray was used to compare gene expression 24 hours following radiation between HPV+ and HPV− cells. Multiple genes in TP53 pathway were upregulated in HPV+ cells (Z score 4.90), including a 4.6-fold increase in TP53 (P < 0.0001). Using immortalized human tonsillar epithelial (HTE) cells, increased radiation sensitivity was seen in cell expressing HPV-16 E6 despite the effect of E6 to degrade p53. This suggested that low levels of normally functioning p53 in HPV+ HNC cell lines could be activated by radiation, leading to cell death. Consistent with this, more complete knockdown of TP53 by siRNA resulted in radiation resistance. These results provide clear evidence, and a supporting mechanism, for increased radiation sensitivity in HPV+ HNC relative to HPV− HNC. This issue is under active investigation in a series of clinical trials attempting to de-escalate radiation (and chemotherapy) in selected patients with HPV+ HNC in light of their favorable overall survival outcome. Cancer Res; 73(15); 1–10. ©2013 AACR.

Introduction

Human papillomavirus (HPV) plays a central etiologic role in an expanding subset of patients with head and neck cancer (HNC; refs. 1, 2). Clinical reports provide clear evidence of improved outcome in patients with HPV-positive (HPV+) HNC versus HPV-negative (HPV−) tumors (3, 4). This result that has been postulated to reflect increased radiation sensitivity in HPV+ tumors (5). However, published results for the few HPV+ cancer cell lines investigated to date are conflicting with some data, suggesting enhanced sensitivity to radiation and some suggesting reduced sensitivity or no effect (6–9). The strength of these data are somewhat limited due to the model systems used: p53-mutated cells, nonhead and neck cancer cell lines, and absence of rigorous validation of HPV status. Clarification of this discrepancy is important to clinical investigators as they consider future clinical trials that incorporate radiation dose reduction and seek underlying mechanisms to explain the markedly improved survival outcomes observed in patients with HPV+ HNC.

The classical HPV− HNC, related to more traditional risk factors such as tobacco and/or alcohol exposure, is driven by a series of mutations in tumor suppressor genes and proto-oncogenes. While many of these mutations occur randomly, recent next-generation sequencing approaches have identified mutations in common genes or their resultant protein pathways including TP53, NOTCH, PIK3CA, and CDKN2A (10–12). In fact, it is estimated that approximately 80% of HPV− HNCs harbor mutations in TP53, resulting in impaired or absent function of its encoded protein p53 (10–12). Alternatively, during the development of HPV+ HNC, an initial viral infection results in expression of virally encoded oncogenes that can have dramatic effects on normal host cellular function. Canonically, the HPV proteins E6 and E7 downregulate the tumor suppressor proteins p53 and Rb, respectively, although their true interactome encompasses a wide variety of cellular targets (13). Recent work has shown that the E6 and E7 proteins act synergistically to induce HNC in a transgenic mouse model system and that additional targets (e.g., p107 and p130) besides Rb are targeted by E7 (14–16). This coordinated perturbation of 2 critical tumor
suppressor pathways results in uncontrolled growth and prolifera-
tion although with a mutational landscape that is signifi-
cantly restricted when compared with HPV− HNC (10–12).

Standard therapy for patients with locally advanced HNC
commonly involves the combination of radiation and cisplatin
chemotherapy. Surgery is often incorporated either in the
initial management or as salvage following definitive radio-
chemotherapy (reviewed in ref. 17). In 2013, despite the signi-
cfic differences in underlying biology and ultimate outcome,
there are no validated differences in treatment approach
based on HPV status, beyond the context of clinical trials. In
addition, there is little preclinical data to support a given treat-
ment approach owing, at least in part, to the lack of preclinical
model systems of HPV+ HNC.

During the last 2 years, we have systematically investigated
the therapeutic sensitivity of a panel of validated HPV+ and
HPV− HNC cell lines, in an effort to generate preclinical data
to support etiology-specific treatment approaches. As consistent
differences in radiation sensitivity between HPV+ and HPV−
cells were identified, we sought to further investigate mechan-
isms underlying these altered responses and to validate the
findings in an in vivo model system.

Materials and Methods

Cell lines and culture conditions

Head and neck cancer lines derived from HPV+ patients:
UM-SCC1, UM-SCC6, UM-SCC22B, and SCC-1483 and from
HPV+ patients: UD-SCC2, UM-SCC47, UPCI-SCC90, and 93-
VU-147T were obtained from indicated sources (Supplemen-
tary Table S1). Standard culture conditions were used (Supple-
mental Table S1). The identity of all cell lines was confirmed via
short- tandem repeat testing within 6 months of cell use.

Immortalized human tonsillar epithelial (HTE) cells were
generated by cotransduction of primary HTE cells with a
pBABE-Hygro-TERT retroviral vector (a gift from Dr. Robert
Weinberg) and a shRNA-p16-Puro-MSCV retroviral vector (a
gift from Dr. Scott Lowe) using transduction techniques as
previously described (18). HTE cells stably carrying LXSN
were cultured in keratinocyte serum-free media (Cat #
17005042, Invitrogen) supplemented with 0.16 ng/mL EGF and
25 μg/mL Bovine Pituitary Extract at 37°C.

Validation of HPV

Southern blot was conducted using 10 μg of BamHI digested
total cellular DNA. DNA was separated on a 1.25% agarose gel,
transferred to Hybond N+ nylon membrane (Amersham) and
crosslinked. DNA probes were made by 5’ end-labeling 10 pmoles of HPV16-specific oligonucleotides (Supplemen-
tary Table S2) in the presence of T4 polynucleotide kinase (New
England Biolabs Inc.) with [γ-32P] ATP (6,000 Ci/mmole) at 37°C
for 1.5 hours. The membrane was prehybridized with Church
hybridization buffer for 15 minutes at 52°C followed by probe
hybridization for 18 hours at 52°C in a hybridization oven.
Membrane was washed with Church wash buffer, exposed to a
storage phosphor screen and scanned using a Typhoon 8610
imaging system (Amersham).

Quantitative reverse-transcriptase PCR (qRT-PCR) was con-
ducted to confirm transcription of HPV-16 E5, E6, and E7 on a
BioRad CFX96 using primers and probes (Supplementary Table S2) purchased from Integrated DNA Technologies, Inc.. Briefly, total RNA was harvested using the miRNeasy with
RNeasy MiniElute Cleanup Kit (Cat# 271004 and 74204, Qiagen)
from confluent plates of both HPV+ and HPV− cell lines.
cDNA was synthesized using the iScript Reverse Transcription
Supermix Kit (Bio-Rad Laboratories) and 1,000 ng of total
RNA. qRT-PCR was conducted by using IQ Multiplex Power-
mix with 10 ng cDNA per 10 μL reaction. GAPDH, HPV-16 E5,
E6, E7, and TP53 transcripts were detected using primers and
probes (Supplementary Table S2) purchased from Integrated
DNA Technologies, Inc.. The thermocycler was programmed
for an initial 95°C for 7 minutes followed by 40 cycles of 94°C
for 15 seconds and 60°C for 30 seconds.

Clonogenic survival assays

Clonal survival of cells following radiation was conducted as
previously described using a JL Shepherd 137Cs irradiator (JL
Shepherd) delivering a dose rate of approximately 400 cGy/
minute (19). After 10 to 15 days, colonies containing more than
50 cells were counted, the surviving fraction was calculated,
and clonogenic survival curves were fit to a linear-quadratic
model, as previously described (19). Clonogenic survival curves
were compared using the extra sum-of-squares F test in
GraphPad Prism version 5.01 (GraphPad Software). Each
point represents the mean surviving fraction calculated from
3 independent experiments done in triplicate for each treat-
ment condition; error bars represent the SD.

Cell-cycle analysis

Untreated and irradiated cells (4 Gy) were harvested by
trypsinization, washed with ice cold PBS, and fixed with 95% 
ethanol overnight at −20°C before DNA analysis. Following
removal of ethanol by centrifugation, cells were incubated with
Pl Master Mix (40 μg/mL propidium iodide and 100 μg/mL RNAse
in PBS) at 37°C for 30 minutes before analysis by FACSCalibur
flow cytometry. Stained nuclei were analyzed for DNA/propidium iodide fluorescence and resulting
DNA distributions were analyzed using Modfit (Verity Software
House, Inc.). Percent change from baseline (unirradiated) is
graphed from samples (n = 3) collected 4, 24, and 48 hours
after radiation.

Immunoblot analysis

Following treatment, cells were lysed with Tween-20 lysis
buffer (50 mmol/L HEPES pH 7.4, 150 mmol/L NaCl, 0.1% Tween-20, 10% glycerol, 1 mmol/L EDTA, 1 mmol/L DTT, 1
mmol/L phenylmethylsulfonylfluoride, and 10 μg/mL leupept-
inc and aprotinin) and were sonicated. Equal amounts of
protein were analyzed by SDS-PAGE (25–75 μg depending
upon target), and then the proteins were transferred to poly-
vinylidene difluoride (PVDF) membranes, were analyzed by
specific primary antibodies, and were detected via incubation
with horseradish peroxidase-conjugated secondary antibodies
using ECL chemiluminescence detection. Antibodies and
sources are listed in Supplementary Table S3.
Apoptosis
Two distinct assays were used to assess apoptosis. A luminescent DEVD cleavage assay for caspase 3/7 activity (ApoTox Triplex Assay, Promega) was conducted in 96-well format. Briefly, cells (2,000/well) were plated in 100 μL media. The plates were irradiated (4 Gy) or mock treated and apoptosis was measured 24, 48, and 72 hours after irradiation. Caspase activity was monitored according to the manufacturer’s directions and normalized to the total number of cells. Baseline activity was subtracted from each time point and the percentage increase over baseline was plotted. All experiments were repeated 3 times and graphs represent the mean of 6 individual replicates per experiment. In addition, apoptosis was detected by flow cytometry via the examination of altered plasma membrane phospholipid packing by lipophilic, fluorescein isothiocyanate (FITC) conjugated Annexin V (Cat# 556547 BD Biosciences) according to manufacturer directions. Both early (Annexin V positive, propidium iodide negative) and late (both positive) cells as well as live (both negative) cells were detected by FACS Calibur flow cytometry and analyzed by FlowJo v9.4.3 (Tree Star, Inc.).

Global gene expression analysis
Global gene expression analysis of HPV+ and HPV– cell lines was conducted using Affymetrix Gene 1.1 ST Array Plate technology (Affymetrix). Briefly, cell lines (n = 8) were harvested as biologic triplicates after mock therapy or 24 hours after a single 4 Gy dose of radiation. Total RNA from each cell line was prepared using Qiagen RNeasy, RNA quality was assessed by bioanalyzer and only samples with a RNA Integrity Number (RIN) more than 9.0 were used. Samples were processed by the University of North Carolina Expression Profiling and SNP Genotyping Core Facility. Normalized data were imported into GeneSifter (Geospiza, Inc.). A pairwise comparison between HPV+ and HPV– cells 24 hours after radiation with the fold change threshold set to 1.5 was conducted. Differences between HPV+ and HPV– cells were viewed by Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and compared by t test with Bonferroni correction.

siRNA knockdown of TP53
TP53-specific siRNA, including scrambled control, was purchased from Invitrogen (Silencer Select siRNA, Cat #4390825), transfected into cell lines using Lipofectamine RNAiMax reagent (Invitrogen). Knockdown of p53 was confirmed by Western blot. Transfected cells were used 48 hours after transfection.

Xenografts
Four to 5-week-old Hsd athymic Nude-Foxn1tm1tm1 female mice were purchased from Harlan Laboratories, housed in filter-topped cages in an aseptic environment, and maintained per defined protocol approved by and in accordance with the University of Wisconsin Animal Care and Use Committee. To establish xenografts, cells (1–2 × 10^6 mixed 1:1 with Matrigel (BD Biosciences) were injected subcutaneously into bilateral flanks. When tumors reached approximately 200 mm³, mice (n = 12/group) were randomly assigned to either mock radiation, or 8 Gy delivered in four 2 Gy fractions delivered over 2 consecutive weeks (Supplementary Materials and Methods). Radiation was delivered using an X-rad 320 biological irradiator (Precision X-ray, Inc.) using a dose rate of approximately 0.6 Gy/minute with customized lead immobilization jigs to shield the majority of the mouse body while leaving the tumor exposed. Tumors were measured, growth curves were generated, and comparisons were done using the extra sum-of-squares f test, as previously described (19). Time to tumor quadrupling was calculated from the first day of treatment, graphed according to the method of Kaplan and Meier, and compared using the log-rank (Mantel-Cox) test using GraphPad Prism.

Results
Validation of HPV status
Following receipt of several HNC cell lines reported to contain HPV that showed no detectable HPV DNA, we were concerned about a potential divergent cell population. Thus, we conducted single-cell isolation and clonal selection of all HPV+ HNC cell lines by plating less than 1 cell/well, visually confirming the presence of single-small colonies with subsequent expansion to a uniform population of cells. All experiments herein used these clonally isolated cellular populations.

Southern blot was used to confirm the presence of HPV DNA in the HPV+ cell lines (UD-SCC2-C6, UM-SCC47-C3, UPCI-SCC90-C35, and 93-VU-147-T-C5) and the absence of HPV in the HPV– cell lines (Fig. 1A). Differences in the restriction digest pattern result from different patterns of integration and was confirmed to match that previously published (20–22). The UD-SCC2 cell line had no known Southern blot published, but karyotype analysis confirmed a population distribution (data not shown) similar to that previously described (23). In addition, qRT-PCR was used to confirm the presence or absence of HPV16 E6 and HPV16 E7 gene expression in HPV+ and HPV– cells, respectively (Fig. 1B).

Increased radiation sensitivity in HPV+ HNC
We used our panel of eight HNC cell lines to investigate radiation sensitivity using clonogenic survival assays. A wide range of sensitivity was seen across cell lines with the surviving fraction after 2 Gy (SF2) ranging from 0.11 to 0.73 (Fig. 1C). HPV+ cell lines showed a significantly greater sensitivity to radiation as shown by average SF2 (HPV+: 0.59 vs. HPV–: 0.22; P < 0.0001).

Altered cell-cycle arrest in HPV+ HNC
The HPV oncoproteins E6 and E7 can have profound effects on cell-cycle regulation (24). To examine whether baseline cell-cycle distribution might contribute to the increased radiation sensitivity observed in HPV+ HNC cells, we used propidium iodide staining to assess baseline and postradiation cell-cycle distribution. At baseline, no difference in cell-cycle distribution was seen between HPV+ and HPV– cells (Fig. 2A, inset; P = ns for G1, G2, and S).

Interestingly, significant differences between HPV+ and HPV– cells were seen in their cell-cycle distribution in response...
Cyclin E, a marker of G1–S remained relatively unchanged in HPV+ cell lines at these same time points.

**Increased apoptosis in HPV+ HNC**

Clinically, patients with HPV+ HNC are reported to show earlier tumor response than those with HPV− HNC (25). This kinetic profile has been associated with apoptotic tumor responses in other types of cancers (26). A caspase activity assay was used to assess apoptosis in HPV+ and HPV− cell lines treated by radiation. While HPV− cells showed only slight increases in apoptosis, HPV+ cells showed robust induction of apoptosis (Fig. 3A). On average, 24 hours after a single 4 Gy dose of radiation HPV− cells showed no increased in caspase activity, whereas HPV+ cells showed an 85% increase ($P = 0.0002$). Apoptosis was also assessed by Annexin V labeling and flow cytometry in HPV+ cells. A significant increase in the percentage of cells positive for Annexin V was seen in 3 of 4
HPV+ cell lines (Fig. 3B). The single cell line with little increase in Annexin V staining was 93-VU-147T. This cell line showed the lowest radiation sensitivity (Fig. 1), and relatively small increases in G2 fraction (Fig. 2A), and small increases in caspase activity (Fig. 3A) following radiation. Interestingly, although it has been reported to harbor wild-type p53 (27), in our hands this cell line was found to have a heterozygous mutation in p53 (c.770T>C; p.L257R, data not shown). All other HPV+ cell lines contain wild-type p53 (21, 27, 28).

**Activation of p53 in HPV+ cell lines**

As there are significant differences between HPV+ and HPV− HNC in terms of global gene expression (24), we conducted Affymetrix microarrays on 8 HPV+ and HPV− HNC cell lines. While a full, unsupervised analysis is ongoing, based upon KEGG analysis, multiple genes in the TP53 pathway were significantly different between HPV+ and HPV− cells 24 hours after radiation (Z score 4.90). For example, levels of TP53 RNA were increased 4.63-fold in HPV+ vs. HPV− cells at baseline (P < 0.0001). Using qRT-PCR of irradiated samples, levels of TP53 RNA were further increased following radiation in HPV+ vs. HPV− cell lines (Fig. 4A).

To determine whether these differences in gene expression resulted in alterations in protein levels, we harvested cellular lysates at baseline, 4, and 24 hours after radiation and assessed levels of p53 and activated, phosphorylated-p53. Absolute levels of p53 varied significantly at baseline such that multiple exposures and ECL reagents were required to identify any p53 protein in UM-SCC47. As expected in a normal epithelial cell line, increased total and phospho-p53 was observed in HTE cells (Fig. 4B, left). While p53 responses in cancer cells can differ from those in normal cells, an increase in activated, phosphorylated-p53 was seen in UM-SCC47, UPCI-SCC90, and 93-VU-147T following radiation (Fig. 4B, right). An increase in total p53 was also seen at these selected time points following radiation in UPCI-SCC90. In HPV− cells, no appreciable p53 was identified in UM-SCC1, UM-SCC6, or UM-SCC22B, whereas SCC1483 showed a slight induction of p53, 4 and 24 hours after radiation (data not shown). Consistent with activation of p53 by radiation, p21 RNA was increased following radiation compared with baseline in 3 of 4 HPV+, but only 1 of 4 HPV−, HNC cell lines (Fig. 4F).

**Increased radiation sensitivity related to HPV E6 expression**

To investigate the individual contributions of HPV oncoproteins, we used a unique system of immortalized HTE cells. Cells stably carrying pLXSN vector alone (+LXSN), or encoding either pLXSN-HPV-16 E6 (+16E6) or pLXSN-HPV-16 E6 and E7 (+16E6E7) were used to conduct colony formation assays. A decrease in clonogenic survival (corresponding to increased sensitivity to radiation) was seen in both 16E6 and 16E6E7 expressing cells (Fig. 4C). Interestingly, little increase in the G2 fraction was seen in HTE-16E6 cells, whereas expression of both E6 and E7 resulted in a significant G2 arrest (Fig. 4D) as seen in the patient-derived SCC cell lines. Both E6 and E6/E7 resulted in an increase in apoptosis as measured by caspase activity (Fig. 4E).

**Knockdown of TP53 results in radiation resistance**

We hypothesized that the wild-type TP53 present in the +16E6 and +16E6E7 cells was being reactivated following radiation, resulting in improved sensitivity to radiation. To test this hypothesis we conducted siRNA-mediated knockdown of p53 in our HTE +16E6 cell line in addition to using 2 HPV+ HNC cell lines. As expected, compared with vector alone, expression of 16E6 resulted in a decrease in detectable p53 (Fig. 5A). Use of a TP53-specific siRNA, but not vehicle or scrambled control siRNA, led to a further reduction in p53 expression to levels that were undetectable (Fig. 5A). Similar decreases in total p53 were seen in 93-VU-147T and UM-SCC47 cells treated with the same siRNA, but not with scrambled control siRNA (data not shown). Using clonogenic survival assays, treatment with scrambled siRNA resulted in a survival fraction similar to that previously seen in untreated cells (Figs. 1C and 4C). Use of TP53-specific siRNA resulted in a significant increase in colony formation consistent with radiation resistance in HTE +16E6, UM-SCC47, and 93-VU-147T cells (Fig. 5B).
In vivo radiation sensitivity

To determine whether the increased radiation sensitivity seen in vitro was also present in vivo, we used a cell line xenograft system. All 4 HPV+ cell lines (Fig. 6A) and all 4 HPV-/C0 cell lines (data not shown) were grown as flank-implanted xenografts. An initial dose-finding experiment was conducted with UM-SCC47 and a dose/fractionation schedule of 2 Gy delivered twice weekly to a total dose of 8 Gy was adopted for all subsequent experiments. Even this relatively low-radiation dose resulted in significant tumor growth delay in 3 of 4 HPV+ cell lines (Fig. 6A). However, the relatively rapid tumor growth rate, coupled with the prolonged time interval between radiation fractions, elicited no overall tumor regression in these xenografts. When a higher daily dose (4 Gy per fraction) was used in the UM-SCC47 cell line, modest tumor regression was seen (data not shown). Interestingly, the slowest growing line,
Figure 5. Critical role for p53 in radiation response of HPV+ HNC. A, expression of HPV16 E6 results in decreased detectable p53 protein, whereas use of p53-specific siRNA, but not scrambled or vehicle control, results in further loss of p53 expression in HTE HPV16 E6 expressing cells (left, 25 μg of total protein), UM-SCC47 cells (center, 75 μg of total protein), and 93-VU-147T cells (right, 25 μg of total protein). E, knockdown of TP53 causes greater colony formation HTE +16E6, UM-SCC47, and 93-VU-147T cells (n = 6 per condition). Compared with unirradiated cells, the surviving fraction of scrambled siRNA pretreated cells was significantly lower than that of TP53-specific siRNA-treated cells (*, P < 0.0001).

93-VU-147T, showed modest tumor regression in the irradiated tumors, but overall tumor growth was not significantly different after 100 days.

To determine whether a significant difference in populations was seen, time to tumor quadrupling from the first day of treatment was calculated for all 8 xenografts. No significant difference in median time to tumor quadrupling was seen between control (mock radiation)-treated HPV+ and HPV− tumors (P = 0.68, data not shown). In HPV− xenografts, no difference between control and radiated tumors was seen (Fig. 6B, P = 0.14). However, consistent with increased radiation sensitivity in HPV+ HNC, xenografts of HPV+ cells showed a significant prolongation in time to tumor quadrupling between control and irradiated tumors (Fig. 6C, P = 0.0003).

Discussion
In early publications suggesting an etiologic link between HPV and HNC, Gillison and colleagues postulated that HPV+ HNC may be more sensitive to radiation than HPV− HNC (1). More recently, clinical trial data show that patients with HPV+...
HNC have markedly improved tumor control and survival outcomes when treated with radiation, and indeed with other treatment approaches (3, 4). However, the very limited experimental data that currently exists is conflicting. For example, one group showed increased radiation sensitivity in 2 putatively HPV+ cell lines compared with one HPV− cell line (6), whereas a second report showed decreased radiation sensitivity for the same HPV+ cells compared with 3 different HPV− cells (7). Cells obtained directly from this group did not confirm the presence of HPV DNA, highlighting the critical importance of confirming the presence of viral DNA in cells during their use. Whether this resulted from loss of viral DNA, selection of a nonviral DNA-containing clonogen, or cell culture contamination is unclear, but may explain the discrepant results previously reported (6, 7). The uncertainty regarding the HPV status in the few globally available HPV+ HNC cell lines led us to acquire cells directly from the original labs that generated them, and conduct Southern blot analysis to confirm the presence of viral DNA. In addition, due to concern regarding potentially mixed population of cells, we conducted single-cell isolation and confirmed the presence of integrated HPV and expression of viral oncoproteins in the isolated cell clones. Only these individually cloned cell lines, which we revalidated by Southern to contain HPV16 with integration patterns or karyotypic properties consistent with the original reported lines, were used in the current study.

Using these well-validated clonal HPV+ HNC cell lines, we show that they are consistently more sensitive to radiation than HPV− HNC cells both in vitro and in vivo. As expected, there is considerable variation in radiation sensitivity in both HPV+ and HPV− cells. This is certainly true in the clinical domain where patients with HNC exhibit a broad spectrum of response to radiation, and we would therefore anticipate a broad response heterogeneity in the preclinical setting as well. Nevertheless, our systematic evaluation of all existing HPV+ HNC cell lines seems to show a strong pattern of increased radiosensitivity compared with HPV− HNC cell lines. This difference could result in a significant improvement in tumor control probability for HPV+ HNC when compounded over 6 to 7 weeks of treatment. Radiation sensitivity seems to associate with prolonged radiation-induced G2 cell-cycle arrest in HPV+ HNC cells and with increased apoptosis-mediated through activation of p53 signaling.

We propose a model (Fig. 7) in which titration of the levels of p53 expression plays a critical role in regulating therapeutic response. In the development of HPV− HNC, TP53, or members of its pathway are commonly mutated, preventing a normal response to radiation-induced DNA damage. However, in HPV+ HNC, the HPV−16 E6 oncoprotein induces degradation of p53, an effect that plays a critical role in the induction of cancer but that also removes selective pressure to develop mutations in TP53 or p53-response pathways. Our data suggests that low levels of wild type, normally functioning, p53 remain in HPV+ cell lines despite the downregulating effects of E6, and that this p53 can be activated by therapeutic stress such as radiation. This apparently protective response of p53 can be overcome by more complete knockdown of p53 by siRNA. We believe that this response results in prolonged G2 arrest, a result likely augmented by the known additional effects of HPV E6 and E7 to bypass G1 checkpoint control (reviewed in ref. 29). Finally, perhaps due to a failure of HPV+ cells to repair DNA damage, cells eventually undergo cell death by apoptosis.

Interestingly, both E6 and E7 are involved in activation and repression of DNA damage response pathways to support viral genome maintenance and amplification in the normal viral life cycle. Further understanding of how these alterations are regulated in HPV-induced cancers may shed additional light on the mechanisms of DNA damage repair from therapeutically induced DNA damage.

Several additional studies have investigated the effects of HPV proteins on radiation sensitivity outside of the head and neck. DeWeese and colleagues showed no effect of E6 or E7 on radiation sensitivity in a study using human colon cancer cell lines (9), whereas Hampson and colleagues used cervical cancer cells engineered to overexpress the HPV oncoproteins and showed that E6 induces radiation resistance in a cell line containing mutant TP53 (30). It is quite likely that the effects of HPV vary based on the cell of origin and that results obtained in colon or cervical cancer may differ from that in head and neck cancer. In fact, clinical data in anal cancer and cervical cancer, 2 malignancies induced by HPV and treated primarily with radiation and concomitant chemotherapy, is suggestive of exactly this phenomenon: target radiation doses for patients with anal cancer are in the range of 40 to 50 Gy (31), whereas patients with cervical cancer are treated with radiation doses of 70 to 80 Gy (32).

We believe that this data has important clinical implications providing supportive data for carefully designed studies investigating the role of therapy de-escalation in patients with HPV+ HNC. While current nonsurgical therapy approaches for these patients most commonly involves concurrent radiation with either cisplatin, or cetuximab, it may be that alternative regimens involving accelerated radiation or alternative concurrent
therapies may be preferable. Ongoing studies including those by the Radiation Therapy Oncology Group and by a number of institutions specifically enrolling patients with HPV+ HNC will provide further insight in this regard. Judicious use of these and novel model systems such as a primary tumorgraft system that we have recently reported (33) may be able to provide preclinical data supportive of specific interventions. In addition, it is hoped that understanding the role of p53 and other pathways in the radiation response of HPV+ HNC will provide insights into methods to optimize therapy for patients with HPV− HNC. For example, consistent with our results, PRIMA-1, a molecule with the ability to reactivitate mutated p53 may have a role in improving outcomes in patients with HPV− HNC (34).

We acknowledge several limitations of our work. Importantly, the in vivo work has been conducted using mice with a compromised immune system. Thus, differences observed in radiosensitivity in vitro and in vivo in our study reflect the inherent radiation sensitivity difference between HPV+ and HPV− HNC cells that is not dependent upon immune surveillance. Spanos and colleagues have suggested that tumor control of mouse tonsillar epithelial cells expressing the human HPV16 E6 and E7 proteins is improved in an immunocompetent mouse compared with a RAG knockout mouse with a compromised immune system (7). It may well be that the presence of a robust immune response further increases tumor control of HPV+ HNC thus magnifying differences in intrinsic radiation sensitivity. However, the consistent findings from both in vitro clonogenic survival assays and in vivo tumor growth delay assays provide support to our conclusions that would not be possible with only one of the chosen systems. In addition, while there are now thousands of patients with HPV+ HNC and biopsy specimens available, there are only a handful of documented HPV+ HNC cell lines available worldwide. We solicited and studied all available cell lines when we commenced this study, but the scarcity of established HPV+ tumor cell lines raises an important question as to why it has been so challenging to isolate and propagate HNC cell lines from patients with HPV+ tumors. Much akin to the rarity of Epstein Barr virus-positive cell lines in nasopharynx cancer, the limited number of HPV+ cell lines hampers progress to investigate new therapeutic approaches for these patients with cancer. In light of this limitation, we have recently established a patient-derived xenograft model system from patients with HPV+ HNC (33) and are hopeful that similar approaches may provide additional insights into the biology of these unique tumors.

In summary, we have shown that compared with HPV− HNC cells, those derived from HPV+ HNC show increased intrinsic sensitivity to radiation. This is associated with prolonged activation of markers of DNA damage, E6- and E7-mediated radiation-induced G2 arrest, and a strong apoptotic response. It seems that residual wild-type p53 plays an important role in modulating this radiation response as further decrease in p53 results in a loss of radiation sensitivity akin to that seen in HPV− cells. Future studies will investigate the role of HPV-proteins in modulating DNA damage repair and understanding the impact of concurrent radiation and chemotherapy on HPV+ HNC.

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

Authors' Contributions
Conception and design: R.J. Kimple, R.Z. Yang, P.M. Harari
Development of methodology: R.J. Kimple, R.Z. Yang, P.M. Harari
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R.J. Kimple, M.A. Smith, G.C. Blitzee, J.A. Martin, R.Z. Yang, C.R. Peet, L.D. Lorenz, K.P. Nickel, A.J. Klingelhutz, P.M. Harari
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R.J. Kimple, J.A. Martin, C.R. Peet, L.D. Lorenz, P.F. Lambert, P.M. Harari
Writing, review, and/or revision of the manuscript: R.J. Kimple, L.D. Lorenz, K.P. Nickel, A.J. Klingelhutz, P.F. Lambert, P.M. Harari
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A.D. Torres, K.P. Nickel
Study supervision: R.J. Kimple, C.R. Peet, P.F. Lambert, P.M. Harari

Grant Support
This study was supported by Kaye Fellowship in Head and Neck Cancer Research (R. Kimple), Radiological Society of North American Research Fellow Grant (R. Kimple), and AACR/Bristol-Myers Squibb Fellowship in Clinical Cancer Research (R. Kimple), K99CA166039 (R. Kimple), R01 CA113448 (P. Harari), and R01 DE01715 (P. Lambert).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received February 28, 2013; revised April 9, 2013; accepted April 26, 2013; published online First June 7, 2013.

References


Enhanced Radiation Sensitivity in HPV-Positive Head and Neck Cancer

Randall J. Kimple, Molly A. Smith, Grace C. Blitzer, et al.

Cancer Res  Published OnlineFirst June 7, 2013.

Updated version Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-13-0587

Supplementary Material Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2013/06/07/0008-5472.CAN-13-0587.DC1

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.