TP53 Disruptive Mutations Lead to Head and Neck Cancer Treatment Failure through Inhibition of Radiation-Induced Senescence

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Abstract

Purpose: Mortality of patients with head and neck squamous cell carcinoma (HNSCC) is primarily driven by tumor cell radioresistance leading to locoregional recurrence (LRR). In this study, we use a classification of TP53 mutation (disruptive vs. nondisruptive) and examine impact on clinical outcomes and radiation sensitivity.

Experimental Design: Seventy-four patients with HNSCC treated with surgery and postoperative radiation and 38 HNSCC cell lines were assembled; for each, TP53 was sequenced and the in vitro radioresistance measured using clonogenic assays. p53 protein expression was inhibited using short hairpin RNA (shRNA) and overexpressed using a retrovirus. Radiation-induced apoptosis, mitotic cell death, senescence, and reactive oxygen species (ROS) assays were carried out. The effect of the drug metformin on overcoming mutant p53-associated radiation resistance was examined in vitro as well as in vivo, using an orthotopic xenograft model.

Results: Mutant TP53 alone was not predictive of LRR; however, disruptive TP53 mutation strongly predicted LRR (P = 0.03). Cell lines with disruptive mutations were significantly more radioresistant (P < 0.05). Expression of disruptive TP53 mutations significantly decreased radiation-induced senescence, as measured by SA-β-gal staining, p21 expression, and release of ROS. The mitochondrial agent metformin potentiated the effects of radiation in the presence of a disruptive TP53 mutation partially via senescence. Examination of our patient cohort showed that LRR was decreased in patients taking metformin.

Conclusions: Disruptive TP53 mutations in HNSCC tumors predicts for LRR, because of increased radioresistance via the inhibition of senescence. Metformin can serve as a radiosensitizer for HNSCC with disruptive TP53, presaging the possibility of personalizing HNSCC treatment. Clin Cancer Res; 18(1); 290–300. ©2011 AACR.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth leading cause of cancer worldwide, with an estimated 49,260 diagnoses each year in the United States alone (1). Although multimodality therapy is important in the management of HNSCC, the eradication of locoregional disease in the primary or postoperative setting is primarily achieved by external beam radiation. As the vast majority of patient deaths from HNSCC are due to locoregional recurrence (LRR), the survival of the patient largely depends on the radiosensitivity the tumor. Unfortunately, there are few biomarkers to determine radioresistance in HNSCC.

One candidate biomarker is TP53, which encodes the p53 protein and is the most commonly altered gene in HNSCC, as shown most recently by our group and others through whole exomic sequencing of a large panel of HNSCC tumors (2). TP53 has been previously investigated as a biomarker for LRR following radiotherapy in HNSCC with mixed results (3–6). A possible explanation for this finding is mutation-specific functionality of the p53 protein. One large study classifying TP53 mutations in HNSCC described TP53 mutation as “disruptive” or “nondisruptive” on the basis of alteration of DNA binding (7). Any mutation in either the L2 or L3 loop of the DNA-binding domain, resulting in a polarity change within the protein, or any

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The clinical setting. Furthermore, we wished to determine whether this form of cell death is associated with cell division due to DNA damage leads to the formation of radiation-induced cell death (19). In this process, abnormal for most tumor cells, apoptosis plays a minimal role in the response is not clear, and there are strong arguments that, known to result in p53 activation and apoptosis; however, of active investigation, with many of the previous studies some models, is dependent upon wild-type TP53. The role of TP53 in kas radiosensitivity is also an area of active investigation, with many of the previous studies producing contradictory results (10–16). Radiation is known to result in p53 activation and apoptosis; however, the contribution of radiation-induced apoptosis to tumor response is not clear, and there are strong arguments that, for most tumor cells, apoptosis plays a minimal role in the radiation response (17; 18). In contrast, it has been hypothesized that mitotic death is the primary mechanism of radiation-induced cell death (19). In this process, abnormal cell division due to DNA damage leads to the formation of large cells with multiple micronuclei (20). It is unclear whether this form of cell death is associated with TP53 (21–24). Furthermore, emerging data indicate that senescence may play a role in the radiation response, which, in some models, is dependent upon wild-type TP53 (25). Regardless, the role of TP53 in radiosensitivity is far from clear.

To investigate the role of TP53 in radiosensitivity, the present study was done to determine whether a classification of TP53 (wild-type, nondisruptive, or disruptive mutation) predicts for radiosensitivity in vitro as well as in the clinical setting. Furthermore, we wished to determine the mechanisms involved in the observed radiation response in vitro and use this knowledge to preferentially radiosensitize HNSCC cells.

**Materials and Methods**

**Clinical samples**

All clinical studies reported in this study have been approved by the Institutional Review Board of the University of Texas MD Anderson Cancer Center. Seventy-four snap-frozen, pretreatment tumor samples were collected from patients with HNSCC at high risk for LRR treated with surgical resection and postoperative radiotherapy from 1992 to 2003. Exons 2-12 of the TP53 gene were sequenced using genomic DNA. Standard Sanger sequencing using BigDye Terminator chemistry (Applied Biosystems Life Technologies) was performed by Beckman Coulter Genomics (Beckman Coulter). Mutations were then classified according to the method of Poeta and colleagues (7). Patients were evaluated every 2 to 3 months for 1 year following treatment, every 3 to 4 months the following year, and every 6 months thereafter.

**Cell culture and constructs**

HNSCC cells were cultured in a 37°C incubator in 5% CO2 atmosphere for this study as described previously (26). Cells stably expressing short-hairpin RNA (shRNA) specific for p53 (shp53) were generated as described previously (27). Briefly, after infection with green fluorescent protein (GFP)-tagged empty lentiviral vector (LVTHM) or encoding an shRNA against p53 (LVTHM-shp53; Addgene), cells were cultured for several passages, and then sorted using flow cytometry. GFP-positive cells were cultured normally. TP53 constructs (C176F, R282W, R175H, and E336X) were generated by extracting RNA from cell lines known to express these mutants. Reverse transcriptase PCR (RT-PCR) was then carried out using TP53-specific primers. The resulting product was purified and inserted into a pBABE retroviral vector containing a puromycin-resistance insert (pBABE-puro; Addgene) using standard cloning techniques. The resulting vectors were verified by Sanger sequencing at the MD Anderson Cancer Center DNA core facility. After transfection and packaging in 293T cells, the viral supernatant was centrifuged at 1,000 rpm to remove cellular debris and added to UMSSC1 cells in combination with polybrene. After 1 passage, the cells underwent selection with puromycin. All cell lines used in this study have been authenticated against the parental recipient cell line via short tandem repeat (STR) analysis.

**Clonogenic assay**

HNSCC cells were seeded in 12-well plates at predetermined densities for different radiation doses to allow for an approximately equal number of resultant colonies. The next day, cells were irradiated using a high-dose-rate 137Cs irradiator (4.5 Gy/min) and cultured for 10 to 14 days to allow for colony formation. Cells were then fixed in a 3% crystal violet/10% formalin solution. Colonies of more than 50 cells were then counted and survival fraction was
determined. All treatments were in triplicate or greater. For metformin (Sigma-Aldrich) or z-Vad-fmk (BD Pharmingen) treatment, cells were pretreated for 2 hours before radiation with either drug or vehicle [PBS and dimethyl sulfoxide (DMSO), respectively] and drug treatment was continued overnight. For N-acetyl cysteine (NAC) treatment, the cells were treated starting 2 hours following radiation and the drug treatment was continued overnight. The cells were then washed and cultured in fresh media for the remainder of the experiment.

**Immunofluorescence**

HNSCC cells were plated on coverslips and treated as indicated. Cells were washed twice with PBS and fixed using a 1:1 mixture of methanol and acetone for 10 minutes. Cells were then washed in TBS–Tween (TBST) and permeabilized using 0.1% Triton-X for 5 minutes. Cells were again washed with TBST and incubated with fluorescein isothiocyanate (FITC)–Phalloidin for 1 hour at room temperature. Cells were washed with TBST and mounted on standard glass slides using 4',6-diamidino-2-phenylindole (DAPI)–Vectashield (Vector Laboratories). For assessment of mitotic death, the number of cells with 1 or more micronucleus per high-power field were counted and reported as a percentage of all the cells observed per high power field.

**Senescence-associated-β-gal staining**

Senescence-associated (SA)-β-gal staining was carried out according to the manufacturer’s instructions (Cell Signaling). Briefly, HNSCC cells plated in 6-well plates were irradiated with 4 to 6 Gy on the next day. Cells were cultured normally until the indicated times post treatment. Cells were then fixed for 10 minutes and stained overnight for SA-β-gal activity at 37°C. Blue-staining cells were scored as senescent and reported as a percentage of all the cells observed per high power field.

**Annexin V staining**

Annexin V staining was carried out according to the manufacturer’s instructions (BD Pharmingen). Briefly, HNSCC cells were cultured normally and treated as indicated. Cells were collected using a plastic scraper and centrifuged at 14,000 rpm at 4°C for 10 minutes. The supernatant was removed, and total protein concentration was then calculated using Bio-Rad Protein Assay (Bio-Rad). Immunoblot analysis was carried out as described previously (26). Membranes were blocked for 1 hour at room temperature using 1% powdered milk in 0.1% Tween 20 in TBS, then incubated overnight with anti-p53 DO-1 (Santa Cruz Biotechnology), anti-β actin (Cell Signalling), or anti-p21 (BD Pharmingen) at room temperature for 30 minutes. Cells were then washed twice in PBS and trypsinized. The resulting cell suspension was washed in PBS and fixed with 70% ethanol at room temperature for 30 minutes. Cells were then washed in PBS and stained with propidium iodide. Cycle detection was then conducted using a Beckman Coulter XL 4 color cytometer, (Beckman Coulter) and the data were analyzed using Flo-Jo software (FloJo).

**Reactive oxygen species measurement**

Intracellular reactive oxygen species (ROS) levels were measured according to previously published protocols using 5-(and-6)-carboxy-2’,7’-dichlorofluorescein (CM-H2DCFDA) dye (28). Briefly, cells were loaded with CM-H2DCFDA for 60 minutes in culture media. Media was changed before irradiation to remove excess dye. Fluorescence was measured using a standard spectrophotometer, normalized to the control condition and total amount of DNA (29). For flow cytometric experiments, cells were incubated with CM-H2DCFDA dye as described and trypsinized, and fluorescence was analyzed using flow cytometry as described above.

**Immunoblotting**

Cells were treated as indicated and washed 3 times with cold PBS. Standard lysis buffer was then added to each plate and incubated on ice for 15 minutes. Cells were then collected using a plastic scraper and centrifuged at 14,000 rpm at 4°C for 10 minutes. The supernatant was removed, and total protein concentration was then calculated using Bio-Rad Protein Assay (Bio-Rad). Immunoblot analysis was carried out as described previously (26). Membranes were blocked for 1 hour at room temperature using 1% powdered milk in 0.1% Tween 20 in TBS, then incubated overnight with anti-p53 DO-1 (Santa Cruz Biotechnology), anti-β actin (Cell Signalling), or anti-p21 (BD Pharmingen) at 4°C. The following day, membranes were washed with 0.1% Tween 20 in TBS and incubated for 1 hour at room temperature with species-specific secondary antibody. For p53 and actin, fluorescence-conjugated secondary antibodies (Invitrogen) were used and signal was analyzed using an Odyssey Infrared Imaging System (LI-COR Biosciences) and the associated software (v3.0). For p21, horseradish peroxidase conjugated anti-rabbit immunoglobulin G (Santa Cruz Biotechnology) was used and signal was generated using the SuperSignal West chemiluminescent system (Pierce Biotechnology).

**p21 transcription**

Induction of p21 transcription was measured through luciferase reporter activity using a vector containing the 2.4-kb p21 promoter and firefly luciferase (pWWP-Luc; ref. 30; Addgene). UMSCC1 (p53-null) cells expressing various TP53 constructs were co-transfected with pWWP-Luc and a constitutively active Renilla luciferase construct using Lipofectamine 2000 (Invitrogen). Cells were irradiated the following day at the indicated doses and incubated for 24 hours before collection. Luciferase
activity was measured with the Promega Dual-Luciferase Reporter Assay System.

Orthotopic mouse model
All animal experimentation was approved by the Animal Care and Use Committee (ACUC) of the University of Texas MD Anderson Cancer Center. The orthotopic mouse model has been previously described (31). Briefly, mice were injected with 100,000 HN 31 cells in the anterior half of the oral tongue on day 0. After tumor growth was noted, tumors were treated with radiation using a Co60 irradiator and custom lead blocks (5 Gy) on day 8 post-injection and/or metformin [250 mg/kg daily, intraperitoneal (IP)]. Each treatment group comprised 10 mice. Mice received a total of 8 daily metformin treatments during the experimental period. Tumor measurements were obtained on days 6, 13, 16, and 20 post injection. Tumor volume was calculated as previously described (31).

Statistics
LRR and overall survival (OS) for the patient population was calculated using the Kaplan–Meier method, and comparisons between groups were determined using log-rank statistics. Multivariate analysis for LRR was carried out using forward step-wise Cox regression analysis. Variables included tumor and nodal stage, surgical margin status or extra-capsular extension, site, gender, smoking history, and TP53 status. ANOVA with post hoc analysis or Student t tests were carried out to analyze in vitro data and tumor volume. All P values are 2-sided. P values less than 0.05 were considered significant.

Results
Disruptive TP53 mutations are associated with increased LRR following PORT
Disruptive TP53 is associated with decreased survival in HNSCC, which we hypothesized was due to higher rates of LRR following PORT. To explore this hypothesis, we sequenced TP53 in HNSCC tumors from 74 patients treated with PORT. Patient characteristics were similar among different TP53 classification groups (Supplementary Table S1). Median follow-up of surviving patients was 154 months (range 82–185). No significant difference in LRR was found between patients with wild-type TP53 and any mutant TP53 (Fig. 1A). However, classification of TP53 as disruptive, nondisruptive, or wild-type, showed that disruptive TP53 is an independent predictor of LRR on multivariate analysis (P = 0.022). Patients with disruptive TP53 had a 5-year freedom from LRR of 41% compared with 64% and 76% in patients with wild-type and nondisruptive TP53, respectively (P = 0.03; Fig. 1B). No significant difference in LRR was seen between patients with wild-type and nondisruptive TP53 (P = 0.48; Fig. 1B). Similarly, the OS rates for patients in this study were predicted by TP53 classification. Specifically, 5-year OS rate for patients with disruptive TP53 mutations was 19% compared with 52% in patients with wild-type TP53 and 41% in patients with nondisruptive TP53 (P = 0.031).

Disruptive TP53 mutations are associated with p53-mediated radioresistance
The finding that disruptive TP53 mutations are associated with a high rate of LRR resulted in the hypothesis that HNSCC tumor cells with disruptive TP53 are intrinsically more radioresistant. To test this hypothesis, we evaluated the radiosensitivity of a panel of 38 HNSCC cell lines of known TP53 status (Supplementary Table S3) and found that cell lines harboring disruptive TP53 mutations were significantly more radioresistant than those with either nondisruptive or wild-type TP53 (Fig. 2A).

To determine whether this observed correlation was due to p53 expression, p53 was silenced using stably expressed shRNA (shp53) in the following HNSCC cell lines: (i) UMSCC1 17A and HN 30 (wild-type TP53); (ii) Detroit (R175H, nondisruptive TP53); and (iii) HN 31 (C176F)
and FADU (R248L; disruptive TP53). Inhibition of p53 expression in cell lines expressing wild-type or nondisruptive TP53 rendered cells more radioresistant (Fig. 2B; Supplementary Table S2). However, inhibition of p53 expression in cell lines expressing disruptive TP53 rendered these cells more radiosensitive (Fig. 2D; Supplementary Table S2). Conversely, UMSCC1 cells, which have no endogenous p53, were engineered to express: (i) wild-type TP53; (ii) nondisruptive TP53 (R175H and R282W); or (iii) disruptive TP53 (C176F and E336X). Disruptive TP53-expressing UMSCC1 cells were found to be radioresistant relative to wild-type TP53- and nondisruptive TP53-expressing cells (Fig. 2C; Supplementary Table S2).

**Alteration of p53 expression does not affect radiation-induced mitotic death or apoptosis**

To explore the means by which disruptive TP53 mutation confers radioresistance in HNSCC cells, we evaluated several modes of cellular response to radiation. Initially, we examined mitotic death, thought to be one of the primary modes of cell death following radiation (19). Mitotic death, as measured by the presence of micronuclei, was significant following radiation, with all HNSCC cell lines tested exhibiting mitotic death in 20% to 30% of irradiated cells. However, there was no correlation between micronuclei formation, TP53 expression, and relative radiosensitivity (Supplementary Fig. S2).

Furthermore, when either annexin V or sub-G1 staining were analyzed, neither of these measures of apoptosis were related to TP53 status (Supplementary Fig. S3). In fact, only a very small proportion of apoptotic cells were observed (~1%–5%), including in those cells shown to be radiosensitive in the clonogenic survival assay. As radiation-induced apoptosis is thought to be caspase-dependent (32), we also treated cells with a pan-caspase inhibitor (z-vad-fmk; BD Pharmingen) to determine the role of apoptosis in this model. Inhibition of caspase activity had almost no effect on clonogenic survival following radiation (Supplementary Fig. S3), supporting the hypothesis that apoptosis does not play a major role in the radiation response.

**Radiation robustly induces SA-β-gal activity in HNSCC cells which is strongly associated with TP53 status and correlates with clonogenic survival**

In cells with wild-type or nondisruptive TP53, treatment with radiation resulted in a decrease in the proportion of cells in S phase and a less prominent G1 arrest (Supplementary Fig. S4). Furthermore, inhibition of p53 expression in HN 30 (wild-type TP53) cells partially abrogated the observed inability to progress through S phase. Because this phenomenon has been previously linked to cellular senescence, we hypothesized a role for altered senescence in the observed differences in radiosensitivity. To evaluate cellular senescence, cells were treated with radiation and stained for the SA-β-gal. As shown in Fig. 3, radiation treatment resulted in significant levels of SA-β-gal activity in cell lines expressing either wild-type (HN 30) or nondisruptive mutant TP53 (Detroit, R175H). Furthermore, in these cells with SA-β-gal staining, morphologic characteristics of cellular senescence were observed, specifically a large, flattened cell with the...
classic "fried egg" appearance on microscopy. Inhibition of p53 expression in both of these cell lines significantly reduced levels of radiation-induced SA-β-gal activity. However, in HN 31 (C176F, disruptive TP53) cells, which have disruptive TP53 and are otherwise isogenic with the HN 30 (wild-type TP53) cell line, inhibition of p53 expression was associated with elevated levels of radiation-induced SA-β-gal activity.

Forced expression of wild-type, as well as 2 nondisruptive, TP53 mutations (R175H and R282W) in p53-null cells, increased radiation-induced SA-β-gal activity; conversely, expression of disruptive TP53 mutations (C176F and E336X) led to decreased SA-β-gal activity compared with empty vector control (Fig. 3B). Furthermore, in experiments comparing micronuclei formation and SA-β-gal staining in the same cell, the main differences between cells of different TP53 statuses appeared to be within the cells staining positive for SA-β-gal alone (Fig. 3D).

The effects of TP53 status on radiation-induced SA-β-gal activity are correlated with p21 expression and mediated by ROS production

In addition to SA-β-gal activity, senescence has been previously linked to induction of p21 expression and ROS production, both of which are believed to be necessary for maintenance of the senescent phenotype (33–36). To determine whether radiation-induced p21 expression correlates

**Figure 3.** TP53 modulates radiation-induced senescence, which correlates with radiosensitivity. A, representative light microscopy showing SA-gal staining. B, percentage of SA-gal–positive cells per total number of cells in a high-power field (hpf) after 4 Gy of radiation for the times indicated in UMSCC1 cells expressing representative wild-type and mutant TP53 constructs and HN 30 (WT), Detroit (R175H), and HN 31 (C176F) where p53 is inhibited. C, representative microscopy showing SA-gal staining, DAPI fluorescence, and GFP. D, percentage of cells SA-gal–positive (S alone), exhibiting micronuclei (M alone), or both at 4 days after 4 Gy of radiation in HN 30 and HN 31 cells where p53 expression is inhibited and UMSCC1 cells are expressing representative wild-type and mutant TP53 constructs. *, significantly elevated over baseline (P < 0.05); †, significantly different from null at the indicated time point (P < 0.05); and #, significantly different from HN 30 control. XRT, X-ray therapy.
with TP53 classification, we assayed p21 protein levels following radiation in cell lines expressing representative TP53 mutations. In cells expressing wild-type TP53 or a nondisruptive TP53 mutation, p21 protein and reporter activity were induced by radiation treatment (Fig. 4A). In contrast, cells expressing disruptive TP53 (C176F) had no induction of p21.

In addition, ROS play a key role in senescence and radiation effect. In the present study, radiation-induced ROS levels correlated with p21 expression, SA-beta-gal activity, and relative radiosensitivity. Specifically, ROS were most highly induced in cells which had greater levels of radiation-induced SA-beta-gal activity (wild type and R175H), whereas little or no ROS induction was seen in cells with disruptive TP53 (C176F; Fig. 4B). Inhibition of ROS using NAC 2 hours following radiation treatment, designed to inhibit secondary ROS production following the initial radiation insult, dramatically decreased senescence in cell lines with normally high levels of radiation-induced SA-beta-gal, but had no effect on cells with disruptive TP53 (Fig. 4C). These results closely correlated with the observed effects of NAC on clonogenic survival following radiation (Fig. 4D).

Metformin preferentially potentiates the effects of radiation in HNSCC cells partially via increased senescence

On the basis of the combined morphologic appearance of irradiated cells, the presence of SA-beta-gal staining, the
characteristic induction of p21 and ROS, and the lack of apoptosis, cells with either wild-type or nondisruptive TP53 mutation appear to be undergoing radiation-induced senescence, whereas cells with a disruptive TP53 mutation are not. Because the induction of senescence may be clinically beneficial, we intended to investigate drug-based therapies designed to this end. As there are no specific senescence-inducing agents currently available, we examined metformin, an antidiabetic agent with minimal clinical toxicity, that has been shown to both induce ROS in some cellular contexts (37) as well as preferentially target cell lines with mutant TP53 (38). In the present study, concurrent radiation and metformin treatment was active against HNSCC cell lines that harbored disruptive TP53 (Supplementary Fig. S5). Conversely, no effect of metformin was seen in cells expressing wild-type TP53 (HN 30 and MCF-7). Furthermore, the addition of metformin dramatically increased ROS in HN 31 cells, which have a disruptive (C176F) TP53 mutation, but had much less effect in HN 30 cells, their wild-type TP53 isogenic counterpart (Supplementary Fig. S5). Similarly, metformin decreased clonogenic survival following radiation and increased radiation-induced SA-β-gal activity in HN 31 cells (C176F, disruptive TP53), but had little effect in HN 30 cells (wild-type TP53; Fig. 5A and B). Furthermore, after inhibition of wild-type p53 expression, metformin was found to potentiate SA-β-gal activity and decrease clonogenic survival (Fig. 5A and B).

Figure 5. Metformin selectively radiosensitizes cells with disruptive TP53 mutations, partially due to altered senescence. A, clonogenic survival after treatment with radiation at the indicated doses together with metformin for 24 hours in HN 30 and HN 31 cells in which p53 expression is inhibited using shRNA (shp53). B, percentage of SA-β-gal-positive cells per total number of cells in a high-power field (hpf) 4 days following 4 Gy of radiation and the indicated doses of metformin. *, significantly different from unirradiated control (P < 0.05); †, significantly changed compared with no metformin treatment (P < 0.05). C, tumor volume in mice with orthotopic tumors derived from HN 31 cells (C176F) after treatment with radiation (6 Gy), metformin (250 mg/kg, intraperitoneal), or both. *, significantly different from radiation alone (P < 0.05); †, significantly different from metformin treatment alone (P < 0.05). D, LRR in patients taking metformin during treatment compared with the remainder of the study population as well as patients matched for tumor and nodal stage, surgical margin status, and TP53 status to the metformin-treated group (P = 0.04).
Discussion

In HNSCC, the majority of patient deaths result from LRR, with up to 60% to 75% dying of their disease (39). The primary method of treating advanced HNSCC involves radiation, either in the definitive or postoperative setting. To date, there are few clinically useful predictive indicators of LRR following radiotherapy. In the current study, we examined the effect of metformin treatment on tumor response to radiation in an orthotopic model of HNSCC described previously (26, 31). As seen in Fig. 5C, the addition of metformin to radiation dramatically decreased tumor growth compared with either radiation alone (P = 0.015) or metformin alone (P = 0.008).

To further investigate the clinical use of metformin, a cohort of patients treated with postoperative radiation therapy (PORT) for HNSCC was identified and the impact of concurrent metformin use was investigated. In addition to the patients in the current study, an additional 30 patients with similar disease characteristics and treatment as well as known TP53 status were evaluated. Although only 10 patients were taking metformin at the time of radiation, these patients had a dramatically lower LRR rate than controls matched for tumor and nodal stage, surgical margin status, and TP53 status (P = 0.04; Fig. 5D). On multivariate analysis controlling for TP53 classification, tumor stage, and surgical margin positivity, metformin use was significantly associated with decreased LRR (P = 0.04) as well as improved OS (P = 0.01). Specifically, 5-year OS rate was 87% in the patients taking metformin compared with 41% in the remaining patients (P = 0.04).

Given the above findings, we examined the effect of metformin treatment on tumor response to radiation in an orthotopic model of HNSCC described previously (26, 31). As seen in Fig. 5C, the addition of metformin to radiation dramatically decreased tumor growth compared with either radiation alone (P = 0.015) or metformin alone (P = 0.008).

Induction of both p21 and ROS appear to be critical mediators of cellular senescence. Induction of p21 alone can lead to increased ROS production (34), and ROS inhibition can reverse senescence in this context. Conversely, ROS have been linked to induction of p21, and a feedback loop between ROS and p21 is proposed to be required for DNA damage-induced senescence (35, 41).

In the proposed model, we found radiation-induced ROS and p21 only in HNSCC cells with wild-type and nondisruptive TP53. Specifically, p21 expression was increased in response to radiation in the context of the nondisruptive TP53 mutation R175H. Previously, R175H has been shown to have little remnant p53-driven transcription (42, 43). Furthermore, in one report, the disruptive mutation, C176F, has been shown to have some p53-dependent transcriptional activity in a yeast-based assay (43); however, this has not been observed in other studies (44, 45). Nonetheless, in the current model, both protein expression and transcription of p21 is induced by radiation in cells expressing either wild-type or R175H TP53. This could be attributable to p53 transcription independent induction of p21 similar to that seen in other models (46, 47) or it could result from residual p53 transcriptional activity. It has been shown recently that only a small subset of p53 transcriptional targets are necessary for induction of senescence; thus, even a small remnant of transcriptional function may be sufficient (48). Further studies are in progress to discern the mechanism behind this phenomenon.

Furthermore, radiation-induced ROS production was inhibited by disruptive TP53. In our study, inhibition of secondary ROS production several hours after the initial cellular insult by radiation had a dramatic effect on cellular senescence. This argues for prolonged ROS production following radiation playing a key role in the maintenance of the senescent phenotype. These ROS are most likely derived from the mitochondria and, in fact, we have previously observed decreased baseline mitochondrial complex activity in cells expressing C176F compared with wild-type TP53 (27). This argues for a suppressive effect on the mitochondria of disruptive TP53, which is relieved by inhibition of p53 expression. This offers a possible link between disruptive TP53 mutations GOF and the inhibition of radiation-induced ROS and senescence. Theoretically, disruptive p53 could interact with known targets of wild-type p53 involved in mitochondrial function, exerting an inhibitory function as opposed to the stimulation seen with wild-type or some nondisruptive forms of TP53.

Our data strongly indicate that disruptive TP53 portends poor clinical outcome in HNSCC associated with increased
metformin exhibit a therapeutically exploitable decrease in metabolic flexibility (27). One agent that can target this process is metformin. Recently, it has been shown that patients taking metformin have improved responses following neoadjuvant chemotherapy (49) and that metformin is a radiosensitizer in vitro (50). Metformin is hypothesized to affect mitochondrial function, and several studies have shown that it induces ROS in certain cellular backgrounds (37). In addition, it has been hypothesized that metformin may induce senescence in cells that are “senescence prone” (51). Interestingly, when we examined the effects of metformin in vitro, radiation-induced senescence and toxicity were potentiated only in cells expressing disruptive TP53, an effect that was modulated by altering p53 expression. This dramatic effect on the efficacy of radiation was also seen in our in vivo orthotopic model, as well as in patients taking metformin at the time of PORT. Although this only included 10 patients in the metformin group, the reduction in LRR was profound and argues for the possibility of a dramatic clinical benefit with targeting of radiation-induced senescence.

The present study is limited by its retrospective nature, although the patient cohort includes a homogenous group of patients treated uniformly with surgery and PORT. Furthermore, the in vitro studies are limited by the analysis of a subset of disruptive and nondisruptive TP53 mutations, and the possibility exists that other disruptive TP53 mutations may behave differently. However, examination of a large panel of HNSCC cell lines confirmed the in vitro predictive value of the disruptive classification. In addition, clinical data showing dramatically increased LRR in patients with disruptive TP53 mutations supports the overall hypothesis. Although the disruptive category is empiric in nature, the fact that senescence appears to be strongly inhibited by these mutations allows further refinement. Specifically, testing of TP53 mutants’ ability to induce senescence could be carried out, refining an already clinically predictive model. Finally, although the clinical data regarding the benefit of metformin is compelling, only a small number of patients were treated with the drug at the time of radiation. Despite the intriguing in vitro, in vivo, and clinical finding of radiation potentiation by metformin, these findings do not provide a definitive conclusion, but rather are hypothesis-generating and require additional validation.

Despite these limitations, we have shown for the first time that disruptive TP53 mutation predicts both LRR following PORT and in vitro response to radiation in HNSCC. In addition, we have shown that this effect is due to altered senescence and can be overcome using metformin, which appears to have dramatic clinical benefit.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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