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Demethylation Therapy as a Targeted Treatment for Human Papillomavirus–Associated Head and Neck Cancer

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Abstract

Purpose: DNA methylation in human papillomavirus–associated (HPV+) head and neck squamous cell carcinoma (HNSCC) may have importance for continuous expression of HPV oncogenes, tumor cell proliferation, and survival. Here, we determined activity of a global DNA-demethylating agent, 5-azacytidine (5-aza), against HPV+ HNSCC in preclinical models and explored it as a targeted therapy in a window trial enrolling patients with HPV+ HNSCC.

Experimental Design: Sensitivity of HNSCC cells to 5-aza treatment was determined, and then 5-aza activity was tested *in vivo* using xenografted tumors in a mouse model. Finally, tumor samples from patients enrolled in a window clinical trial were analyzed to identify activity of 5-aza therapy in patients with HPV+ HNSCC.

Results: Clinical trial and experimental data show that 5-aza induced growth inhibition and cell death in HPV+ HNSCC. 5-aza reduced expression of HPV genes, stabilized p53, and induced p53-dependent apoptosis in HNSCC cells and tumors. 5-aza repressed expression and activity of matrix metalloproteinases (MMP) in HPV+ HNSCC, activated IFN response in some HPV+ head and neck cancer cells, and inhibited the ability of HPV+ xenografted tumors to invade mouse blood vessels.

Conclusions: 5-aza may provide effective therapy for HPV-associated HNSCC as an alternative or complement to standard cytotoxic therapy. *Clin Cancer Res; 23(23); 7276–87. ©2017 AACR.*

Introduction

There is increasing evidence that human papillomavirus (HPV)-associated cancers represent a unique disease entity separate from HPV-negative (HPV−) tumors. Demographic data reveal that HPV+ HNSCC occurs in younger patients with less tobacco and alcohol exposure (1, 2). HPV+ head and neck primary tumors are usually smaller, as compared with HPV− HNSCC; however, patients with HPV-associated tumors are more likely to present with metastatic disease in regional lymph nodes (3, 4). In addition, HPV+ HNSCC responds better to chemotherapy and radiation than HPV− HNSCC, and HPV positivity is associated with improved overall survival (5). At the molecular level, HPV+ and HPV− head and neck cancers have different mutational landscapes and different protein expression profiles (6–10). HPV− HNSCCs contain mutations and genetic alterations classically associated with tobacco and alcohol exposure, including loss-of-function mutations of *TP53* (84%) and loss or inactivating mutations of *CDKN2A* (54%). In contrast, genetic alterations in these genes are extremely rare in the HPV+ subset (*TP53*, 3%; *CDKN2A*, 0%; ref. 6), which instead is associated with a mutational signature typical of apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3 (APOBEC3) antiviral proteins (6, 11–13). In addition, The Cancer Genome Atlas (TCGA) head and neck study found that TNF receptor–associated factor 3 (*TRAF3*) and *CYLD* genes are commonly mutated and deleted in HPV+ HNSCC (6, 8, 14). Finally, HPV− HNSCC have relatively low levels of DNA methylation, which is correlated with genomic instability (15), whereas HPV+ HNSCC harbor distinctly hypermethylated genomes (7, 8, 16, 17).

Demographic, clinical, molecular, and epigenetic differences between HPV+ and HPV− HNSCC suggest two distinct cancers, yet HPV− status is currently only used for prognosis and not to guide treatment (18). A majority of HNSCC patients are treated with radiation and platin-based chemotherapy, which is associated with deleterious and lifelong side-effects. Despite the relatively good prognosis associated with HPV+ HNSCC, 25%–30% of patients with HPV-positive tumors experience recurrent or metastatic disease, for which treatment options are limited.

The molecular and epigenetic differences between HPV+ and HPV− tumors provide an opportunity for therapies that exploit the interaction of the HPV genome and host that result in hypermethylation of cancer cell genomes. 5-Azacytidine (5-aza)

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Translational Relevance

The human papillomaviruses (HPV) are the etiologic agents of 60% to 70% of head and neck squamous cell carcinoma (HNSCC) arising from the oropharynx. Approximately 70% of patients with HPV$^+$ HNSCC are cured with aggressive therapy that may result in lifelong side effects. Upon recurrence, treatment options and survival are limited. Unique molecular and epigenetic features of HPV$^+$ HNSCC provide an opportunity for targeted therapies. Here, we show that 5-azacytidine (5-aza), a demethylating agent that is used to treat patients with hematologic malignancies, inhibited tumor growth and reduced HNSCC invasion in mouse models. 5-aza decreased expression of HPV genes accompanied by increased expression of p53 and apoptosis in both preclinical models, as well as following 5 to 7 days of 5-aza patient therapy in a window trial. These data suggest that demethylation therapy may be effective for treating HPV$^+$ HNSCC and that a larger clinical trial is warranted.

and its structural analogue, 5-aza-2′-deoxycytidine (decitabine), are FDA-approved DNA-demethylating agents used for therapy of myelodysplastic syndromes (MDS) and acute myelogenous leukemia (AML; refs. 19, 20). As cytidine analogues that incorporate into newly synthesized DNA, these drugs inactivate DNA methyltransferase 1 (DNMT1), resulting in global demethylation of the cellular genome (21). Although known to demethylate DNA, the link between demethylation-induced transcription and the therapeutic effects of these drugs is not well established (21). On one hand, demethylation can re-activate tumor suppressors that were repressed by promoter methylation, resulting in tumor suppressor–induced cancer cell death (22). On the other hand, demethylation of CpG loci within gene bodies can paradoxically decrease gene transcription (23). Furthermore, 5-aza is incorporated into RNA (24, 25), where it can alter mRNA stability (26) and therefore change expression independently of its effect on transcription. Finally, 5-aza and decitabine cause DNA damage and activate the DNA damage response in some cells; however, the nature of this DNA damage has not been fully characterized and is likely cell type–dependent (27).

Exploration of demethylating drugs for treatment of solid tumors is in the early stages with the highest response rates to decitabine occurring in cervical (36%) and pleural (14%) malignancies, but decitabine has not yet been tested in HNSCC (28). Here, demethylation therapy was explored as a novel, targeted treatment for HPV$^+$ HNSCC. We assessed the cellular response and effect on metastatic potential following demethylation therapy in HPV$^+$ HNSCC cells, xenograft mouse models, and in clinical trial specimens from head and neck cancer patients (29). We demonstrated that HPV$^+$ HNSCC cells were more sensitive to 5-azacytidine than were HPV$^-$ negative cells, and that sensitivity was at least partially due to reactivation of p53 in HPV$^+$ tumors. 5-azacytidine treatment correlated with decreased expression of HPV genes and activated caspases in HPV$^+$ HNSCC cells and patient tumors. In addition, 5-aza therapy significantly delayed growth of xenografted HPV$^+$ HNSCC tumors and decreased levels and activity of matrix metalloproteinase enzymes suggesting that 5-azacytine may inhibit malignant spread.

Materials and Methods

Cell lines, constructs, and chemicals

We used HPV$^-$ (SCC61, SCC25, JHU012, and FaDu) and HPV$^+$ (SCC090, UMSCC47, UMSCC104) HNSCC cell lines. All HPV$^-$ cells were cultured in DMEM/F12 medium supplemented with 0.4 μg/mL hydrocortisone, and all HPV$^+$ cell lines were grown in DMEM with nonessential amino acids. All media were supplemented with 10% FBS (Invitrogen), 50 μg/mL penicillin, and 50 μg/mL streptomycin (Invitrogen). All cell lines have been tested negative for Mycoplasma and microsatellites authenticated.

To establish primary HNSCC cultures, surgical specimens were collected in PBS from consented patients within 30 minutes of resection. Tissue was cut into approximately 5-mm^3 pieces, disinfected by immersion in 70% ETOH for approximately 1 minute, rinsed with PBS four times, and digested in 0.05% trypsin-EDTA supplemented with Collagenase type 1A (200 U/mL; Sigma C-9722) in a vented flask at 37°C with 5% CO$_2$ for 10–20 minutes. Digestion was stopped by adding 1 volume of FBS (Sigma). After centrifugation at 1,500 rpm for 5 minutes, the supernatant was aspirated, the cells were resuspended in keratinocyte serum-free medium with supplements and 10% FBS, strained through a 100-μm nylon cell strainer (BD Falcon; Becton Dickinson Labware), plated in keratinocyte serum-free medium with supplements (Gibco/Invitrogen) and 10% FBS onto 0.1% gelatin (Millipore) coated plates, and grown at 37°C in a 5% CO$_2$ incubator. The next day, cells were washed with PBS and grown in keratinocyte serum-free medium with supplements until they reached approximately 90% confluence. After that, the cells were detached with 0.05% trypsin-EDTA, the reaction was stopped with defined trypsin inhibitor (Gibco), and the cells were plated onto uncoated plates in keratinocyte serum-free medium with supplements and used in experiments.

Control and p53 shRNAs in a retroviral vector were from Origene. P53 luciferase reporter, p-super and p-super p53 shRNA-expressing vectors were a gift from Galina Selivanova (Karolinska Institute). Cells were transfected using Lipofectamine 2000 (Invitrogen) according to manufacturer's recommendations.

Control and p53 shRNA constructs were transfected into Phoenix, and supernatant containing viral particles was harvested for the infection of cells in the presence of 5 μg/mL of polybrene (EMD Millipore).

P53 reporter

UMSCC47, UNC7, and SCC35 cells were grown in 6-well plates and transfected with p53 luciferase reporter plasmid; 6 hours later, the cells were treated or not with 3 μmol/L of 5-aza. The cells were collected 24 or 48 hours after treatment and lysed in Luciferase Cell Culture Lysis Reagent (Promega). Ten micrograms of each cell lysate and 100 μL of Luciferase Assay Reagent (Promega) were used to measure luminescence in the plate-reading luminometer.

5-Azacytidine, zeocin, carboplatin, and pifithrin-α were obtained from Sigma.

Immunoblotting

Cells were collected by trypsinization and lysed in radioimmunoprecipitation assay (RIPA) lysis buffer (Sigma) with the addition of protease inhibitors (Roche) and phosphatase inhibitors (Sigma) for 15 minutes on ice as described previously (30, 31).

Frozen tissue was cut into small (∼1–3 mm) pieces and homogenated in RIPA buffer. Insoluble material was removed by centrifugation at 14,000 rpm for 15 minutes at 4°C. Proteins were separated in 10% Tris-glycine polyacrylamide gels (Mini-PROTEAN; Bio-Rad) and electrophoretically transferred onto polyvinylidene difluoride membranes. Membranes were blocked with 3% BSA in PBS and incubated with antibodies against p53, GAPDH and PARP-1 (Santa Cruz Biotechnology), and DNMT1 from Cell Signaling Technology. After incubation with primary antibodies, membranes were washed, incubated with secondary DyLight anti-mouse and anti-rabbit antibodies (Thermo Scientific), and signals was visualized using a Bio-Rad imager.

Zymography. A total of 1×10^5 of UMSCC47 or SCC090 cells were plated in growth media in 12-well plates. The next day, the cells were treated or not with 5-aza in 0.5 mL of DMEM supplemented with 1% FBS. Twenty-four or 48 hours after treatment, the media were collected, and the cells were trypsinated, collected, and counted using Trypan Blue (Invitrogen). Volumes of media corresponding to 1×10^4 live cells were mixed with 2XNovex Tris-Glycine SDS Sample Buffer (Invitrogen), incubated for 10 minutes at room temperature, and loaded in Novex 10% Zymogram (Gelatin) Protein Gels (Invitrogen). After electrophoresis, gels were incubated in $1\times$ Zymogram Renaturing Buffer (Invitrogen) for 30 minutes at room temperature with gentle agitation, and equilibrated in $1\times$ Zymogram. They were exposed to developing buffer (Invitrogen) for 30 minutes at room temperature with gentle agitation, developed at 37°C in fresh developing buffer overnight, and stained with SimplyBlue Safestain (Invitrogen).

CCL5 ELISA. Human RANTES/CCL5 ELISA Kit was obtained from Sigma. A total of 1×10^5 of UMSCC47 cells were plated in growth media in 12-well plates. The next day, the cells were treated or not with 5-aza in 0.5 mL of DMEM supplemented with 1% FBS. Twenty-four, 48, or 72 hours after treatment, the media were collected, and the cells were trypsinized, collected, and counted using Trypan Blue (Invitrogen). ELISA was performed according to the kit instructions, and the results were normalized to the number of cells.

Caspase activity assay

Frozen tissue was cut into small (∼1–3 mm) pieces, homogenated in cell lysis buffer, and sonicated on ice. Insoluble material was removed by centrifugation at 14,000 rpm for 15 minutes at 4°C. Caspase-3/7 activity was assessed using 50 μg of lysates and Caspase-3 Activity Assay Kit (Cell Signaling Technology) according to the manufacturer's instructions.

Survival assay

All cells lines, except SCC090 and primary cultures, were seeded in 12-well plates at a density of 1,000 cells/well in duplicate and treated with increasing doses of 5-aza the following day. SCC090 and primary cultures were plated at a density of 10,000 cells/well. After 7 days, the cells were trypsinized and counted using Trypan Blue Solution (Invitrogen) to determine the number of live cells. Alternatively, we used CellTiter-Glo reagent (Promega). The data presented were obtained from at least two independent experiments. Two-tailed *t* test was used to determine statistical significance of the differences between untreated cells and cells treated with the various concentrations of 5-aza.

RNA extraction and quantitative RT-PCR

Total RNA was extracted from cells, blood, or tissue by Qiagen RNA extraction kit and cDNA was synthesized using iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions. Quantitative real-time reverse transcription (qRT-PCR) was done using iQ SYBR Green Supermix (Bio-Rad) and primer pairs indicated in the Supplementary Table S1 on the iCycler iQ Real-Time PCR Detection System (Bio-Rad). Each qRT-PCR reaction was done in at least duplicate, and the $\Delta\Delta C_t$ method was used to analyze the data. RT-PCR primers for *MMP1* and *MMP10*, *IFIT1*, *IFIT3*, and *CCL5* were from Origene.

Illumina microarray

A total of 1×10^6 of UMSCC47, SCC090, or U2OS cells were treated or not with 3 μmol/L of 5-aza in independent triplicate for 48 hours; RNA was extracted and analyzed on Human HT gene expression array at Yale Genomics facility. The data have been deposited in the ArrayExpress database under accession number E-MTAB-6055.

DNA purification

DNA from cells and tumors was isolated using Qiagen DNeasy Blood & Tissue Kits according to the suggested protocol.

Line-1 methylation

We used Global DNA Methylation LINE-1 Kit from Active Motif to assess Line-1 methylation status in cells and tumors.

In vivo experiments

The *in vivo* study was approved by the local animal experimental ethical committee. Exponentially growing UMSCC47 or SCC090 cells were injected subcutaneously into the sacral area of female NUDE mice. Each mouse was inoculated with 2×10^5 cells in 50% Matrigel and 50% PBS at a volume of 100 μL. Body weight, tumor growth, and general behavior were monitored. Tumor volumes were measured every 3 days. Mice were sacrificed when the tumor exceeded a size of 1 cm^3.

Patient population

We used tumor specimens derived from research-consented patients with HNSCC enrolled in a window 5-aza trial (29).

IHC

Specimens were fixed with 10% formalin and embedded in paraffin per routine of the surgical pathology division. Sectioning, hematoxylin & eosin staining, and immunostaining with the antibody against Ki67 were performed by the Yale Pathology Core Facility.

Statistical analysis

Two-way ANOVA analysis was used to compare 5-aza–treated and untreated mouse groups. Other statistical analyses were done using Fisher exact and *t* test.

Results

HPV$^+$ head and neck cancer cells are sensitive to 5-aza partially due to stabilization and activation of p53

To determine whether HNSCC cells were sensitive to 5-aza, two HPV$^+$ HNSCC cell lines and one HPV$^+$ primary culture were compared with three HPV$^-$ HNSCC cell lines and one HPV$^-$
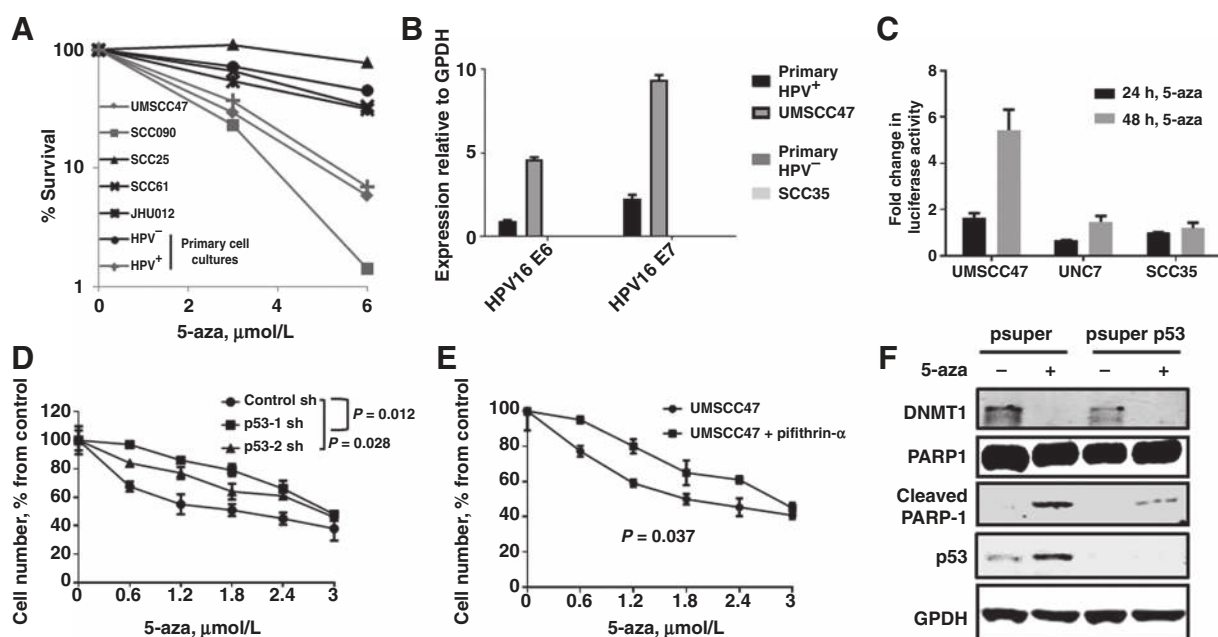
Figure 1.
HPV$^+$ head and neck cancer cells are sensitive to 5-aza partially due to stabilization and activation of p53. **A,** HPV$^+$ and HPV$^-$ head and neck cancer cells (cell lines and primary cells derived from patient specimens) were treated with increasing concentration of 5-aza; cell viability was assessed by Trypan blue exclusion 7 days after the treatment. Results are from two independent experiments. **B,** Expression of HPV16 E6 and E7 genes in the primary cultures; HPV$^+$ UMSCC47 and HPV$^-$ SCC35 cells were used as positive and negative controls, respectively. **C,** Fold induction in p53 transactivation activity (luciferase) in p53 luciferase reporter expressing HPV$^+$ (UMSCC47) and HPV$^-$ (UNC7 and SCC35) cells treated with 3 μmol/L of 5-aza as compared with untreated cells. Data from three independent experiments are presented. **D,** Survival of UMSCC47 cells, expressing control or two different p53 shRNAs and treated with increasing concentrations of 5-aza. Data from two independent experiments are presented. **E,** Survival of UMSCC47 cells pretreated with p53 inhibitor pifithrin-α and treated with 5-aza. Data from two independent experiments are presented. **F,** DNMT1, p53, and PARP-1 immunoblot of lysates from UMSCC47 expressing control (psuper) of p53 (psuper p53) shRNAs cells treated or not with 5-aza.

primary culture. The HPV status of primary cultures was confirmed by HPV16 E6 and E7 expression (Fig. 1B), and their squamous epithelial etiology was validated by the expression of cytokeratins (Supplementary Fig. S1A). Growth inhibitory activity of 5-aza was found in both HPV$^+$ and HPV$^-$ cells, but HPV-associated cell lines and primary culture were markedly more sensitive (Fig. 1A, HPV$^+$ in gray).

Restoring p53 protein expression and tumor suppressor functions has been suggested as a promising strategy to combat HPV$^+$ cancer, and the 5-aza structural analogue 5-aza-2′-deoxycytidine (decitabine) was recently found to increase p53 protein levels in HPV$^+$ cervical and head and neck cancer cells (32). p53 is a powerful transcription factor able to transactivate proapoptotic and growth-inhibitory genes. In addition, p53 induces apoptosis through transcriptional repression of another set of genes, and through direct activation of the mitochondrial apoptotic pathway (33, 34). To determine whether 5-aza treatment enhanced p53 transactivation function, cells transiently transfected with a p53-responsive luciferase reporter were treated with 5-aza for 24 and 48 hours. After 24 and 48 hours of treatment, HPV$^+$ UMSCC47 cells had increased p53 reporter activity. After 48 hours, 5-aza–treated HPV$^+$ HNSCC UMSCC47 cells had markedly increased p53-dependent luciferase activity (∼6-fold), whereas HPV-negative and p53 wild-type UNC7 cells had a more modest increase (less than 2-fold; Fig. 1C). As expected, SCC35 cells with mutant p53 did not induce luciferase.

To determine whether increased sensitivity of HPV$^+$ HNSCC to 5-aza relied on activation of p53, UMSCC47 cells were infected with retroviral constructs expressing control or two different p53 shRNAs. Depletion of p53 (Supplementary Fig. S1B) decreased sensitivity of UMSCC47 cells to 5-aza treatment (Fig. 1D), as did pretreatment of the cells with p53 inhibitor pifithrin-α (35), which inhibits p53 transactivation function (Fig. 1E). In line with elevated p53 transactivation activity in HPV$^+$ UMSCC47 cells (Fig. 1C), 5-aza stabilized p53 protein level in these cells, and as expected, p53 shRNA expression decreased p53 levels regardless of 5-aza therapy (Fig. 1F). Increased PARP-1 cleavage was found following 5-aza treatment of UMSCC47 cells, and PARP-1 cleavage was reduced following p53 depletion by shRNA (Fig. 1F). Together, these data suggest that apoptosis is at least partially responsible for reduced survival of HPV$^+$ UMSCC47 cells after 5-aza, and that apoptotic cell death is partially driven by p53. Upon incorporation into DNA, 5-aza traps DNMT1 on chromatin thus removing it from the soluble fraction. The absence of DNMT1 in 5-aza–treated samples confirms 5-aza incorporation (Fig. 1F).

5-aza treatment reduces expression of all HPV genes in head and neck cancer cells

As p53 protein in HPV$^+$ cells is kept at low levels through proteasomal degradation as a result of the HPV E6 oncoprotein,
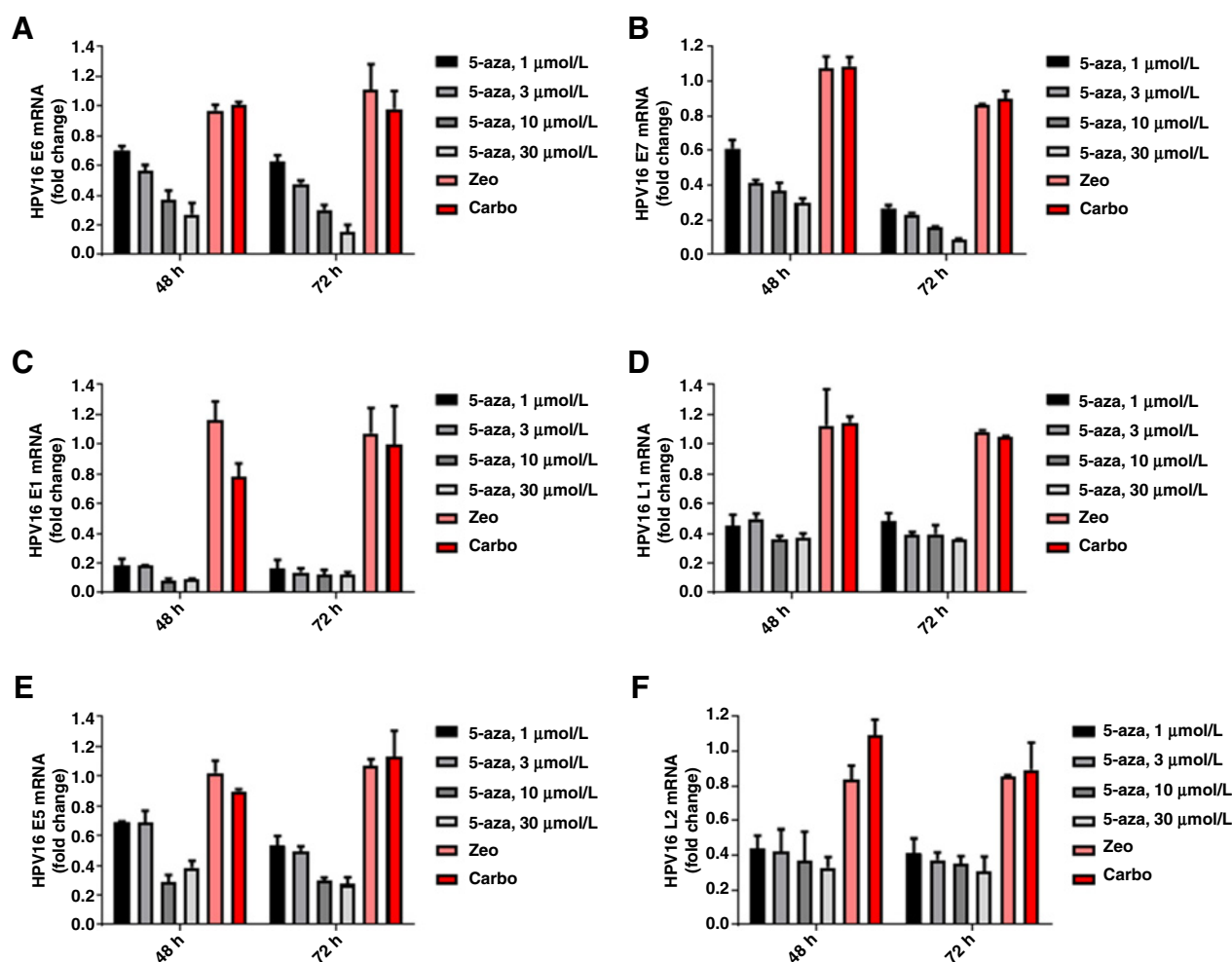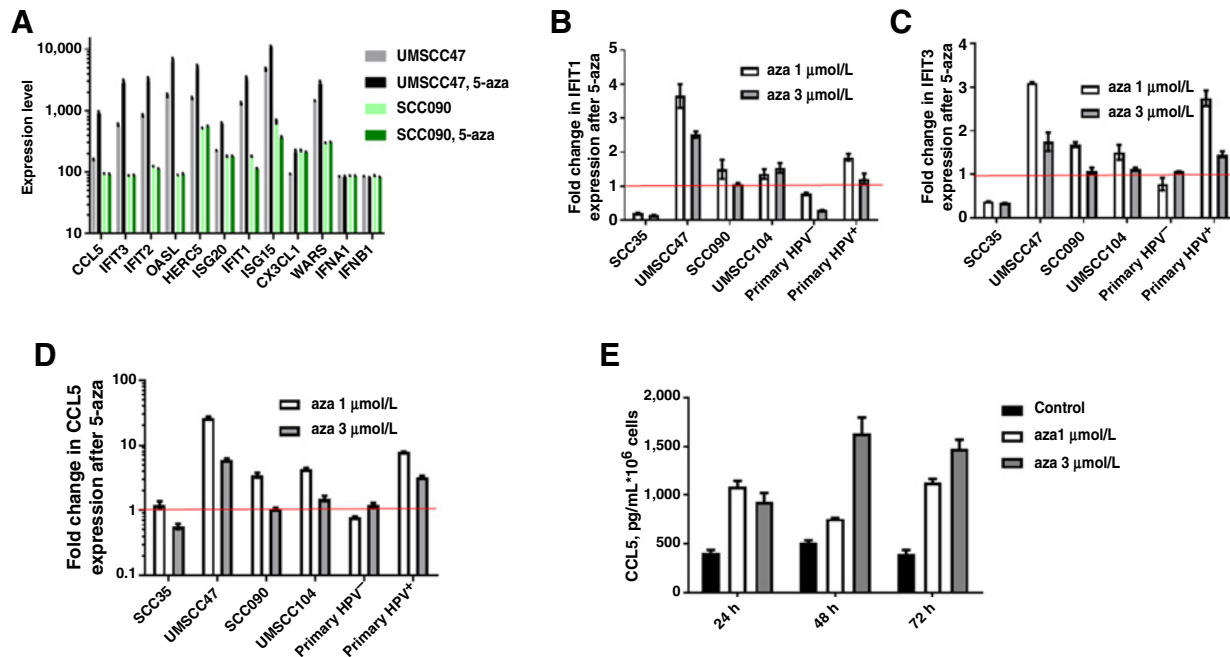
Figure 2.
5-aza treatment reduces expression of all HPV genes in head and neck cancer cells. Fold change in expression of HPV16 genes 48 and 72 hours after treatment with 5-aza, 20 μg/mL of zeocin or 3 μg/mL of carboplatin. GAPDH was used as the control gene and data from three independent experiments are presented.

we determined whether the increase in p53 levels following 5-aza therapy was accompanied by changes in *E6* expression. Consistent with a recent report using decitabine as the demethylating agent (32), 48 or 72 hours of 5-aza treatment decreased HPV *E6* expression in HPV+ HNSCC cells (Fig. 2A). High concentrations of demethylating drugs were shown to induce DNA damage in cells (27). To test whether DNA damage after 5-aza treatment contributed to 5-aza–induced p53 upregulation, we treated UMSCC47 cells with radiomimetic drug zeocin, or 1 or 3 μmol/L of 5-aza—the concentrations that significantly downregulated HPV *E6* expression. As expected, zeocin induced DNA damage, as indicated by increased phosphorylation of H2AX, and upregulated p53 protein (Supplementary Fig. S2). Both concentrations of 5-aza trapped DNMT1 to DNA and increased p53 levels, but did not induce DNA damage (Supplementary Fig. S2). In contrast to zeocin, 5-aza did not phosphorylate p53 at Ser15 (Supplementary Fig. S2).

The major HPV oncogenes, *E6* and *E7*, are critical for HPV tumorigenesis and for evasion of apoptosis by infected cells

through E6- and E7-dependent inhibition of p53 and Rb family members, respectively (36). As was observed with HPV *E6*, 5-aza therapy also decreased expression of the *E7* oncogene (Fig. 2B). Although not well studied, expression levels of HPV genes in addition to *E6* and *E7* have been reported to alter cellular proliferation, viability, and/or survival of HPV+ cancer cells (37, 38). Expression analyses revealed that 5-aza treatment decreased expression of all HPV genes in UMSCC47 cells (Fig. 2C–F) and in the HPV+ head and neck cancer cell line SCC090 (Supplementary Fig. S3). HPV+ UMSCC47 cells were also treated with DNA damaging agents (carboplatin and zeocin) to determine whether decreased survival or DNA damage was sufficient to downregulate HPV gene expression. Carboplatin and zeocin therapy did not noticeably change *E6* or *E7* expression (Fig. 2A and B, red). Likewise, expression of other HPV genes was not markedly altered by carboplatin or zeocin (Fig. 2C–F, red). These data suggest that inhibition of HPV genes expression is specific to 5-aza and is not a general effect of DNA damage or cellular toxicity.

Figure 3.
5-aza activated IFN response in some HPV$^+$ head and neck cancer cells. **A,** Expression of IFN-responsive genes in HPV$^+$ SCC090 or UMSCC47 cells, treated or not with 3 μmol/L of 5-aza for 48 hours, as determined in Illumina microarray. Fold change in expression of IFIT1 (**B**), IFIT3 (**C**), or CCL5 (**D**) genes 48 hours after treatment with 1 or 3 μmol/L of 5-aza in HPV$^+$ (UMSCC47, UMSCC104, SCC090, and a primary culture) and HPV$^-$ (SCC35 and a primary culture) head and neck cancer cells. GAPDH was used as the control gene, and data from two independent experiments are presented. **E,** CCL5 protein detected by ELISA in a growth media of UMSCC47 cells treated or not with 1 or 3 μmol/L of 5-aza for 24, 48, or 72 hours.

5-aza induced IFN response in some HPV$^+$ head and neck cancer cells

Next, we hypothesized that p53 is not the only cytotoxic pathway upregulated in HPV$^+$ cells after 5-aza treatment, and that additional mechanisms may contribute to the increased sensitivity of HPV$^+$ HNSCCs to demethylation. To determine these additional pathways, we identified genes whose expression was changed after 48 hours of 3 μmol/L 5-aza treatment in HPV$^+$ head and neck cancer cell lines UMSCC47 and SCC090 using Illumina microarray gene expression profiling. We found that many genes upregulated after 5-aza treatment in UMSCC47 cells, but not in SCC090 cells, were known transcriptional targets of type I IFNs (Fig. 3A). To validate the microarray result, we treated UMSCC47, SCC090, an additional HPV$^+$ head and neck cancer cell line UMSCC104, HPV$^-$ head and neck cancer cells SCC35, as well as HPV$^+$ and HPV$^-$ primary cultures, with 1 or 3 μmol/L of 5-azacytidine for 48 hours and performed qRT-PCR to determine expression of *IFIT1*, *IFIT3*, and *CCL5* genes. Interestingly, 1 μmol/L of 5-aza induced expression of these three genes in all HPV$^+$, but not HPV$^-$ cells, although very modest (less than 2 times) upregulation of *IFIT1* and *IFIT3* was noticed in SCC090 and UMSCC104 cells (Fig. 3B and C). In contrast, 3 μmol/L of 5-aza significantly elevated expression of *IFIT1* and *IFIT3* only in UMSCC47 cells. The inverse correlation of gene expression levels in HPV$^+$ cells and 5-aza concentration was also observed for *CCL5* (Fig. 3D), and again, the most pronounced change in *CCL5* expression after 5-aza treatment was found in UMSCC47 cells. In addition, we detected increased release of CCL5 in a

growth media of UMSCC47 cells after 24, 48, or 72 hours of treatment with 5-aza (Fig. 3E).

5-aza delays HPV$^+$ tumor growth and inhibits proliferation in a mouse model

To investigate activity of 5-aza against HPV$^+$ tumors *in vivo*, mice bearing HPV-associated head and neck UMSCC47 or SCC090 xenografts were treated with 2 mg/kg of 5-aza. 5-aza treatment significantly suppressed the growth of tumors without causing weight loss in treated mice (Fig. 4A and B). All mice were sacrificed on the same day when the largest tumor approached 1 cm^3. Subsequently, mice bearing UMSCC47 tumors were sacrificed 3 days after the last dose of 5-aza and mice harboring SCC090 tumors were sacrificed on the day of their final dose. Upon sacrifice of mice, tumors were collected and stained with Ki-67 as a marker of proliferation. 5-aza inhibited proliferation in HPV$^+$ xenografts as measured by the percentage of cells expressing Ki-67 (Fig. 5). Proliferation at the periphery of the tumor was inhibited in UMSCC47 from 70% to 15% and in SCC090 from 83% to 32% (Fig. 5). The timing of the last dose of 5-aza did not seem to greatly alter its effect on proliferation when comparing these two xenografted cell lines.

5-aza inhibits expression and activity of MMPs and inhibits invasion in a mouse model

Cancer cell invasion and intravasation represent a first and limiting step during metastasis that is strictly dependent on the function of matrix metalloproteinases (MMP) to degrade the
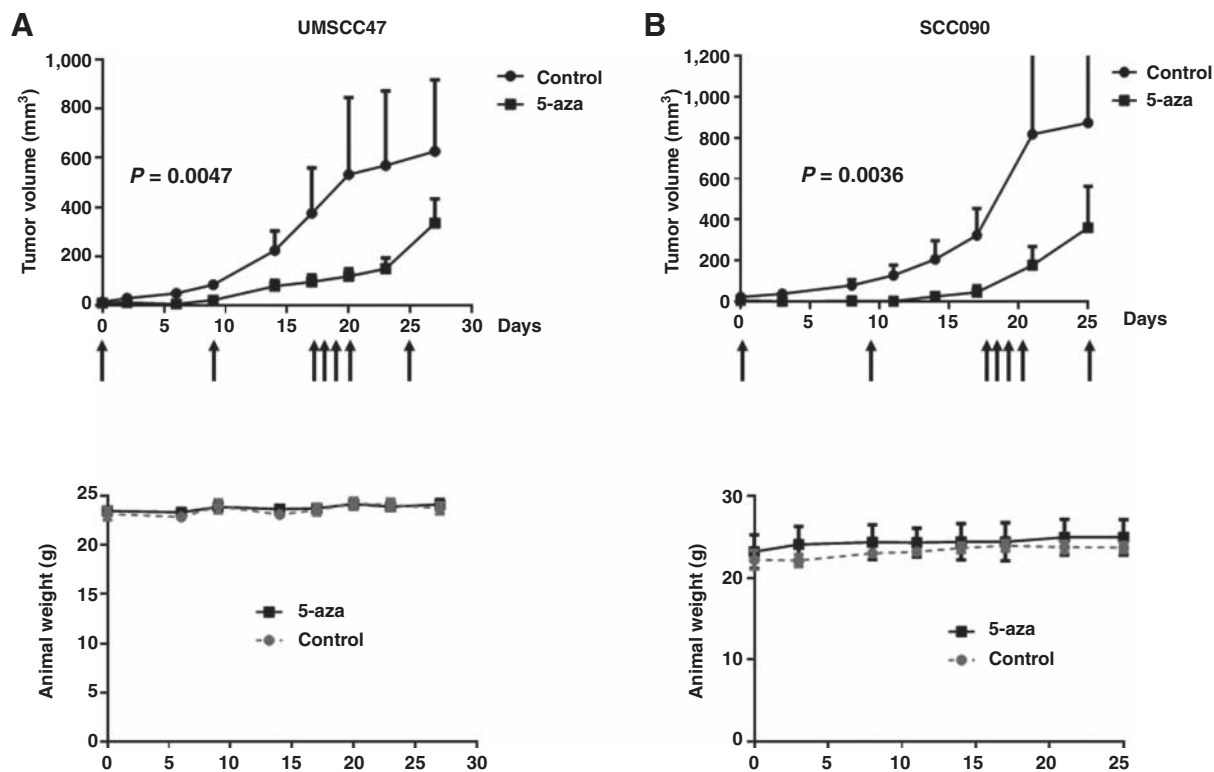
Figure 4.
5-aza delays HPV[+] tumor growth in mice. HPV[+] UMSCC47 (**A**) or SCC090 (**B**) cells were inoculated into nude mice. When tumors became palpable, mice were treated with 2 mg/kg of 5-aza or vehicle (5 mice in each group) at days indicated with arrows. Tumor volume (*P* value calculated with ANOVA; top) and animal weight (bottom) are presented.

extracellular matrix (39). MMPs 1 and 10 have been shown to promote tumor cell invasion and metastasis in diverse cancer types, including head and neck cancers regardless of HPV status (40, 41). To begin determining whether demethylation may alter cancer cell behaviors in addition to proliferation and survival, HPV[+] UMSCC47 xenografted tumors from Fig. 4 were analyzed for expression of *MMP10*. Treatment with 5-aza significantly decreased levels of *MMP10* mRNA in tumors (Fig. 6A). Results were confirmed in cell culture, where 48 hours of 5-aza inhibited expression of *MMPs 1* and *10* in two HPV[+] HNSCC cell lines (Fig. 6B and C) and in an HPV[+] head and neck primary culture (Supplementary Fig. S4). 5-aza treatment also decreased secretion of active MMPs into culture media from both UMSCC47 and SCC090 cells, as measured by degradation of gelatin (Fig. 6D and E) and casein (Supplementary Fig. S5A), which are substrates for both MMP1 and MMP10 (42). Gelatin is also an ideal substrate for MMP2 and MMP9; however, we did not find any significant changes in the expression of *MMP2* or *9* in HPV[+] head and neck cancer cells after 5-aza treatment (Supplementary Fig. S5B).

Given that 5-aza treatment decreased MMP expression and secretion from HPV^{+} cells, we performed preliminary experiments to determine whether demethylation therapy may alter the ability of HPV[+] xenografted tumors to invade mouse blood vessels by assessing expression of human *GAPDH* in cDNA isolated from blood collected from UMSCC47 tumor-bearing mice. Human *GAPDH* mRNA was found in blood of 4 of 5

control mice, but in only one 5-aza–treated mouse (Fig. 6F). We realize that increased human *GAPDH* mRNA in blood could result from exosomes or free RNA; however, given the effect of demethylation on *MMP* expression, this result is consistent with decreased numbers of circulating tumor cells in xenograft bearing mice following 5-aza therapy. Additional studies will be required to further characterize this effect.

5-aza downregulates HPV gene expression, stabilizes p53, and induces apoptosis in HPV[+] HNSCC in patients

Based largely on the cellular and *in vivo* preclinical data presented above, a window clinical trial was opened at the Yale Cancer Center to assess the tumor cell activity and safety of single-agent 5-aza administered at 75 mg/m^2/d for 5 or 7 days in patients with HPV[+] and HPV[−] HNSCC (29). To determine whether 5-aza treatment induced DNA demethylation in head and neck tumors, we analyzed methylation status of transposable Long Interspersed Nuclear Element 1 (*Line-1*), which is used as a marker for global DNA methylation. CpG islands in *Line-1* sequences were significantly demethylated in all five HPV[+] and one HPV[−] patient tumors tested (Supplementary Fig. S6A). *Line-1* demethylation levels after 5-aza treatment in tumors were comparable with *Line-1* demethylation in HPV^{+} UMSCC47 cells treated with 5 μmol/L of 5-aza for 72 hours (Supplementary Fig. S6B). In agreement with results obtained in HPV[+] head and neck cancer cells (Fig. 2), 5-aza treatment of patients resulted in decreased expression of HPV
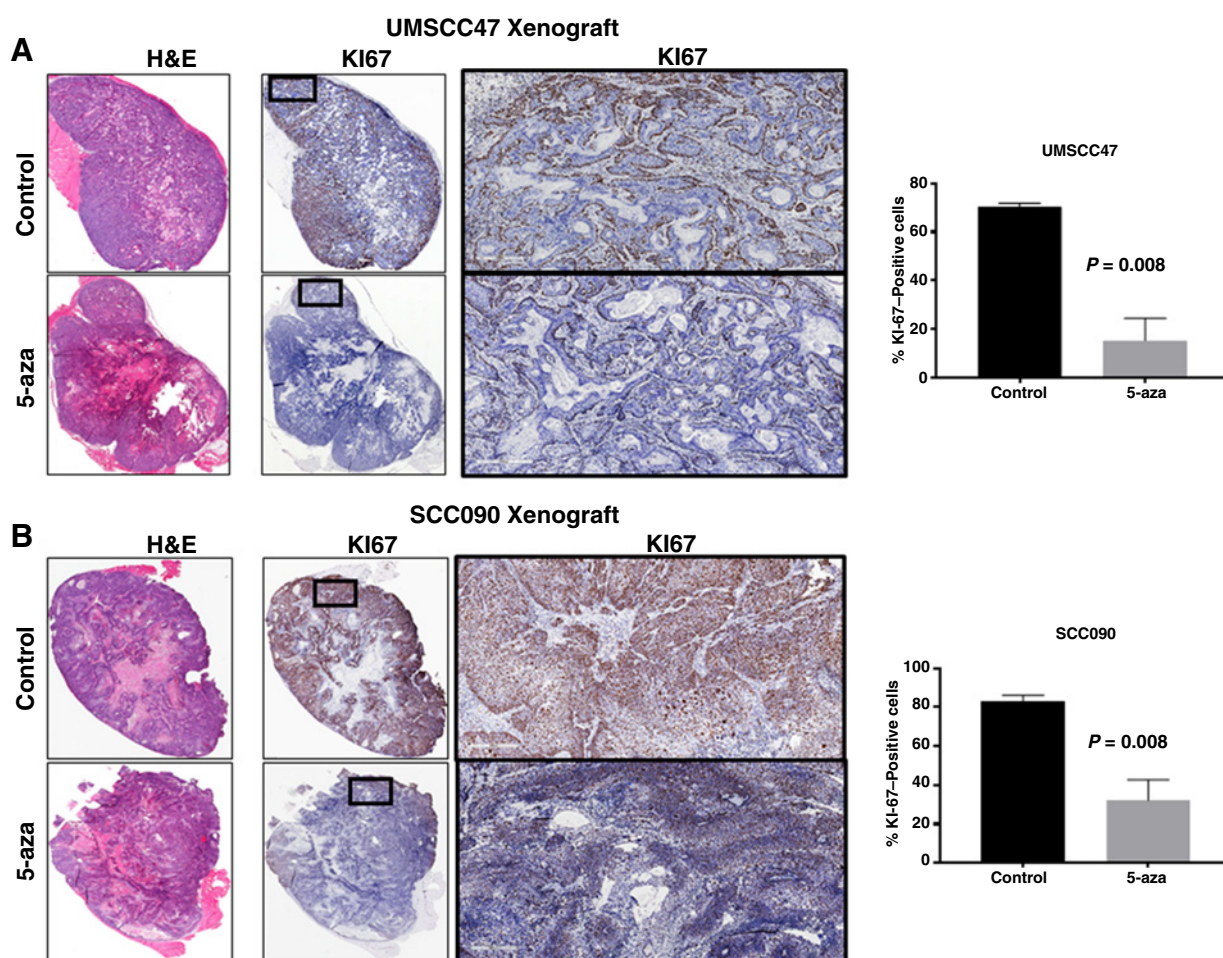
Figure 5.
5-aza constrains proliferation in HPV$^+$ tumors. H&E and KI-67 staining of representative tumors from Fig. 4. **A,** UMSCC47 xenograft. **B,** SCC090 xenograft. Proliferative index was calculated from five different high-power fields at the periphery, with at least 100 cells counted at each field.

oncogenes *E6* and *E7* in 5 of 5 tumors tested (Fig. 7A). Five or 7 days of patient treatment with 5-aza also significantly increased caspase-3/7 activity (cleavage of the fluorescent substrate AC-Devd-AMC) in 4 of 5 HPV$^+$ tumors, but not in an HPV$^-$ tumor (Fig. 7B). As observed in cell lines, 5-aza therapy resulted in marked increases in p53 protein levels in all HPV$^+$ tumors tested (mean of 18-fold, Fig. 7C). Consistent with 5-aza–induced inhibition of MMPs expression and activity in HPV$^+$ cells and xenografted tumors (Fig. 6), we detected substantial reduction of *MMP1* and *MMP10* mRNA levels in tumors from HPV$^+$ patients after 5-aza treatment (Fig. 7D). In contrast, 5-aza markedly upregulated expression of both *MMPs* in HPV-negative HNSCC. A similar 5-aza–induced increase in *MMP1* mRNA levels was detected in HPV$^-$ head and neck cancer cell lines (Supplementary Fig. S7).

Molecular analyses of tumors derived from patients treated with 5-aza for a short window are consistent with preclinical experiments using cell lines, cell cultures, and xenografts. Together, these data provide strong evidence that 5-aza was toxic to HPV$^+$ HNSCC and resulted in decreased proliferation and increased apoptosis. Mechanistically, 5-aza therapy inhibited expression of

HPV genes and increased p53 protein levels, resulting in p53-mediated cell death (confirmed only *in vitro*).

Discussion

New therapies are needed for HNSCC, and HPV$^+$ disease is a priority because of its rapidly increasing incidence. The Cancer Genome Atlas (TCGA) and others have cataloged molecular distinctions between HPV$^+$ and HPV$^-$ HNSCC, and these differences can be mined to identify new targets. We took advantage of an epigenetic distinction of HPV$^+$ HNSCC to test the effects of demethylation therapy in cell lines, primary cultures, xenograft mouse models, and in tumors from head and neck cancer patients treated with a short course of 5-azacytidine. Drug therapy to decrease HPV gene expression may be effective to treat HPV$^+$ cancers, as depletion of E6 and E7 resulted in reduced cell growth and increased apoptosis. IFNα, -β, and -γ have been used to treat HPV diseases, but their effect on HPV gene expression is variable with increases in some cells and decreases in others (43). Consistent with a recent report showing that demethylation decreased *E6* and *E7* expression (32), we showed that 5-aza decreased
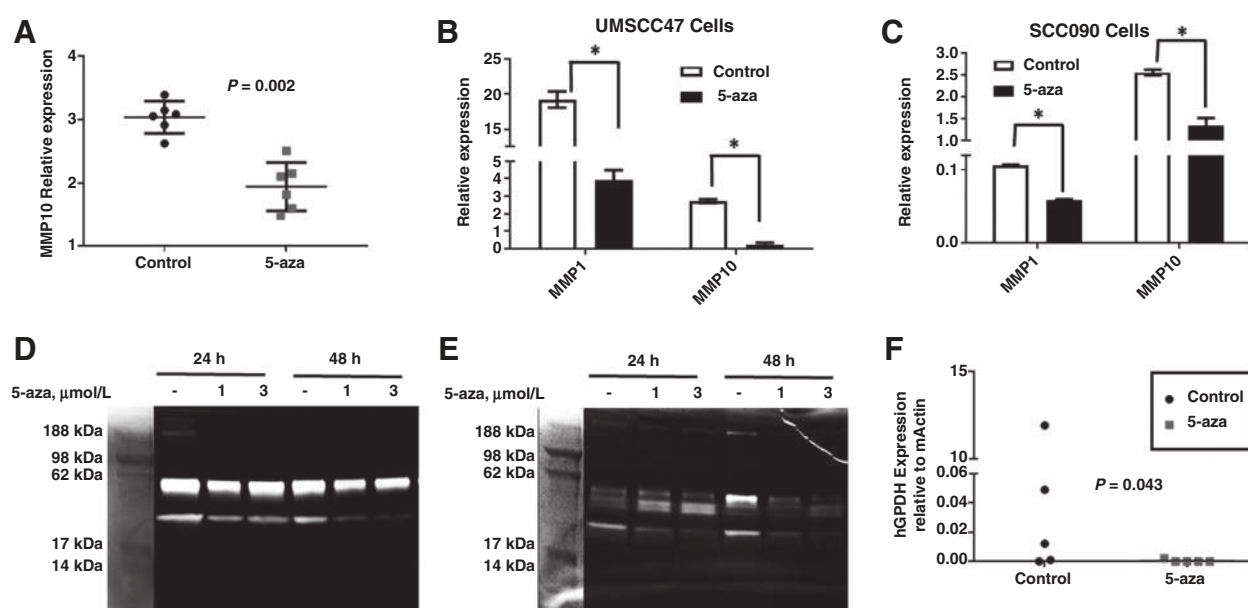
Figure 6.
5-aza inhibits MMPs and detection of cancer-derived cDNA in blood. **A,** Expression of MMP10 in UMSCC47 tumors derived from mice treated or not with 5-aza. GAPDH was a reference gene for qRT-PCR. MMP1 and MMP10 mRNA levels in UMSCC47 (**B**) or SCC090 (**C**) cells treated or not with 3 μmol/L of 5-aza for 48 hours as determined by qRT-PCR with GAPDH as a reference gene. Zymogram gel showing activity of proteases that use gelatin as a substrate in growth medium from UMSCC47 (**D**) or SCC090 (**E**) cells treated or untreated with 5-aza. **F,** Expression of human GAPDH relative to mouse actin gene (%) in blood collected from UMSCC47 tumor-bearing mice on day 25 (P value calculated with two-tailed t test). *, P < 0.05.

expression of all HPV16 genes, including the major oncoproteins E6 and E7 in HPV+ HNSCC (Figs. 2 and 7). In cervical cancer, E6 and E7 expression has been linked to hypermethylation and tumor invasiveness (44–46). Although uterine cervical cancer and HNSCC are distinct diseases, demethylating the host genome in HPV-associated tumors may begin to reverse the fundamental steps of HPV-driven carcinogenesis and remove protective measures that allow the expression of viral genes. Among several interesting implications, demethylation treatment may even play a role in preventing HPV+ carcinogenesis if patients can be identified at the stage of active HPV oropharyngeal infection or premalignancy.

Mechanistically, it is unclear how demethylation alters HPV gene expression, but effects on expression of cellular transcription factors or transcription repressors following demethylation could mediate downregulation of HPV gene expression. Recently, modulation of transcriptional factor activator protein-1 (AP-1) composition was shown to alter expression of HPV genes (47). AP-1 consists of several proteins from Jun, Fos, and ATF families that form homo- or heterodimers. Two AP-1–binding sites located in the HPV upstream regulatory region (URR) were identified (48), and it remains to be tested whether 5-aza treatment initiates changes in AP-1 content that may in turn affect expression of HPV genes. In addition, demethylation could alter many factors shown to restrict HPV transcription including miRNAs, apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC), and innate immune effectors (reviewed in ref. 49).

The finding that p53 activity is stimulated by 5-aza treatment (Fig. 1) is exciting and further speaks to the potential role of 5-aza in the treatment of HPV+ HNSCC, as reactivation of p53 has long

been an attractive goal in cancer therapy (50). Although p53 is mutated in 84% of HPV− HNSCC tumors, it is rarely mutated in HPV+ HNSCC and is instead suppressed by the HPV E6 viral oncoprotein (6). Thus, in HPV+ HNSCC, 5-aza treatment leads to the stabilization of wild-type p53 (Figs. 1 and 7), with subsequent apoptosis (Figs. 1 and 7) and inhibition of proliferation (Figs. 1, 4, and 5). Mechanistically, the 5-aza–induced p53 reactivation is likely secondary to the downregulation of HPV E6 and release of p53 suppression, as low concentrations of 5-aza (1 or 3 μmol/L) significantly inhibited expression of HPV E6 (Fig. 2), trapped DNMT1 to DNA, upregulated p53 (Fig. 1; Supplementary Fig. S2), but did not induce DNA damage (Supplementary Fig. S2) that was shown to activate p53 in HPV+ cells (30). However, DNA damage, induced by increasing concentrations of 5-aza (27), can further contribute to p53 stabilization. We recently reported that HPV is not integrated in a significant portion of HPV+ HNSCC suggesting that HPV episomal maintenance is required in these tumors (8, 14). p53 activity in cells with mutated E6 prevents HPV genome replication (51), and likewise inhibition of E6 expression by demethylation may similarly limit HPV genome replication in cells lacking integration. Reactivation of p53 through decreased E6 expression provides yet another axis through which HPV+ HNSCC cells may be specifically targeted by demethylation.

Interestingly, 5-aza treatment induced expression of type I IFN genes in some, but not all, HPV+ cells tested (Fig. 3). Given that (i) increasing 5-aza concentrations from 1 to 3 μmol/L diminished induced expression of IFN target genes (Fig. 3), while no significant toxicity was observed in HPV+ cells after 1 μmol/L of 5-aza (Fig. 1), (ii) the absence of detectable changes in IFNα and IFNβ expression after 5-aza (Fig. 3), and (iii) the selectivity of IFN
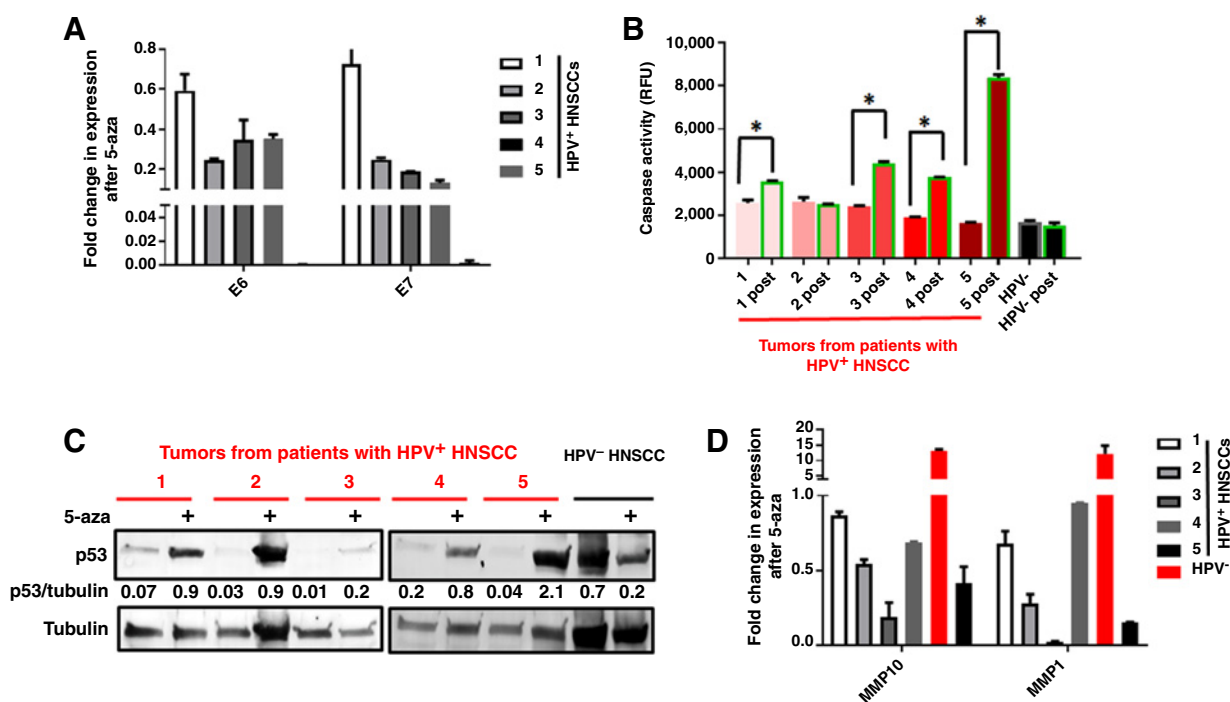
Figure 7.
5-aza downregulates HPV and MMP expression, stabilizes p53, and induces apoptosis in HPV$^+$ HNSCC in patients. **A,** Fold change in expression of HPV E6 and E7 in tumors from 5 different patients after 5 or 7 days of 5-aza treatment. **B,** Caspase-3/7 activity in tumors from 5 HPV$^+$ and 1 HPV$^-$ patients after 5-aza treatment. Data represents assay performed in duplicate. **C,** p53 protein levels in tumors from patients after 5-aza treatment. The ratio of p53 to tubulin loading control is calculated. **D,** Fold change in expression of MMP1 and MMP10 in tumors from 6 different patients with HNSCC (5 HPV$^+$ and 1 HPV$^-$) after 5 or 7 days of 5-aza treatment. *, $P < 0.05$.

pathway activation to some HPV$^+$ cells, we do not believe that concentration-limited activation of IFN response substantially contributed to 5-aza toxicity in HPV$^+$ cells. Moreover, treatment of p53-null, but not p53 wild-type cells with the 5-aza analogue 5-aza-dC was shown to result in cytotoxic IFN response due to transcriptional derepression of repetitive elements (TRAIN; ref. 52), whereas HPV$^+$ cancer cells nearly always harbor wild-type p53, which is induced and activated by 5-aza treatment (Figs. 1, 7; Supplementary Fig. S2). Nevertheless, 5-aza–induced local production of cytokines, including CCL5 (Fig. 3), which are known to attract T cells (53, 54), suggests possibly beneficial, at least for some patients, combination of 5-aza with immunotherapy (anti-PD-L1/PD-1), as the number of tumor-infiltrating CD8$^+$ T cells was found to predict response to anti-PD-1/PD-L1 treatment. This hypothesis is currently being investigated in the laboratory.

One key to improve patient survival in head and neck cancer is prevention or management of distant hematogenous metastases. MMPs promote angiogenesis and tumor growth, but most importantly, the ability of MMPs to degrade the extracellular matrix is the primary way for tumor intravasation as the first step in metastasis. The finding that 5-aza reduced expression of *MMPs* 1 and 10 and their activity in HPV$^+$ HNSCC cells (Fig. 6) and patient tumors (Fig. 7), suggest that 5-aza therapy may decrease spread of these tumors. Initial exploration of this idea in mice revealed that 5-aza treatment greatly diminished the detection of human cDNA in the blood of xenografted mice (Fig. 6F). Because 5-aza significantly inhibited HPV$^+$ HNSCC tumor growth in these

mice (Fig. 4), it is possible that decreased blood-borne human mRNA in treated mice was indicative of decreased tumor size, rather than of a reduced ability of cancer cells to penetrate mouse blood vessels. While decreased expression and activity of MMPs in 5-aza–treated cells and tumors provides a mechanistic explanation for reduced metastatic potential, the ability of 5-aza to prevent HPV$^+$ head and neck cancer metastatic spread needs to be validated further.

A major aim of these studies was to develop and advance a treatment for HPV$^+$ HNSCC that carries less morbidity than current treatment paradigms. Findings reported here reveal that 5-aza treatment stabilized p53 and reduced the expression of HPV genes and *MMPs* in HPV$^+$ head and neck tumors from patients enrolled in the 5-aza window clinical trial. The activation of caspases in 4 out of 5 tested HPV$^+$ tumors following treatment (Fig. 7B) indicates that 5-aza induced tumor apoptosis after only 5 days of treatment. The major side effects seen after extended 5-aza and decitabine treatments in MDS and AML are associated with bone marrow suppression. Although not tested, it is possible that head and neck cancer patients will not experience these effects due to their underlying pathology not involving hematologic abnormalities and due to a potential shorter treatment regimen. These results are promising and suggest that demethylation therapy should be further explored in HPV$^+$ HNSCC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Acknowledgments

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