Downregulation of SMG-1 in HPV-Positive Head and Neck Squamous Cell Carcinoma Due to Promoter Hypermethylation Correlates with Improved Survival

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Abstract

Purpose: Human papillomavirus (HPV) is linked with a subset of head and neck squamous cell carcinomas (HNSCC). HPV-positive HNSCCs show a better prognosis than HPV-negative HNSCCs, which may be explained by sensitivity of the HPV-positive HNSCCs to ionizing radiation (IR). Although the molecular mechanism behind sensitivity to IR in HPV-positive HNSCCs is unresolved, DNA damage response (DDR) might be a significant determinant of IR sensitivity. An important player in the DDR, SMG-1 (suppressor with morphogenetic effect on genitalia), is a potential tumor suppressor and may therefore be deregulated in cancer. No studies have yet been conducted linking defects in SMG-1 expression with cancer. We investigated whether deregulation of SMG-1 could be responsible for defects in the DDR in oropharyngeal HNSCC.

Experimental Design: Expression and promoter methylation status of SMG-1 were investigated in HNSCCs. To identify a functional link between HPV infection and SMG-1, we transfected the HPV-negative cells with an E6/E7 expression construct. SMG-1 short hairpin RNAs were expressed in HPV-negative cells to estimate survival upon IR.

Results: Forced E6/E7 expression in HPV-negative cells resulted in SMG-1 promoter hypermethylation and decreased SMG-1 expression. Due to promoter hypermethylation, HPV-positive HNSCC cells and tumors express SMG-1 at lower levels than HPV-negative SCCs. Depletion of SMG-1 in HPV-negative HNSCC cells resulted in increased radiation sensitivity, whereas SMG-1 overexpression protected HPV-positive tumor cells from irradiation.

Conclusions: Levels of SMG-1 expression negatively correlated with HPV status in cancer cell lines and tumors. Diminished SMG-1 expression may contribute to the enhanced response to therapy exhibited by HPV-positive HNSCCs.

Introduction

SMG-1 belongs to a family of phosphoinositide 3-kinase–related kinases (PIKK). Initially, it was described as the main kinase involved in nonsense-mediated mRNA decay (NMD)—the process of eliminating mRNAs that contain premature termination codons to prevent the accumulation of truncated proteins (1). In addition, it was found that SMG-1 maintains telomere integrity (2), protects against TNF-α–induced apoptosis (3), regulates lifespan, and oxidative stress resistance in Caenorhabditis elegans (4), has an essential role in embryogenesis (5), activates p53 and plays an important role in the DNA damage response (DDR) network (6–8). SMG-1 displays functional overlap with ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and RAD3-related (ATR) and cooperates with these 2 major stress-responsive kinases within the DDR network. Others and we (Gubanova and Helleday; unpublished data) have shown that SMG-1 is crucial for signaling irradiation-induced DNA damage by regulating the G1 checkpoint (6). Depletion of SMG-1 in human osteosarcoma cells results in enhanced sensitivity to ionizing radiation (IR). Finally, SMG-1 was suggested to function as a tumor suppressor, particularly in hypoxic tumors (9). However, up to date there is no data showing SMG-1 functional mutations, deletions, or reduced expression in human cancer.
Translational Relevance

Oropharyngeal squamous cell carcinoma (SCC) accounts for about 10% of head and neck cancer and is divided into human papillomavirus (HPV)–positive and HPV-negative subgroups. Patients with HPV-positive tumors respond better to radiation therapy and have survival advantage compared with patients with HPV-negative SCC. Although the mechanisms underlying radiosensitivity of HPV-positive tumors are not resolved, they may be connected to the ability of cancer cells to respond to DNA damage. Here, we found that expression of a potential tumor suppressor, SMG-1, is diminished in HPV-positive SCCs due to SMG-1 promoter hypermethylation. Low SMG-1 expression correlates with positive HPV status and improved patient survival. Because depletion of SMG-1 in head and neck cancer cells results in their increased sensitivity to radiation, underexpressed SMG-1 may contribute to the enhanced response to therapy by HPV-positive HNSCCs. SMG-1 levels in biopsies may be used as a marker to predict tumor radiosensitivity, allowing personalized therapy.

In this study, we hypothesize that the sensitivity of HPV-positive HNSCCs to chemo- and radiotherapy may be directly connected to the ability of cancer cells to respond to DNA damage. We determine expression levels of 3 PIKK family members, orchestrating the whole DDR cascade, ATM, ATR, and SMG-1. We show that in oropharyngeal HNSCC only levels of SMG-1 expression negatively correlated with HPV status. SMG-1 downregulation in HPV-positive HNSCC is explained by SMG-1 promoter hypermethylation, which is the result of HPV-16 E6 and E7 proteins expression. Given that cells with decreased SMG-1 expression are more sensitive to irradiation, low SMG-1 levels may contribute to the enhanced response to therapy exhibited by HPV-positive HNSCC patients.

Materials and Methods

Cell lines and constructs

We used 3 HPV-negative (JHUI012, SCC61, and SCC25), 2 HPV-positive (SCC090 and UMSCC47) HNSCC cell lines and normal human keratinocytes immortalized by expression of hTERT. SCC61 and SCC25 cell lines were cultures in Dulbecco’s modified Eagle’s medium (DMEM)/F12 medium supplemented with 0.4 μg/mL hydrocortison, SCC090 cells were grown in DMEM, UMSCC47 in DMEM with nonessential amino acids, JHUI012 were cultured in RPMI, and keratinocytes—in keratinocyte serum-free medium with supplements (GIBCO/Invitrogen) in a humidified atmosphere of 5% CO₂ at 37°C. All medium types, except of keratinocyte serum-free medium, were supplemented with 10% FBS (Invitrogen), 50 μg/mL penicillin, and 50 μg/mL streptomycin (Invitrogen).

LXSN and HPV16 E6/E7-containing LXSN replication-incompetent MLV retroviral constructs were obtained from Dr. John H. Lee. Human keratinocytes were purchased from Dr. J. Rheinwald.

SMG-1 expressing vector was a gift from Dr. Abraham R. Dr. J. Rheinwald.

For establishing SCC61 vector and SCC61-E6/E7 cells, parental SCC61 cells were transfected with LXSN and 16 E6/E7 LXSN using Lipofectamine 2000 (Invitrogen), stable transfectants were selected with 400 μg/mL of Hygromycin B (Invitrogen) for 2 weeks.

Unique short hairpin RNA (shRNA) constructs against SMG-1 and HPV-16 E6 and HusSH shRNA retroviral cloning vector were obtained from OriGene, packed in 293T retroviral packaging cells (ClonTech) using Lipofectamine and Plus reagent from Invitrogen. JHUI012, SCC61, SCC25, and UMSCC47 cells were transduced with SMG-1 shRNAs and E6 shRNAs, respectively, using high titer of viral particles in the presence of Polybrene (Sigma).

Azacitidine was obtained from Sigma.

Zecoxin was obtained from Invitrogen.

Patient population

We used a collection of tumor specimens derived from 40 previously untreated, research-consented patients with SCC of the oropharynx. This collection contained HPV-positive (23 patients) and HPV-negative (17 patients) groups.

Head and neck cancer is the sixth most common cancer worldwide (10, 11). These cancers originate in mucosa lining the oral cavity, oropharynx, hypopharynx, larynx, sinusonal tract, and nasopharynx. About 10% of all cases arise in the oropharynx. The most common type of head and neck cancer is squamous cell carcinoma (SCC). Several reports have been published connecting head and neck SCC (HNSCC) with human papillomavirus (HPV) infection (12–14). It is estimated that about 60% of oropharyngeal SCCs are positive for the most common type of virus, HPV 16, and HPV is now recognized as the primary agent causing this type of head and neck cancer (15).

Patients with HPV-positive HNSCC are clinically distinct from those with HPV-negative cancer. They tend to be younger (16–18), nonsmokers, and nondrinkers (11, 19). In general, HPV-positive HNSCC patients are highly curable with IR with or without chemotherapy (11, 17, 20). On the contrary, HPV-negative cancers are resistant to therapy and patients show poor survival. Currently, it is not known what makes HPV-positive HNSCCs more sensitive to the treatment. Finding the molecular pathways responsible for this sensitivity will help identify therapeutic targets that could be used in targeted therapy for HPV-negative and improved therapy for HPV-positive cancers. Published data on the impact of HPV oncoproteins E6 and E7 in in vitro cell sensitivity to radiation is controversial. Few studies suggest that HPV 16–positive cervical cancer cells (21) and SCC cells (22), as well as HPV-negative cells overexpressing E6/E7 (23) are chemo- and radioresistant. On the other hand, in vivo data showing that HPV-positive HNSCC patients respond better to radiation treatment and have better prognosis is growing.
Clinical annotation for these groups included follow-up information on disease recurrence and death.

Western blotting

Cells were collected by trypsinization and lysed in radioimmunoprecipitation assay lysis buffer (Sigma) with addition of protease inhibitors (Roche) and phosphatase inhibitors (Sigma) for 30 minutes on ice. Insoluble material was removed by centrifugation at 14,000 rpm for 15 minutes at 4°C. Proteins were separated in 4% to 20% Tris-glycine polyacrylamide gels (Mini-PROTEAN; Bio-Rad) and electrophoretically transferred onto polyvinylidene fluoride membrane. Membranes were blocked with 5% dry milk in PBS and incubated with antibodies against SMG-1 (Q25; Cell Signaling), p53 DO-1 and actin (Santa Cruz Biotechnology), PARP-1 (BD Biosciences), γH2AX (Abcam), PCNA (proliferating cell nuclear antigen; Sigma), and α-tubulin (Sigma). After incubation with primary antibodies, the membranes were washed in PBS and incubated with secondary horseradish peroxidase–coupled goat anti-mouse and anti-rabbit antibodies. The signal was visualized using enhanced chemiluminescent substrate (Thermo Scientific).

Tumor lysates were made using 1 mmol/L NaVO3, 1 mmol/L DTT, with addition of protease inhibitors (Roche), phosphatase inhibitors (Sigma), and 100 μL phenylmethylsulfonylfluoride all in a total volume of 10 mL 0.5% NP-40 lysis buffer. Two hundred microilters of lysis buffer was added to each tube containing macrodissected tumor tissue. Samples were then sonicated and placed on a rotator for 30 minutes, centrifuged 15 minutes at 14,000 rpm at 4°C. Supernatant was transferred to a fresh 1.5-mL Eppendorf tube, protein quantitated, aliquoted, and stored at −80°C.

RNA extraction and quantitative real-time reverse transcription PCR

Total RNA was extracted by Roche RNA extracting 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 qSTAR primer pairs for SMG-1, ATM, and TP53 (OriGene Technologies) on an iCycler iQ Real-Time PCR Detection System (Bio-Rad). Primers for HPV-16 E6 were as follows: forward: CTCTGAATTCGCCACCATGCAACAAAAGAAAAGCTTGA, reverse: CCCTCGAGGTATCTCCATGTATTACATCA. A housekeeping gene, GUSB, encoding β-glucuronidase was amplified in parallel and used as an endogenous control to quantify relative gene expression. RNA extracted from normal uvula and keratinocytes was used as a calibrator, to which the RNA extracted from patient material and HNSCC cell lines were compared, respectively. Each qRT-PCR reaction was done in duplicate at least, and the ΔΔCt method was used to analyze the data.

Immunohistochemistry of tissue microarrays

Tissues from 34 patients, retrieved from needle core biopsies and placed in paraffin blocks, were used to construct tissue microarray (TMA). All the specimens were inspected by pathologist and were confirmed to contain at least 70% of SCC. Normal paraffin embedded tonsillar tissue was used as a control. One mm punches from each patient block and the control block were obtained and placed in a paraffin donor block using a Manual Tissue Arrayer-1 from Beecher Instruments, Inc. provided by the Translational Pathology Shared Resource (THAP) at the Vanderbilt Ingram Cancer Center.

Tissue sections were deparaffinized by two 5-minute incubations in xylene, two 5-minute incubations in 100% ethanol, two 5-minute incubations in 95% ethanol, one 5-minute incubation with 85%, 70%, 50%, and 30% ethanol, two 5-minute incubations in ultrapure water. Staining was done using Pierce Peroxidase Detection Kit (Thermo Scientific) and anti–SMG-1 antibody according to manufacture instructions.

SMG-1 promoter methylation

Genomic DNA was purified with DNeasy Blood and Tissue Kit (Qiagen) and prepared for methylation analysis with EpiTect Methyl DNA Restriction Kit (SABiosciences; see details in the text). Methylation status of the SMG-1 promoter was detected using EpiTect Methyl DNA Methylation qPCR Primer Assays from SABiosciences and calculated according to manufacturer's instruction.

Statistical analyses

The Kaplan–Meier method was used to generate recurrence-free survival and overall survival curves, and log-rank test analysis was used to compare HPV-positive and HPV-negative patient groups. Other statistical analyses were done using Fisher exact and χ² for trend tests.

Clonogenic survival assay

Cells were seeded in 6-well plates in duplicates at different densities depending on the cell line and treated with increasing doses of IR the following day. The following number of cells per well was seeded for each cell line: 500 for JHU012, SCC61, SCC25, and UMSCC47, 5,000 for SCC090, and 1,000 for JHU012 and SCC61 transduced with SMG-1 shRNAs and control vector. The colonies were fixed and stained with methylene blue in methanol after 10 to 14 days depending on the cell line. Colonies consisting of at least 30 cells were counted. The data presented in Fig. 5 was obtained from 2 independent experiments.

Reverse phase protein array

The reverse phase protein array (RPPA) procedures for antibody staining and signal detection were done as described previously (24, 25). Briefly, cellular proteins lysates were denatured and diluted in five 2-fold serial dilutions in dilution buffer. Serial diluted lysates were arrayed on nitrocellulose-coated slides (Grace Biolab) using Aushon 2470 Arrayer (Aushon BioSystems). Each sample was robotically printed in 5-fold serial dilutions on multiple slide including positive and negative controls.
Each slide was probed with a validated primary antibody plus a biotin-conjugated secondary antibody. Only antibodies with a Pearson correlation coefficient between RPPA and Western blotting of greater than 0.7 were used in RPPA study.

The signal obtained was amplified using a Dako Cytomation–catalyzed system (Dako) and visualized by DAB colorimetric reaction. The slides were scanned, analyzed, and quantified using a customerized software Microvigen (Vigenetech Inc.) to generate spot intensity.

Flow cytometry

Cells were collected, washed in PBS, and fixed in ice-cold 70% ethanol over night at −20°C. Ethanol was removed by centrifugation and the cells were rehydrated in PBS and pelleted. The cell pellets were resuspended in 25 μg/mL propidium iodide (PI; Sigma) solution in PBS containing 100 μg/mL RNase A (Invitrogen) and stained for 30 minutes at room temperature. The DNA content was analyzed by FACS-Calibur flow cytometer (BD Biosciences), and data analysis was done using WinMDI software version 2.9.

Apoptosis detection

Cells were collected, washed in PBS, and stained with fluorescein isothiocyanate Annexin V Apoptosis Detection Kit II (BD Biosciences) according to manufacturer’s instructions.

Results

SMG-I, but not ATM and ATR, is expressed at lower levels in HPV-positive than in HPV-negative HNSCC

Forty diagnostic biopsies from oropharyngeal HNSCC patients, 23 HPV-positive, and 17 HPV-negative were available for our analysis. Characteristics of patients including age at diagnosis, smoking status, and tumor staging are summarized in Table 1. All patients, for which disease stage was known (35 of 40 patients), had advanced disease of stage III or IV. Mean time of follow-up was 32 months for HPV-positive patients (ranging from 2–68 months) and 17 months for HPV-negative patients (ranging from 2–42 months). Three patients were lost to follow-up and 4 died due to cancer-unrelated causes. HPV status of each patient sample was determined by PCR amplification of L1, E6, and E7 genes.

Table 1. Patients characteristics

<table>
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<tr>
<th>Characteristics</th>
<th>Total</th>
<th>HPV-positive</th>
<th>HPV-negative</th>
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<td>No. of cases</td>
<td>40 (100%)</td>
<td>23 (57.5%)</td>
<td>17 (42.5%)</td>
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<td>Sex</td>
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<tr>
<td>Male</td>
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<td>20 (87%)</td>
<td>12 (70.6%)</td>
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<td>Female</td>
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<td>3 (13%)</td>
<td>5 (29.4%)</td>
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<tr>
<td>Age at Dx</td>
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<tr>
<td>Median</td>
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<td>57</td>
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<tr>
<td>Mean</td>
<td>58.2</td>
<td>55.6</td>
<td>61.8</td>
<td>0.2499</td>
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<td>Staging*</td>
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<td>T1–T2</td>
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<td>15 (65.2%)</td>
<td>5 (29.4%)</td>
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<td>1 (5.9%)</td>
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<td>23 (65.7%)</td>
<td>12 (70.6%)</td>
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<td>9 (39.13%)</td>
<td>10 (58.82%)</td>
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<td>9 (22.5%)</td>
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<td>6 (35.29%)</td>
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<tr>
<td>RT/Chemo</td>
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<td>3 (13.04%)</td>
<td>2 (11.76%)</td>
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<td>5 (29.42%)</td>
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<td>1 (5.88%)</td>
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<td>2 (11.76%)</td>
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<td>10 (25%)</td>
<td>5 (21.74%)</td>
<td>5 (29.42%)</td>
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</table>

* Four HPV-negative patients had unknown T stage, N stage and stage group, and one patient had unknown N stage and stage group.

n/a—no available information.
E7 genes, as well as analysis of E6, E7, and p16 expression by qRT-PCR (data not shown).

To analyze the levels of the SMG-1 protein in head and neck tumors, we carried out immunohistochemical staining of TMA, which contained cores from 34 HNSCC specimens. TMA was assessed by the pathologist and at least 70% of tumor cells content was confirmed for all samples. SMG-1 staining intensity was scored as negative (no signal), weak (weak intensity in less than 50% of tumor cells), moderate (strong intensity in less than 40% of tumor cells), or strong (strong intensity in the majority of tumor cells; Fig. 1A). We divided HNSCC tumors into 3 groups according to the intensity of the staining: negative, weak, and moderate/strong. Among the HPV-negative specimens, the distribution of negative, weak, and moderate to strong staining was quite equal (28.6%, 35.7%, and 35.7%, respectively). On the contrary, the majority of HPV-positive cases had negative and weak SMG-1 staining (45.0% and 40.0%, respectively), whereas a minor percentage (15.0%) had moderate to strong staining (Fig. 1B). Where detected, SMG-1 staining was both nuclear and cytoplasmic. To confirm immunohistochemical results, we used protein lysates from 4 randomly chosen HPV-positive and 4 HPV-negative primary oropharyngeal HNSCC tumors for Western blot analysis. We detected SMG-1 protein in all 4 HPV-negative tumors. On the contrary, 3 of 4 HPV-positive tumors did not express any detectable SMG-1 (Fig. 1C).

SMG-1 expression was also determined in 2 HPV-positive (SCC090 and UMSCC47) and 3 HPV-negative (SCC61, SCC25, and JHU012) head and neck cancer cell lines. As shown in Supplementary Fig. S1, SMG-1 expression was lower in HPV-positive cells.

Next we analyzed expression levels of 2 essential DNA damage sensor kinases coordinating DDR network, ATM, and ATR in 25 HPV-positive and 16 HPV-negative oropharyngeal HNSCCs using RPPA. ATR protein is equally
expressed in both types of tumors (Supplementary Fig. S2). However, expression of ATM kinase was slightly higher in HPV-positive HNSCCs (mean = 0.162 ± 0.068 vs. mean = −0.026 ± 0.158; Supplementary Fig. S2).

We assessed the expression of SMG-1 gene in 22 HPV-positive and 18 HPV-negative oropharyngeal head and neck tumors by quantitative real-time PCR relative to SMG-1 expression in sample obtained from normal uvula. As shown in Fig. 1D left, HPV-positive primary tumor cells express SMG-1 at lower level (mean = 1.253 ± 0.27SE) than HPV-negative cells (mean = 2.068 ± 0.47SE). On the contrary, ATM is expressed at the same level in HPV-positive (mean = 0.781 ± 0.273SE) and HPV-negative (mean = 0.759 ± 0.325SE) tumors (Fig. 1D right).

These results suggested that among the 3 kinases playing major roles in DDR and maintenance of genome stability, SMG-1 protein and gene expression is uniquely lower in HPV-positive versus HPV-negative HNSCC.

SMG-1 expression is regulated by the E6/E7 oncoproteins

The main transforming genes of HPV are E6 and E7. E6 induces degradation of p53 through ubiquitin-mediated proteolysis, leading to loss of p53 activity (26, 27). Thus, cells expressing E6 cannot activate p53-mediated response to DNA damage and are therefore susceptible to genomic instability (14). The viral oncoprotein E7 binds and inactivates Rb, causing the cell to enter S-phase, leading to cell-cycle disruption, proliferation, and malignant transformation (28, 29). As the level of SMG-1 was found to be lower in HPV-positive samples, as compared with HPV-negative tumors, we speculated that SMG-1 expression may be regulated by the HPV oncoproteins E6 and/or E7. To test this hypothesis, we transfected the HPV-negative squamous carcinoma cell line SCC61 and normal human keratinocytes with HPV-16 E6/E7 expressing vector or empty vector (30), and analyzed SMG-1 mRNA and protein levels by quantitative RT-PCR and Western blot. Transient expression of E6/E7 in HPV-negative cells resulted in a decreased SMG-1 mRNA level (Fig. 2A and data not shown) and protein level (Fig. 2B and data not shown) in both normal keratinocytes and cancer cells. Expression of HPV-16 E6/E7 in transfected cells was confirmed by E6 real-time PCR (Supplementary Fig. S3). In addition, transduction of 2 different E6 shRNAs to HPV-positive cells UMSCC47 resulted in increased expression of SMG-1 (Fig. 2C left panel). Efficient downregulation of E6 was confirmed by E6 real-time RT-PCR (Fig. 2C right panel) and by upregulation of p53 protein level (Fig. 2C left panel).

Next, we established SCC61 cells stably expressing E6/E7 proteins (SCC61 E6/E7) and confirmed expression of E6/E7 by RT-PCR for E6 (Fig. 2D left panel). As shown in Fig. 2D, SMG-1 is downregulated on the protein (right panel) and RNA (middle panel) levels in these cells as compared with...
SCC61 stably expressing empty vector (SCC61v). Interestingly, we found that in addition to expected decrease in p53 protein level, TP53 expression was downregulated in SCC61 E6/E7 cells (Supplementary Fig. S4).

Taken together, these results allowed us to conclude that SMG-1 is regulated by expression of HPV-16 E6/E7 on the transcriptional level.

Expression of HPV-16 E6/E7 leads to SMG-1 promoter methylation

Aberrant DNA promoter methylation is a common feature of many human cancers, including HNSCC, and is an important mechanism of tumor suppressor genes silencing in head and neck cancer (31–33). Genome-wide gene expression profiling highlighted several hundred genes that are differentially expressed in HPV-positive and HPV-negative oropharyngeal cancers (34). Recently, higher DNA methylation in genic and LINE-1 regions has been found in HPV-positive versus HPV-negative cell lines (35). Taking into account that SMG-1 promoter region (sequence located approximately 1.3 kb upstream and up to 200 bp downstream of the transcription initiation site) has a high GC content (64.6%), we were interested to test whether downregulation of SMG-1 mRNA expression is due to promoter hypermethylation. First, we treated SCC61v and SCC61 E6/E7 cells with demethylating agent azacitidine and analyzed SMG-1 expression in quantitative real-time PCR. Treatment with azacitidine did not cause any changes in SMG-1 expression in SCC61v cells but restored SMG-1 mRNA level in SCC61 E6/E7 cells back to the level observed in SCC61v cells (Fig. 3A). In addition, treatment with azacitidine upregulated SMG-1 mRNA in HPV-positive cell lines, UMSCC47 and SCC090, but not in HPV-negative cells, SCC25 and SCC61 (Supplementary Fig. S5).

Intriguingly, although treatment with azacitidine did not change TP53 expression in SCC61v cells, it increased TP53 mRNA in SCC61 E6/E7 cells (Supplementary Fig. S6).

Next, we addressed a question whether expression of HPV-16 E6 and E7 proteins results in direct methylation of SMG-1 promoter. We used the EpiTect Methyl DNA Restriction Kit (SABiosciences), containing methylation-sensitive and methylation-dependent restriction enzymes, to prepare genomic DNA from SCC61v and SCC61 E6/E7 cells for SMG-1 promoter methylation analysis. Nondigested DNA served as a control. The product of the methylation-sensitive restriction contained hypermethylated DNA sequences, whereas the product of the methylation-dependent restriction contained unmethylated DNA. Double restriction reaction was used to detect the background and the success of both single restrictions. SMG-1 promoter methylation was assessed in quantitative RT-PCR with PCR primers corresponding to distinct CpG island in the SMG-1 promoter region (SABiosciences). This assay revealed that in SCC61v cells 19.19% of DNA in SMG-1 promoter was hypermethylated; however, percentage of hypermethylation in this region was increased up to 50% in SCC61 cells upon exogenous expression of E6 and E7 proteins (Fig. 3B).

Using the same method we investigated SMG-1 promoter methylation in oropharyngeal HNSCCs. Three HPV-negative head and neck tumor samples showed 2.25%, 2.37%, and 10.02% of hypermethylated DNA in SMG-1 promoter. Remarkably, SMG-1 promoter was hypermethylated by 50% and 35.18% in 2 HPV-positive tumors (Fig. 3C left panel), which corresponded to lower relative SMG-1 mRNA expression determined by real-time PCR (Fig. 3C right panel).
HPV status correlates with improved recurrence-free and overall survival and low SMG-1 expression levels

Our data suggest that SMG-1 expression is repressed in HPV-positive HNSCC cell and tumors and that SMG-1 promoter hypermethylation may largely explain this expression pattern. SMG-1 is a multifunctional protein, playing a major role in several biologic processes, and a potential tumor suppressor (1, 3, 4, 9). What is the biologic outcome of low SMG-1 expression in a subset of head and neck cancer? Kaplan–Meier analyses of 37 oropharyngeal HNSCC patients were carried out. Recurrence-free and overall survival (Fig. 4A and 4B, respectively) were determined on the basis of HPV (left panel) status and SMG-1 expression (right panel). Fold change in SMG-1 expression relative to normal uvula of less than 0.5 was considered as “low SMG-1,” whereas relative expression of 0.5 and above was determined “moderate-high SMG-1.” Low SMG-1 group includes 8 of 22 HPV-positive (36.36%) and 2 of 18 (11.11%) HPV-negative primary tumors. As expected, HPV positivity strongly correlated with improved overall survival, reinforcing the notion that even advanced stage HPV-positive HNSCC patients are highly curable with chemoradiation. On the contrary, HPV-negative cancers are resistant to therapy and patients show poor survival (17, 20). Importantly, low SMG-1 expression level correlated with positive HPV status and improved patient overall and recurrence-free survival. There were neither recurrences nor deaths observed among patients expressing low SMG-1 levels.

Downregulation of SMG-1 in HNSCC cells results in increased sensitivity to IR due to elevated induction of apoptosis

To investigate whether low SMG-1 level directly contributes to increased sensitivity of HPV-positive HNSCC to IR, we first analyzed the response of HPV-positive and HPV-negative head and neck cancer cells to different doses of radiation in clonogenic survival assay. As shown in Fig. 5A, HPV-positive cells, UMSCC47 and SCC090, which express low levels of SMG-1 (Supplementary Fig. S1), were the most sensitive to increasing doses of radiation, whereas HPV-negative cell lines, SCC61, JHU012, and SCC25, expressing higher levels of SMG-1 (Supplementary Fig. S1) were more resistant to IR.

We realized that the response of cell lines with completely different genetic background may not be perfectly correlated with the level of a single protein. To clarify this point, we expressed in 2 HPV-negative SCC cell lines, JHU012 and SCC61, 2 different SMG-1 shRNAs (termed sh1 and sh2) or empty vector, and assessed sensitivity of the cells to radiation in clonogenic survival assay. Transduction of both shRNAs resulted in a decreased expression of SMG-1 in JHU012 and SCC61 cells (Fig. 5B). All 4 shRNA–transduced cells, JHU012 SMG-1 sh1, JHU012 SMG-1 sh2, SCC61 SMG-1 sh1, and SCC61 SMG-1 sh2 showed significantly reduced clonogenic survival upon radiation compared with empty vector–transduced cells (Fig. 5C and D), in accordance with previously published data obtained from osteosarcoma cells U2OS (6).

In an attempt to understand whether reduced clonogenic survival upon DNA damage of SMG-1–depleted
cells is due to increased induction of apoptosis, we transduced JHU012 cells with 2 different SMG-1 shRNAs or empty vector and treated them with radiomimetic drug zeozin for 16 hours. Zeozin treatment resulted in increased level of DNA damage marker, phosphorylated H2AX (Supplementary Fig. S7B) in SMG-1–depleted cells, and enhanced induction of apoptosis as assessed with Annexin V staining (Supplementary Fig. S7A). Analysis of PARP-1 cleavage in zeozin-treated SCC25 cells showed that cell death induced by zeozin is accompanied by elevated activation of caspases in SMG-1–depleted cells indicative of advanced apoptosis (Supplementary Fig. S7C).

We next determined whether forced expression of SMG-1 in HPV-positive cells resulted in protection from DNA damage–induced cell death. HPV-positive cells, UMSCC47, were transfected with either empty vector or SMG-1 construct (Supplementary Fig. S8B) and irradiated with 10 Gy. Cell death was assessed with PI staining, cell-cycle analysis, and quantification of subG1 population. Twenty-four hours after radiation treatment, we detected 32.4% subG1 population in vector-transfected cells, whereas only 15.3% dead cells were observed in the SMG-1–transfected set (Supplementary Fig. S8A).

These data showed that SMG-1 expression level is important determinant of head and neck cancer cells sensitivity to IR and radiomimetic drugs.

**Discussion**

For many years SMG-1 has been known as a critical component of an evolutionary conserved mRNA surveillance mechanism, NMD. In addition to its role in NMD, SMG-1 was later discovered to be involved in maintenance of genome integrity via genotoxic stress response pathways (6–8). SMG-1 is activated not only in response to errors in mRNA processing but also in response to DNA double-strand breaks caused by IR and UV light. SMG-1 helps cells survive IR, and its depletion leads to increased cells sensitivity to radiation in human osteosarcoma cells (6).

Our aim with this study was to investigate whether deregulation of SMG-1 expression could be responsible for defects in the DDR in a human disease. For this purpose, we chose to study oropharyngeal HNSCC, which accounts for about 10% of all HNSCCs. This type of HNSCC is divided into HPV-positive and HPV-negative subgroups. Interestingly, patients with HPV-positive oropharyngeal tumors respond better to chemo- and radiotherapy and have better
overall and disease-free survival compared with patients diagnosed with HPV-negative SCC (11, 20). This observation may be directly connected to the ability of cancer cells to respond to DNA damage. Therefore, we aimed to investigate expression levels of the proximal stress–responsive kinases that are essential for the DDR signaling cascade ATM, ATR, and SMG-1 in HNSCCs from both HPV-positive and HPV-negative groups.

Examination of HPV-negative and HPV-positive oropharyngeal HNSCC revealed that protein and mRNA levels of SMG-1, but not ATM and ATR, were lower in HPV-positive tumors (Fig. 1, Supplementary Fig. S2). In addition, low SMG-1 expression was determined in HPV-positive head and neck cancer cell lines and in HPV-negative HNSCC and normal cells transfected with HPV-16 E6/E7 expression vector (Supplementary Fig. S1–S3).

The cytotoxicity of radiation therapy is mediated by the generation of DNA damage, in particular DNA double-strand breaks, which requires a functional DDR for signaling and repair. Disrupting the DDR and double-strand break repair causes radiosensitivity (36). Others and we (Gubanova and Hellday; unpublished data) have shown that SMG-1 is an important component of the DDR induced by IR (6). Hence, we propose that downregulation of SMG-1 in HPV-positive HNSCC may contribute to increased sensitivity of this type of cancer to radiation therapy. We realize that SMG-1 is not the only player in a complicated DDR network and there may be other contributing factors that increase IR sensitivity, but indeed, our data obtained from 37 oropharyngeal HNSCC patients showed that low SMG-1 expression correlated with positive HPV status and improved patient survival (Fig. 4). Remarkably, there were no recurrences or deaths detected among patients expressing low SMG-1 levels. Through manipulating of SMG-1 level in HNSCC cells, we found that low SMG-1 level results in elevated sensitivity to IR due to increased induction of apoptosis (Fig. 5, Supplementary Fig. S7), which is in line with previously published data (6). Importantly, forced SMG-1 expression in HPV-positive cells resulted in protection from cell death induced by irradiation (Supplementary Fig. S8). Potentially, SMG-1 expression level in biopsies may be used as an additional marker to help predict tumor radiosensitivity to allow personalized therapy.

Aberrant genome hypermethylation is an important mechanism of HNSCC progression. HPV-positive and HPV-negative oropharyngeal HNSCC show distinct epigenetic profiles with hypermethylation in genomic and LINE-1 regions in HPV-positive head and neck tumors (35). In our study, analysis of methylation status identified SMG-1 promoter hypermethylation in HPV-positive HNSCCs and in HPV-negative head and neck cancer cells following exogenous expression of HPV-16 E6 and E7 proteins (Fig. 4).

Moreover, treatment with demethylating agent azacitidine upregulated SMG-1 mRNA in SCC61 cells stably expressing HPV-16 E6 and E7, but did not change SMG-1 expression in cells expressing empty vector. In this investigation, we did not address the question of whether expression of E6 or E7 singly would result in SMG-1 promoter hypermethylation and lower gene expression. However, Fig. 2C shows that downregulation of E6 in HPV-positive cells UMSSC47 upon transduction with 2 different E6 shRNAs led to upregulation of SMG-1 protein level, indicating that HPV-16 E6 is at least partially responsible for aberrant SMG-1 expression in HPV-positive oropharyngeal head and neck cancer.

Interestingly, despite the well-known fact that p53 is downregulated on the protein level by E6 ubiquitin ligase in HPV-positive cells, SCC61 E6/E7 cells displayed lower TP53 mRNA expression as compared with SCC61v cells, suggesting involvement of transcriptional regulation. Moreover, treatment with azacitidine restored expression of TP53 in SCC61 E6/E7 cells to levels observed in cells expressing empty vector (Supplementary Fig. S3). Whether TP53 expression is regulated through promoter methylation in HPV-positive cells deserves further investigation.

In summary, in this study, we show that the newest and the least studied member of the PIKK family, potential tumor suppressor, SMG-1, is underexpressed in HPV-positive oropharyngeal HNSCC cells and tumors as compared with HPV-negative ones. To our knowledge, this is the first article showing aberrant expression of SMG-1 in human cancers. We provide direct evidence that expression of HPV-16 E6 and E7 results in SMG-1 promoter hypermethylation. In our expression array study, we found that more than 1,200 genes were downregulated and about 650 genes were upregulated in SCC61 E6/E7 cells compared with SCC61v cells (data not shown). Whether and how expression of E6 and E7 induces massive changes in genome methylation and whether expression of E6 or E7 alone is the cause of increased SMG-1 promoter methylation is a subject of our separate ongoing study.

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

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SMG-1 Is Downregulated in HPV-Positive Head and Neck Cancer

References


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