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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Running title: SMG-1 is downregulated in HPV-positive head and neck cancer.

Keywords: SMG-1, methylation, radiation, survival, HPV.

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Conflicts of interest: the authors report no conflicts of interest

Words count: 4774
Total number of figures and tables: 6 (1 table and 5 figures). 8 supplementary figures.
TRANSLATIONAL RELEVANCE

Oropharyngeal squamous cell carcinoma (SCC) accounts for about 10% of head and neck cancer and is divided into HPV-positive and HPV-negative subgroups. Patients with HPV-positive tumors respond better to radiation therapy and have survival advantage compared to 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 the 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 expression correlates with positive HPV status and improved patient survival. Since 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.
ABSTRACT

Purpose: Human papillomavirus (HPV) is linked with a subset of head and neck squamous cell carcinomas (HNSCCs). 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 HNSCC 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 shRNAs 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, while 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 (PI3-kinase)-related kinases (PIKKs). Initially it was described as the main kinase involved in nonsense mediated RNA decay (NMD) - the process of eliminating mRNAs that contain premature termination codons in order to prevent the accumulation of truncated proteins (1). In addition, it was found that SMG-1 maintains telomere integrity (2), protects against tumor necrosis factor-α (TNFα)-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 network (6-8). SMG-1 displays functional overlap with ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and RAD3-related (ATR) and cooperates with these two 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. 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.

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, sinonasal tract, and nasopharynx. About 10% of all cases arise in 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 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), non-smokers and non-drinkers (11, 19). In general, HPV-positive HNSCC patients are highly curable with ionizing radiation 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.

In this study, we hypothesize that the sensitivity of HPV-positive HNSCCs to chemo- and radiotherapy may be directly connected to the ability of the cancer cells to respond to DNA damage. We determine expression levels of three PIKKs 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 three HPV-negative (JHU012, SCC61, and SCC25), two 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 DMEM/F12 medium supplemented with 0.4 µg/ml hydrocortison, SCC090 cells were grown in DMEM, UMSCC47 in DMEM with non-essential amino acids, JHU012 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%
fetal bovine serum (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.

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 Hygromicin B (Invitrogen) for two weeks.

Unique 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. JHU012, 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.

Zeozin was obtained from Invitrogen.

**Patient population**

We used a collection of tumor specimens derived from 40 previously untreated, research-consented patients with squamous cell carcinoma of the oropharynx. This collection contained HPV-positive (23 patients) and HPV-negative (17 patients) groups. Clinical annotation for these groups included follow-up information on disease recurrence and death.

**Western blotting**

Cells were collected by trypsinization and lysed in RIPA lysis buffer (Sigma) with addition of protease inhibitors (Roche) and phosphatase inhibitors (Sigma) for 30 min. on ice. Insoluble material was removed by centrifugation at 14 000 rpm for 15 min. at 4°C. Proteins were separated in 4-20% Tris-glycine polyacrylamide gels (Mini-PROTEAN; Bio-Rad) and electrophoretically transferred onto polyvinylidene fluoride (PVDF) 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), H2AX (Abcam), PCNA (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 1mM NaVO3, 1mM DTT, with addition of protease inhibitors (Roche), phosphatase inhibitors (Sigma), and 100μl PMSF all in a total volume of 10ml 0.5% NP-40 lysis buffer. 200ul of lysis buffer was added to each tube containing macrodissected tumor tissue. Samples were then sonicated and placed on a rotator for 30 min., centrifuged 15 min. at 14000rpm at 4 ºC. Supernatant was transferred to a fresh 1.5ml Eppendorf tube, protein quantitated, aliquoted, and stored at -80ºC.

RNA extraction and quantitative real-time reverse transcription-PCR (qRT-PCT)
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. qRT-PCR was performed using iQ SYBR Green Supermix (Bio-Rad) and qSTAR primer pairs for SMG-1, ATM and p53 (OriGene Technologies, Rockville, MD) on an iCycler iQ Real-Time PCR Detection System  (Bio-Rad). Primers for HPV-16 E6 were: forward: CTCTGAATTCGCCACCATGCACAAAGAGAACTGCA, reverse: CCCTCGAGGTATCTCCATGCATGATTACA. A housekeeping gene, GUSB, encoding beta-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 performed 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 de-paraffinized by two 5 min. incubations in xylene, two 5 min. incubations in 100% ethanol, two 5 min. incubations in 95% ethanol, one 5 min. incubation with 85%, 70%, 50%, and 30% ethanol, two 5 min. incubations in ultrapure water. Staining was performed 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 manufacture 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 performed using Fisher’s exact and Chi-square for trend tests.

Clonogenic survival assay

Cells were seeded in six-well plates in duplicates at different densities depending on the cell line and treated with increasing doses of ionizing radiation the following day. The following number of cells/well was seeded for each cell line: 500 for JHU012, SCC61, SCC25 and UMSCC47, 5000 for SCC090, and 1000 for JHU012 and SCC61 transduced with SMG-1 shRNAs and control vector. The colonies were fixed and stained with methylene blue in methanol after 10-14 days depending on the cell line. Colonies consisting of at least 30 cells were counted. The data presented on Fig. 5 was obtained from two independent experiments.

RPPA

The RPPA procedures for antibody staining and signal detection were performed
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 customized-software Microvigene (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 min. at room temperature. The DNA content was analyzed by FACSCalibur flow cytometer (BD Biosciences) and data analysis was performed using WinMDI software version 2.9.

Apoptosis detection

Cells were collected, washed in PBS and stained with FITC Annexin V Apoptosis Detection Kit II (BD Biosciences) according to manufacture instructions.

RESULTS

SMG-1, 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 to 68 months) and 17 months for HPV-negative patients (ranging from 2 to 42 months). Three patients were lost to follow-up and four died due to cancer-unrelated causes. HPV status of each patient sample was determined by PCR amplification of L1, E6 and 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 performed immunohistochemical staining of tissue microarray, 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 the 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 three 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), while a minor percentage (15.0%) had moderate to strong staining (Fig 1B). Where detected, SMG-1 staining was both nuclear and cytoplasmic. In order to confirm IHC 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 tumor. In contrary, 3 out 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 on Suppl. Fig 1, SMG-1 expression was lower in HPV-positive cells. Next we analysed expression levels of two essential DNA damage sensor kinases coordinating DDR network, ATM and ATR, in 25 HPV-positive and 16 HPV-negative oropharyngeal HNSCCs using reverse phase protein array (RPPA). ATR protein is equally expressed in both types of tumors (Suppl. Fig. 2). However, expression of ATM
kinase was slightly higher in HPV-positive HNSCCs (mean=0.162+/−0.068 versus mean=−0.026+/−0.158) (Suppl. Fig. 2).

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 on 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 suggest that among three 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 to 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 (Suppl. Fig.3). In addition, transduction of two 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 to 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 (Suppl. Fig 4).

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 in the presence of E6 and E7 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 (Suppl. Fig. 5).

Intriguingly, although treatment with azacitidine did not change TP53 expression in SCC61v cells, it increased TP53 mRNA in SCC61 E6/E7 cells (Suppl. Fig 6).
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. Non-digested DNA served as a control. The product of the methylation-sensitive restriction contained hypermethylated DNA sequences, while the product of the methylation-dependent restriction contains 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 two 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 biological processes, and a potential tumor suppressor (1, 3, 4, 9). What is the biological outcome of low SMG-1 expression in a subset of head and neck cancer? Kaplan-Meier analyses of 37 oropharyngeal HNSCC patients were performed. Recurrence-free and overall survival (Fig 4A and 4B, respectively) were determined based on 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 out of 22 HPV-positive (36.36%) and 2 out 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*

In order to investigate whether low SMG-1 level directly contributes to increased sensitivity of HPV-positive HNSCC to ionizing radiation, 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 (Suppl Fig 1), were the most sensitive to increasing doses of radiation, while HPV-negative cell lines, SCC61, JHU012, and SCC25, expressing higher levels of SMG-1 (Suppl Fig 1) were more resistant to IR.

We realize 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 two HPV-negative SCC cell lines, JHU012 and SCC61, two 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 four 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 to empty vector transduced cells (Fig 5C and 5D) in accordance with previously published data obtained from osteosarcoma cells U2OS (6).
In 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 two different SMG-1 shRNAs or empty vector and treated them with radiomimetic drug zeozin for 16h. Zeozin treatment resulted in increased level of DNA damage marker, phosphorylated H2AX (Suppl. Fig. 7B) in SMG-1 depleted cells and enhanced induction of apoptosis as assessed with Annexin V staining (Suppl. Fig. 7A). Analysis of PARP-1 cleavage in zeozin treated SCC25 cells demonstrated that cell death induced by zeozin is accompanied by elevated activation of caspases in SMG-1 depleted cells indicative of advanced apoptosis (Suppl. Fig. 7C).

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 (Suppl. Fig. 8B) and irradiated with 10Gy. Cell death was assessed with propidium iodide staining, cell cycle analysis and quantification of subG1 population. 24 hours after radiation treatment we detected 32.4% subG1 population in vector-transfected cells, while only 15.3% dead cells were observed in the SMG-1-transfected set (Suppl. Fig. 8A).

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 radio therapy, and have better overall and disease-free survival compared to 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, Suppl. Fig. 2). 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 (Suppl. Fig 1 and Fig 2 and 3).

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 Helleday, unpublished data) have shown that SMG-1 is an important component of the DDR induced by ionizing radiation (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 expression correlated with positive HPV status and improved patient survival (Fig 4). Remarkably, there were no recurrences nor 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 ionizing radiation due to increased induction of apoptosis (Fig 5, Suppl. Fig. 7), 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 (Suppl. Fig. 8). 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 -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 the present 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 UMSCC47 upon transduction with two 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 to 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 (Suppl. Fig 3). Whether TP53 expression is regulated through promoter methylation in HPV-positive cells deserves further investigation.

In summary, in the present 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 to HPV-negative ones. To our knowledge, this is the first report demonstrating 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 1200 genes were downregulated and about 650 genes were upregulated in
SCC61 E6/E7 cells compared to 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.

ACKNOWLEDGMENTS

We thank Dr. Ely K.A. for TMA assessment, Dr. John H. Lee for LXSN and 16 E6/E7 LXSN constructs, Dr. Abraham R. for SMG-1 expressing vector, David Sidransky, Tomas Carey, and Robert Ferris for JHU012, UMSCC47, and SCC090 cells.

We are grateful to department of Genetics, Microbiology and Toxicology at Stockholm University for providing E. G. with International Collaboration Travel Grant to support research visit to Department of Otolaryngology at Vanderbilt University. We thank Department of Otolaryngology at Vanderbilt University for supporting this project. RPPA analysis was supported by MD Anderson Head and Neck SPORE P50CA097007.

The authors declare that they have no conflicts of interest

GRANT SUPPORT

An endowment provided to the Barry Baker Laboratory for Head and Neck Oncology, Department of Otolaryngology at Vanderbilt University, MD Anderson Head and Neck SPORE P50CA097007, the Swedish Cancer Society, and Stockholm University supported this work

REFERENCES


FIGURE LEGENDS

Figure 1. SMG-1, but not ATM, is expressed at lower levels in HPV-positive primary oropharyngeal HNSCC
(A) IHC analysis of tissue microarray (TMA) of primary HNSCC using antibodies against SMG-1. Representative images of TMA cores are presented, showing negative, weak, moderate and strong staining intensities. (B) Percentage of specimens with different SMG-1 staining intensities. Chi-square analysis was used to calculate p-value. (C) Western blot showing SMG-1 levels in lysates from HPV-positive and HPV-negative HNSCC tumors. (D) Scatter plots of expression levels of SMG-1 mRNA (left) and ATM mRNA (right) relative to normal uvula in HPV-positive and HPV-negative HNSCC tumors as assessed by qRT-PCR analysis.

Figure 2. Regulation of SMG-1 expression by HPV-16 E6/E7
(A) SCC61 cells were transiently transfected with empty vector or construct expressing HPV-16 E6/E7 (SCC61 E6/E7) and SMG-1 mRNA levels relative to normal human keratinocytes were analyzed by qRT-PCR. (B) Western blot showing SMG-1 protein levels in keratinocytes transfected with vector expressing HPV-16 E6/E7 or empty...
vector. (C) UMSCC47 cells were transduced with two individual E6 shRNAs (sh1 and sh2) or empty vector and analyzed by Western blotting (left panel). Downregulation of E6 was confirmed by qRT-PCR (right panel). (D) Left panel shows expression of E6 in SCC61 cells stably transfected with E6/E7 expression vector (SCC61 E6/E7) compared to cells harboring empty vector (SCC61v) as confirmed by qRT-PCR. Middle panel shows relative to parental SCC61 cells SMG-1 mRNA levels in SCC61 E6/E7 and SCC61v as assessed by qRT-PCR analysis. Right panel depicts Western blot showing SMG-1 protein levels in SCC61 E6/E7, SCC61v, and parental SCC61 cells. Error bars represent SD.

Figure 3. Expression of HPV-16 E6/E7 leads to SMG-1 promoter methylation

(A) SCC61 cells expressing HPV-16 E6/E7 (SCC61 E6/E7) or empty vector (SCC61v) were treated with azacitidine (aza) and analyzed by qRT-PCR for SMG1 expression. Data was normalized to value obtained for SCC61v cells. Error bars represent SD. (B) Percentage of hypermethylation in the SMG-1 promoter in SCC61v and SCC61 E6/E7 cells measured by the EpiTect Methyl DNA Restriction Kit. (C) Percentage of hypermethylation in the SMG1 promoter in HPV-positive and –negative oropharyngeal HNSCC patients measured using the same method as in B (left panel). SMG-1 mRNA expression in these patients was determined by qRT-PCR (right panel).

Figure 4. HPV correlates with improved survival and low SMG-1 expression levels

Kaplan-Meier curves showing disease-free survival (A) and overall survival (B) of patients with HPV-positive and HPV-negative HNSCC (left panel), as well as patients with low and moderate-high SMG-1 expression (right panel).

Figure 5. SMG-1 downregulation in HNSCC cells results in increased sensitivity to IR.

(A) Clonogenic survival assay following treatment with increasing doses of ionizing radiation of HPV-positive (UMSCC47 and SCC090) and HPV-negative (JHU012, SCC61 and SCC25) HNSCC cell lines. Error bars represent SD. (B) Western blot showing SMG-1 depletion by two individual shRNAs (sh1 and sh2) in JHU012 and
SCC61 cells compared to cells transduced with empty vector. (C) Clonogenic survival assay following treatment with increasing doses of ionizing radiation of JHU012 cells described in B. (D) Clonogenic survival assay following treatment with increasing doses of ionizing radiation of SCC61 cells described in B. Error bars represent SD.
Figure 2

A

B

C

D

SMG-1 α-tubulin

E6/E7

vector

p=0.014

3 2 1 0

SMG-1 mRNA (fold change)

SCC61 E6/E7

SCC61

E6 shRNA

sh1

sh2

p=0.0066

1.2 1 0.8 0.6 0.4 0.2 0

SMG-1 mRNA (fold change)

SCC61

p53 PCNA

vector

HPV-16 E6 mRNA

SMC1 v SCC61 E6/E7

HPV-16 E6 mRNA

SMC1 v SCC61 E6/E7
Figure 4

- Low SMG1 vs. High SMG1
- Percent recurrence free

- HPV+ vs. HPV-

- Percent survival

- P = 0.011

- P = 0.0057

- P = 0.224

- P = 0.0045
Figure 5

Vector
SH2
SH1

B

SCC61
JHU012

D

JHU012 vector
JHU012 shRNA1
JHU012 shRNA2

SCC61 vector
SCC61 shRNA1
SCC61 shRNA2

A

100 10 1 0.1

0 2 4 6 8

0.1 1 10 100

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Table 1. Patients characteristics

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<th>HPV-negative</th>
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<tr>
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<tr>
<td>T1-T2</td>
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<td>5 (21,74%)</td>
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* 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
Downregulation of SMG-1 in HPV-positive head and neck squamous cell carcinoma due to promoter hypermethylation correlates with improved survival

Evgenia Gubanova, Brandee T. Brown, Sergey V. Ivanov, et al.

Clin Cancer Res  Published OnlineFirst January 13, 2012.

Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-11-2058

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