American Journal of BioMedicine
AJBM 2014;**2**(2): 199–212
Research Article doi: 10.18081/2333-5106/014-08/199-212

Mechanistic links between HPV16/18 E6 and lung cancer

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Abstract

Lung cancer has emerged as a global public health issue. Recent studies have consistently linked the presence of HPV16/18 E6 to the development of lung cancer as oncoproteins. HPV16/18 E6 and E7 have been shown to regulate the expression of various target genes and proteins including p53/pRb, VEGF, HIF-1α, cIAP-2, and hTERT, and contribute to cell proliferation, angiogenesis, and cell immortalization. We hypothesized that HPV16/18 E6 links to development of lung cancer through activation of HIF-1α/ VEGF signaling. Lung cancer patients eligible for surgical treatment were tested for the presence of HPV16/18 E6 in excised tumor tissue. HPV16/18 E6 detection and genotyping was performed using facility of a polymerase chain reaction and immunohistochemistry. Of the 292 tumor samples tested, 98 samples showed presence of HPV16/18 E6, and significantly, high in nonsmoker patients. Furthermore, overexpression of HPV16/18 E6 associated with increased level of HIF-1α and VEGF, that demonstrated by using reporter gene assays. We conclude that HPV16/18 E6 is strongly associated with lung cancer and might represent novel therapeutic targets to manage lung cancer.

Key words: Lung cancer, HPV16/18 E6, HIF-1α, VEGF, Immunohistochemistry

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Received 11 April 2014; accepted July 20, 2014, Published August 27, 2014
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Introduction

Lung cancer has emerged as a global public health issue. Recent studies have consistently linked the presence of HPV16/18 E6 to the development of lung cancer as oncoproteins [1-4]. HPV16/18 E6 and E7 have been shown to regulate the expression of various target genes and proteins including p53/pRb, VEGF, HIF-1α, cIAP-2, and hTERT, and contribute to cell proliferation, angiogenesis, and cell immortalization. We hypothesized that HPV16/18 E6 links to development of lung cancer through activation of HIF-1α/ VEGF signaling [5-7].

Human papilloma viruses, belonging to the Papillomaviridae family, are DNA viruses and are strictly host specific and exquisitely tissue tropic, having a preference to infect cutaneous or internal mucosal surfaces [8]. Nearly 200 subtypes of HPV have been identified which are categorized as high risk (HR) and low risk (LR) based on their oncogenic potential. HPV infects

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the keratinocytes found in the basal layer of the skin (stratum germinativum). Several investigations have convincingly proved the presence of human papilloma virus in the lesions of upper aerodigestive tract (UADT). We have previously described the distribution of HPV types in oral cancer [3] as well as in the larynx [9].

The process of HPV-induced cell transformation is a combined manifestation of several discrete cellular, genetic, and molecular alterations accumulated in the mucosal tissue, termed "condemned mucosa syndrome," which later progresses onto invasive cancer [10]. The viral proteins E6 and E7 contribute predominantly to the process of carcinogenesis and further tumor progression. These oncoproteins interact with critical cell cycle regulators to hamper their activity ensuing deregulation of the cell cycle machinery leading to uncontrolled cell proliferation. The virally encoded E6 binds to a cellular ubiquitin/protein ligase, E6-AP, and to p53 resulting in ubiquitination of p53 leading to its proteolytic degradation [11].

On the other hand, the E7 oncoprotein binds to the pRb dissociating the transcription factor E2F from the pRb/E2F complex, resulting in the transcriptional activation of several genes which facilitate cell proliferation. Rb/E7 complex formation is important for E7-induced cell transformation [12].

Materials and Methods

Study Subjects

Lung tumors were enrolled from 292 NSCLC patients who were treated with surgical resection at the Division of Thoracic Surgery, Taichung Veterans General Hospital (Taichung, Taiwan) between 1997 and 2012. This study is approved by division of Thoracic Surgery, Columbia University. The tumor type and stage of each collected specimen were histologically determined according to the World Health Organization classification system. Cancer relapse data were obtained by chart review and further confirmed by two clinical physicians.

Immunohistochemistry

Formalin-fixed and paraffin-embedded specimens were sectioned at a thickness of 3 μm. The detailed procedures were described previously [13]. For antigen detection, sections were heated in a microwave oven twice for 5 minutes in citrate buffer (pH 6.0) and then incubated with a monoclonal anti–NKX2-1 (SC-53136; at a dilution of 1:100) and FOXM1 antibody (GTX-100276; GeneTex, Irvine, CA; at a dilution of 1:100) for 60 minutes at 25°C.

Western Blot Analysis

Cells were washed twice on ice with phosphate-buffered saline (PBS) before adding protein lysis buffer [1 × protease inhibitor cocktail (Roche, Basel, Switzerland), 1.5 mM EDTA, 1 mM DTT, 10% glycerol, 25 mM Hepes, pH 7.6]. The protein concentration was determined by the Bradford assay (BioRad, Hercules, CA) using BSA as a standard. Total protein (20 μg) was resolved by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) for

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subsequent Western blot analysis using antibodies against the following proteins [diluted in Tween–Tris-buffered saline: 0.02% Tween-20 in 100 mM Tris-Cl (pH 7.5), 1:1000 as indicated]: monoclonal anti-p53 (DO7; Dako, Carpinteria, CA), anti–HPV 16 E6 (sc-1586; Santa Cruz Biotechnology, Dallas, TX), anti–HPV 18 E6 (sc-1584; Santa Cruz Biotechnology), anti–HPV 16 E7 (sc-6981; Santa Cruz Biotechnology), anti–HPV 18 E7 (GTX40864; Gene Tex), anti-SP1 (sc-14027; Santa Cruz Biotechnology), anti–α-tubulin (sc-5286; Santa Cruz Biotechnology), anti-Rb (sc-102; Santa Cruz Biotechnology), anti–phosphorylated Rb (pRb; sc-12901; Santa Cruz Biotechnology), anti–β-catenin (sc-7199; Santa Cruz Biotechnology), anti–c-Myc (sc-40; Santa Cruz Biotechnology), anti-vimentin (sc-6260; Santa Cruz Biotechnology), anti-Snail (sc28199; Santa Cruz Biotechnology), anti-Nanog (sc-3769; Santa Cruz Biotechnology), anti-E2F1 (GTX11837; GeneTex), anti-Oct4 (100622; GeneTex), anti-FOXM1 (GTX100276; GeneTex), anti–NKX2-1(699P; Thermo Scientific, Pittsburgh, PA), anti–E-cadherin (80098; BD Biosciences, San Jose, CA), and MMP2 (Lot 804P703C; NeoMarkers, Woburn, MA). The gel was transferred to a Hybond-C Extra membrane (GE Healthcare Life Science, Piscataway, NJ) and immunoblotted with primary antibody, as indicated in the figure legends. Anti-mouse or rabbit IgG conjugated to HRP was used as the secondary antibody for detection using an ECL Western Blot Detection System.

Plasmid Construction

The NKX2-1 cDNAs were cloned into pcDNA3.1 Zeo(+) (Invitrogen, Carlsbad, CA). The FOXM1-Luc and NKX2-1-Luc plasmids were constructed by inserting *Kpn*I/*Xho*I fragments into a *Kpn*I/*Xho*I-treated pGL3 vector (Promega Corp, Madison, WI). The small hairpin NKX2-1 (shNKX2-1; TRCN0000275519), shMZF1 (TRCN0000017137), shLOX (TRCN0000045991), shFOXM1 (TRCN0000273982), shNEDD4 (TRCN0000004967), shβ-catenin (TRCN0000314991), shCaveolin1 (TRCN0000007999), and pLKO.1 vector plasmids were purchased from the National RNAi Core Facility, Academia Sinica. HPV 16 E6 and HPV 18 E6 cDNAs and HPV 16 E6 and HPV 18 E6 shRNAs were as previously described [19], [20]. HPV 16 E7 and HPV 18 E7 shRNA template were constructed using two complementary oligos that, when partially annealed, create a loop region with a sequence complementarity to HPV 16 E7 and HPV 18 E7 mRNA. The oligos contained 19 to 20 nucleotides of the HPV 16 E7 and HPV 18 E7 sequence, as follows: HPV 16 E7 shRNA forward, 5′-gatc**aggaggatgaaatagatgg**tttcaagagaa TTTCAAGAGAA-3′; HPV 16 E7 shRNA reverse, 5′-agctaaaaa**aggaggatgaaatagatggt**tctcttgaaa-3′; HPV 18 E7 shRNA forward, 5′-gatc**gtgttgtaagtgtgaagcc** ttcaagagag-3′; HPV 18 E7 shRNA reverse, 5′-agctaaaaa - **gtgttgtaagtgtgaagcc**tctcttgaag-3′.The shRNA template was cloned into the pcDNA-HU6 vector as previously described [21]. The various concentrations of expression plasmids, as indicated, were transiently transfected into the lung cancer cells (1 × 10^6) using the Turbofect reagent (Fermentas, Pittsburgh, PA). After 48 hours, the cells were harvested, and the whole-cell extracts were assayed in subsequent experiments.

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Xenograft Tumors in Nude Mice

Female immunodeficient nude mice (BALB/c nu/nu mice) that were 5 weeks old and weighed 18 to 22 g were injected with PBS and the stable clones of TL-1/NC, TL-1/shE6, TL-1/shE6+FOXM1, TL-1/shFOXM1, GNM/NC, GNM/shE6, GNM/shE6+FOXM1, and GNM/shFOXM1 through the tail vein (10^6 cells in 0.1 ml of PBS). After 42 days, the mice were sacrificed, and their lungs were removed and fixed in 10% formalin. The number of lung tumor metastasis was counted under a dissecting microscope.

Statistical Analysis

The Student's *t* test and Chi-square test were applied for continuous or discrete data analysis. This analysis was performed using SPSS software (version 13.0; SPSS Inc, Chicago, IL). For survival data, statistical differences were analyzed using the log-rank test. Survival curves were plotted using the Kaplan-Meier method, and the variables related to survival were analyzed using Cox's proportional hazards regression model with SPSS software.

Results

HPV genomic DNA causes *A3B* upregulation.

We first tested whether high-risk HPV genomes could trigger *A3B* upregulation. Normal immortalized keratinocytes (NIKS) were transfected with full-length HPV16 or HPV18 genomes. Pools of transfectants were selected and expanded to allow for establishment of the viral genomes as nuclear plasmids and viral gene expression, and then reverse transcription-quantitative PCR (RT-qPCR) was used to quantify *A3B* mRNA levels. In comparison to a control vector-transfected pool of NIKS established in parallel, *A3B* mRNA levels were induced significantly by transfection of either HPV16 or HPV18 genomes (Fig. 1A). HPV18 genomic DNA consistently caused higher levels of *A3B* induction, routinely 5- to 10-fold above the negative control.

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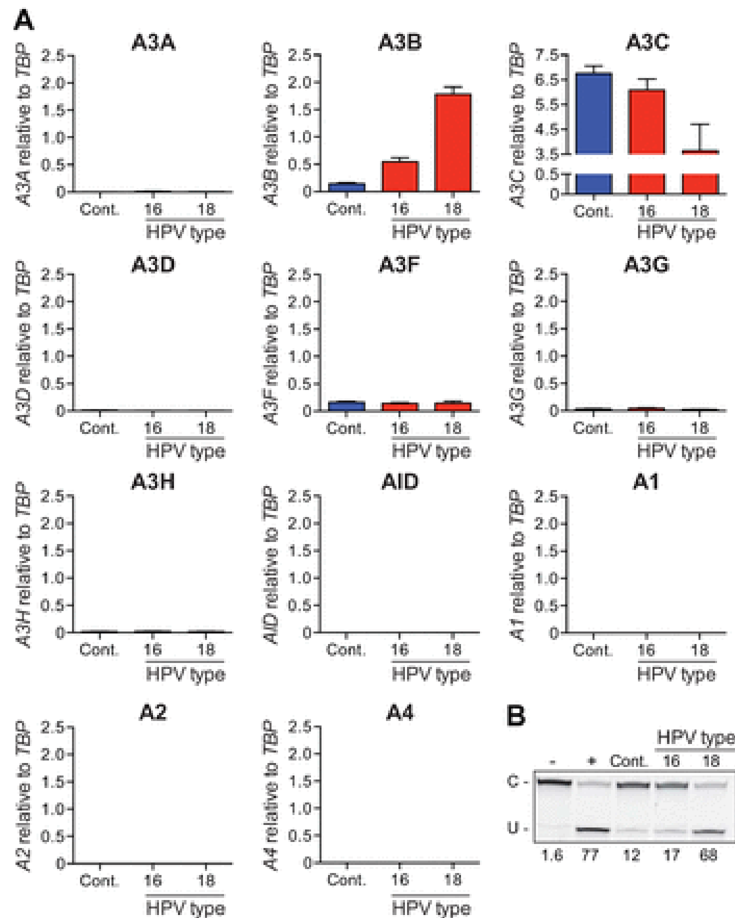

Figure 1.

APOBEC3B upregulation by transfection of full-length HPV genomes. (A) Histograms reporting *APOBEC* family member mRNA levels in NIKS transfected with a full-length HPV16 or HPV18 or a control plasmid (Cont.). Each histogram bar shows the mean expression level of each *APOBEC* family member normalized to *TBP* (error bars report standard deviations from triplicate assays). (B) Image of the results of a representative DNA cytosine deaminase assay performed with cell extracts from the same cells as in panel A. The single-stranded DNA substrate was treated with reaction buffer as a negative control (−) and recombinant APOBEC3A as a positive control (+).

HPV E6 is sufficient for *A3B* upregulation

Considering that the viral oncoproteins are invariably expressed in HPV-positive tumors, we next tested for a possible role of E6 in *A3B* upregulation. NIKS were transfected either with the full-length HPV18 genome or with a full-length HPV18 genome containing a stop codon within the E6 open reading frame (HPV18 E6-STOP) (41). As shown above, transfection with the wild-type HPV18 genome resulted in a significant upregulation of *A3B* mRNA levels. However, most of this effect was lost upon transfection with the HPV18 genome containing an E6-STOP mutation, indicating that E6 is required for induction of *A3B* (Fig. 2A). E6 mRNA levels were also reduced likely due to nonsense-mediated decay (Fig. 2B). To reconfirm the correlation between upregulation of *A3B* mRNA levels and enzymatic activity, DNA deaminase assays

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were performed using cell extracts. As expected, the DNA cytosine deaminase activity induced by transfection with wild-type HPV18 genome was ablated by inactivation of E6 (**Fig. 2C**).

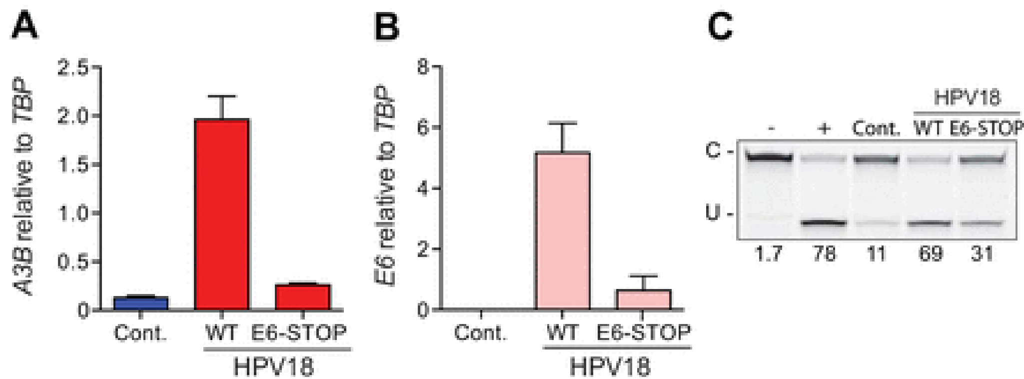

Figure 2.

HPV18 *E6* is necessary for APOBEC3B upregulation. (A) *A3B* and (B) *E6* mRNA levels in NIKS transfected with full-length HPV18 (WT), with HPV18 with a stop codon truncating the *E6* open reading frame (E6-STOP), or with a control plasmid (Cont.). Each histogram bar shows the mean mRNA expression level normalized to *TBP* (error bars report standard deviations from triplicate assays). (C) Image of the results of a representative DNA cytosine deaminase assay performed with cell extracts from the same cells as in panels A and B. The single-stranded DNA substrate was treated with reaction buffer as a negative control (−) and recombinant APOBEC3A as a positive control (+).

E6 is required for endogenous *A3B* expression in HPV-positive cancer cell lines. To test if endogenous *E6* could contribute to upregulation of endogenous *A3B*, we depleted the HPV early transcript from the HPV16-positive CaSki cell line. Two different small interfering RNAs (siRNAs) were used to interfere with *E6* expression. In each instance, the level of *E6* depletion was proportional to the decrease in endogenous *A3B* mRNA levels with an approximately 3-fold reduction in *E6* mRNA levels and a corresponding 3-fold reduction in *A3B* mRNA levels (Fig. 3). These results indicate that endogenous *E6* contributes to upregulation of endogenous *A3B.*

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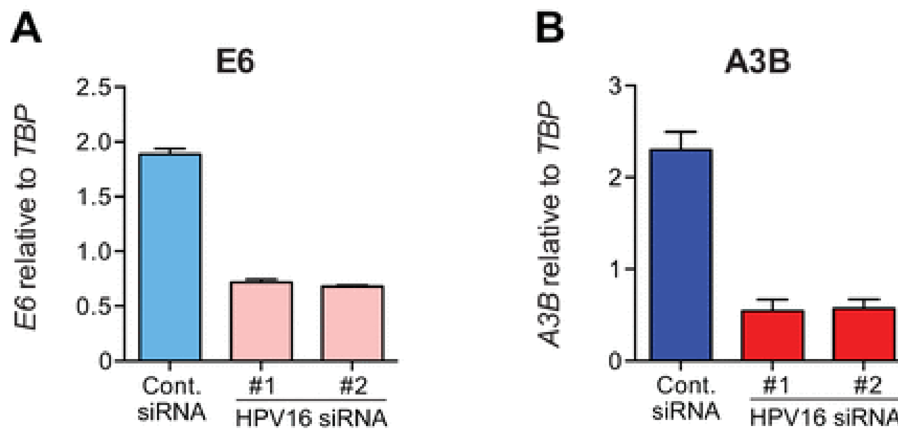

Figure 3.

HPV *E6* knockdown reduces endogenous *A3B* expression. *E6* mRNA levels (A) and *A3B* mRNA levels (B) in CaSki cells transfected with siRNA targeting the HPV16 early transcript (#1 or #2) or with a nontargeting control siRNA (Cont.). Each histogram bar shows the mean *E6* or *A3B* expression level relative to *TBP* (error bars report standard deviations from triplicate assays).

E6-Induced FOXM1 Expression Is Responsible for HPV-Mediated Xenograft Metastatic Lung Tumor Formation in Nude Mice

We examined whether E6-induced FOXM1 expression could be responsible for HPV-infected tumor progression in nude mice. Four nude mice in each group were injected with E6-knockdown GNM or TL-1, FOXM1-knockdown GNM or TL-1, or ectopic FOXM1 expression in E6-knockdown GNM or TL-1 stable clones. The expression of E6 and FOXM1 in E6-knockdown, FOXM1-knockdown, and E6/FOXM1-knockdown cells and the further ectopic FOXM1 expression in E6-knockdown stable clones were confirmed by Western blot analysis (Figure 6*A*, *upper panel*). The change of FOXM1-mediated downstream genes *Nanog*, *Oct4*, and *c-Myc* in GNM and TL-1 cells subjected to different treatments was further evaluated by real-time polymerase chain reaction (PCR), indicating that these gene expression levels in both cells were markedly decreased by E6 knockdown, FOXM1 knockdown, and thiostrepton treatment. However, the decrease of these three gene expressions by E6 knockdown in both cells was reversed by ectopic FOXM1 expression. These results suggest that the expression of Nanog, Oct4, and c-Myc elevated by E6-mediated FOXM1 may be responsible for cell invasiveness and stemness in E6-positive oral and lung cancer cells.

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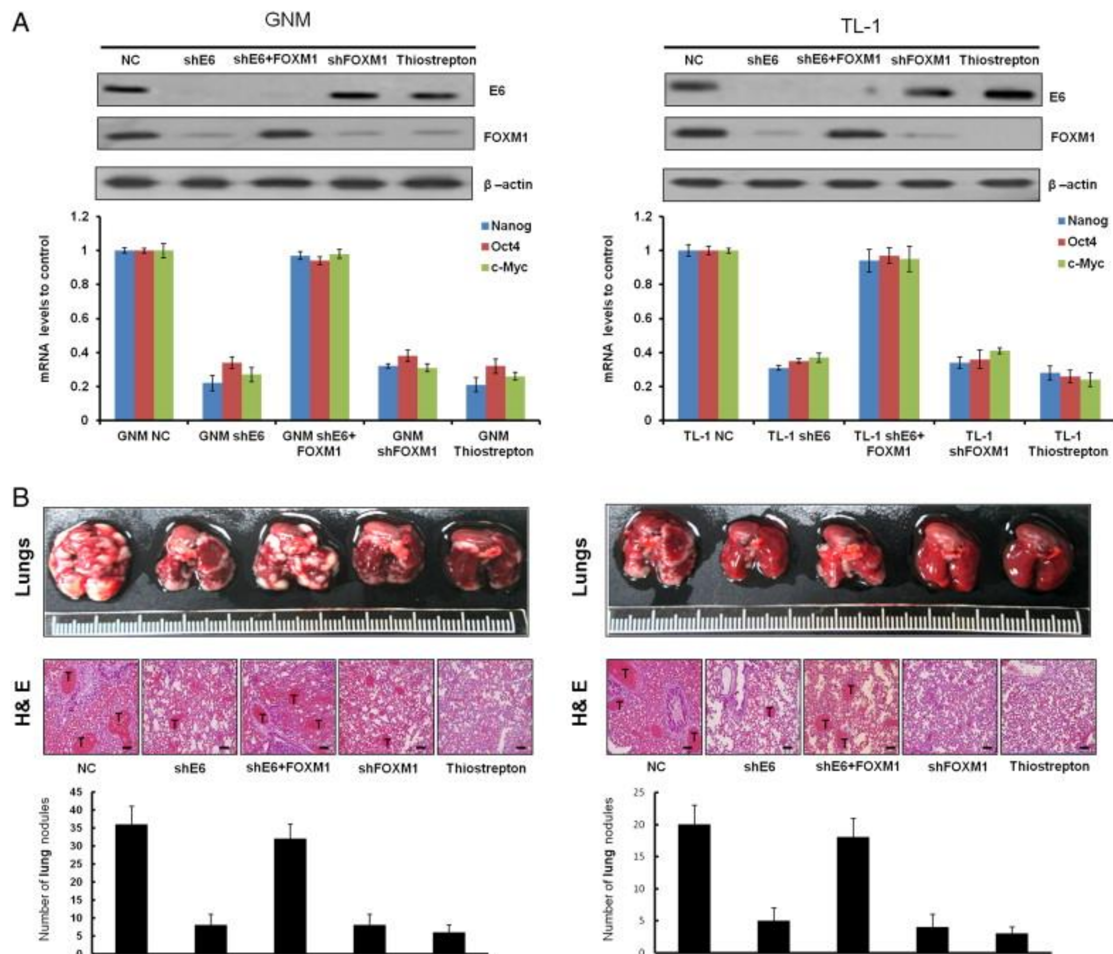

Figure 4.

E6-induced FOXM1 expression is responsible for HPV-mediated metastatic lung tumor formation in nude mice. An *in vivo* metastasis assay was conducted by injecting nude mice with TL-1 (shGFP) and GNM (shGFP), a stable GNM (shE6) and TL-1 (shE6) clone (1×10^6 cells per mouse), or PBS through the tail vein. The mice were sacrificed, and the lungs were excised on day 42. Representative lungs bearing metastatic xenograft tumors were examined by hematoxylin and eosin staining, indicated with a “T”. The scale bar represents a length of 100 μm (right). Data are presented as means ± SEMs; data were compared between groups using the *t* test, and $^*P < .05$ was considered to be statistically significant (the comparator was the control).

β-Catenin Activation Is Responsible for Invasiveness and Stemness Caused by the E6-Induced FOXM1 through the MZF1/NKX2-1 Axis

FOXM1 promotes β-catenin nuclear localization and controls Wnt target gene expression, stemness, and glioma tumorigenesis [14]. Therefore, activation of the Wnt/β-catenin signaling pathway is expected to be responsible for cell invasiveness and stemness mediated by E6-induced FOXM1 expression. Western blot analysis showed that nuclear β-catenin expression levels were elevated by ectopic FOXM1 expression in E6-knockdown GNM cells; however, nuclear β-catenin expression levels were reduced by FOXM1 silencing in E6-overexpressing OECM-1 cells (Figure 5*A*, *middle panel*). Wnt/β-catenin downstream gene—*cyclin D1* and *c-*

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Myc—expressions were consistent with nuclear β-catenin expression in both cell types (Figure 5*A*, *upper panel*). The transcription factor 4 reporter activity (TOPFlash) was significantly elevated by FOXM1 overexpression in E6-knockdown GNM cells and reduced by FOXM1 knockdown in E6-overexpressing OECM-1 cells. However, the transcription factor 4 reporter activity (FOPFlash) was unchanged in both cell types with the same treatment (Figure 5*A*, *lower panel*). We examined whether an increase in nuclear β-catenin expression by E6-mediated FOXM1 could be responsible for cell invasiveness and stemness. As expected, cyclin D1 and c-Myc expressions were decreased by β-catenin silencing in E6-knockdown GNM cells with ectopic FOXM1 expression (Figure 5*B*, *upper panel*). A Boyden chamber assay showed that the invasiveness was markedly decreased by E6 knockdown; intriguingly, the invasiveness was nearly restored by ectopic FOXM1 expression in E6-knockdown GNM cells, as compared with VC cells (Figure 4*B*, *upper right panel*). Moreover, the increase in invasiveness caused by ectopic FOXM1 expression was rescued by β-catenin silencing in E6-knockdown GNM cells with ectopic FOXM1 expression. Interestingly, stemness-related c-Myc, Nanog, and Oct4 were elevated by ectopic FOXM1 expression in E6-knockdown GNM cells (Figure 5*B*, *upper left panel*). The representative invasiveness and sphere cells are shown in Figure 4*B* (*lower panel*). E6-mediated cell invasiveness due to increased FOXM1-mediated β-catenin nuclear translocation was observed in the increase of sphere formation efficacy in GNM cells (Figure 5*B*, *upper right panel*). Concomitantly, the Nanog, Oct4, and c-Myc proteins and their mRNA expressions were dose-dependently decreased by FOXM1 inhibitor (thiostrepton) in E6-overexpressing OECM-1 cells (Figure 5*C*). Similar observations to those seen in GNM oral cancer cells were also seen in TL-1 lung cancer cells (Figure S1). These results clearly indicate that the increased nuclear translocation of β-catenin due to E6-induced FOXM1 expression through the MZF1/NKX2-1 axis is responsible for invasiveness and stemness in HPV-positive oral and lung cancer cells.

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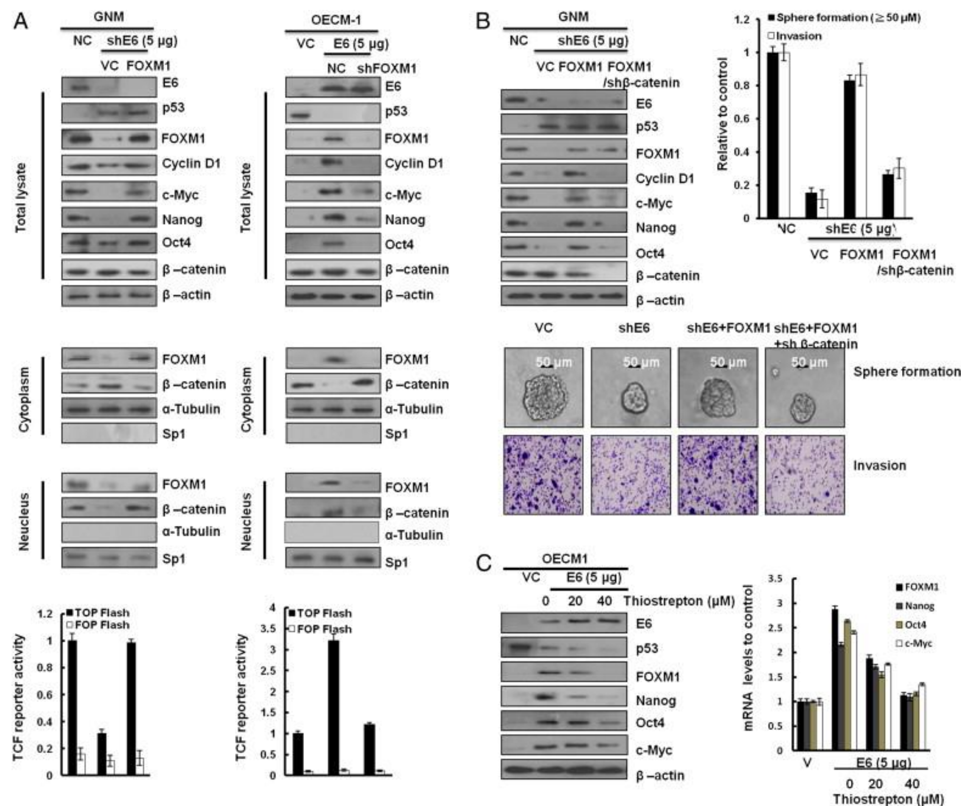

Figure 5.

E6-induced FOXM1 expression promotes cell invasiveness and stemness through activating β-catenin/TCF signaling pathway in OCSCC cells. (A) Western blot analysis of the total levels of E6, p53, FOXM1, cyclin D1, c-Myc, Nanog, Oct4, and β-catenin; cytoplasmic and nuclear levels of β-catenin and FOXM1 in GNM and OECM-1 cells that were transfected with a vector (control, NC, or VC), FOXM1, shFOXM1, E6, or shE6 plasmids, respectively. β-Actin, α-tubulin, and SP1 were used as the protein loading controls of whole-cell extracts, as well as cytoplasmic and nuclear proteins. TOPFlash and FOPFlash activity levels were determined through luciferase reporter activity assay. FOPFlash was used as a negative control. β-Gal served as an internal control. Values are given as means ± SEMs for triplicate samples. (B) Western blot analysis of the total levels of E6, p53, FOXM1, cyclin D1, c-Myc, Nanog, Oct4, and β-catenin in GNM cells that were, respectively, transfected with a vector (control, NC, or VC), shE6, FOXM1, and shβ-catenin plasmids. The sphere formation and invasive cells were photographed (bottom) and quantified (upper right) The scale bar represents a length of 50 μm. (D) Western blot analysis of the total levels of E6, p53, FOXM1, cyclin D1, c-Myc, Nanog, Oct4, and β-catenin in OECM-1–E6 cells on treatment with thiostrepton at various dosages. Quantitative real-time PCR (right) was used to analyze the levels of FOXM1, Nanog, Oct4, and c-Myc mRNA expression. The data shown are the means ± SDs of three independent experiments.

Discussion

To date, >100 studies (including case reports) have analyzed HPV DNA in lung cancers (7). Although the majority of these studies have reported the prevalence of HPV16 and/or 18 DNA and a few publications have shown evidence of HPV oncogene (E6 and E7) expression [15], definite evidence of a causal relationship is still missing.

Previously published meta-analyses of HPV in lung cancer show that HPV16 and HPV18 are the two most common genotypes detected in lung tumors worldwide [16]; however, the characteristics of patients with these tumors were not determined. This study is the first

international pooled analysis of individual data received from various research groups and was focused to define the demographic, behavioral and clinical characteristics of lung cancer cases with HPV16 and/or 18 DNA.

We have also for the first time reported and compared published data from five North American studies with other parts of the world.

It is plausible that differences in the geographic location, study size, demographic and clinical makeup of each study could contribute to heterogeneity in HPV prevalence between studies. Furthermore, the majority of studies included in this pooled analysis were small (<100 cases) and may be less representative samples, thus contributing to heterogeneity in HPV prevalence. Therefore, for each geographic region, the prevalence of HPV 16/18 was adjusted for each study as well as potential confounders yet variations in HPV16/18 prevalence remained.

The highest prevalence of HPV16/18-positive lung cancers was observed in South and Central American, whereas North American and Asian studies had similar prevalence and European studies had significantly lower prevalence of HPV 16/18 [17].

Similar observations were made for HPV 16 and HPV 18 independently. However, within each geographic region, distinct differences in the prevalence of HPV 16 and HPV18 genotypes were noted with the exception of Asia and North America. In North America, although the prevalence of each genotype was low, the prevalence of HPV 18 was slightly higher than that of HPV 16.

In contrast, for Asia, Europe and South and Central America, if any HPV DNA detected in lung cancer, HPV 16 appeared to be more prominent than HPV18. In Europe, the prevalence of HPV18 was near zero. Factors that may be responsible for variability in HPV prevalence could be sexual behaviors of participants, genetics and possible environmental contamination as argued in the study [18].

In addition to geographic location, the recent meta-analysis by other reported that the heterogeneity between studies might be related to differences in histologic type [19]. In this study, there were no differences in the proportion of histologic types in North America compared with the rest of the world and we observed no association of HPV 16/18 status with histology. Although South and Central American studies had the highest HPV prevalence, there were only two studies from this region; therefore, this finding will need to be confirmed by additional studies.

Second to South and Central America, we observed that Asia had the highest prevalence of HPV 16 and 18. A significant association of HPV 16/18 DNA was also observed with race, where non-White lung cancer patients were more likely to have HPV-positive tumors compared with White lung cancer patients [20].

There may be a number of reasons for this observed difference in HPV prevalence some of which might include differences in sexual practices or differences in susceptibility. Further investigation of the potential reasons for the racial difference in the association of HPV16/18 is needed.

Other study [21] evaluated HPV status in 112 non-small-cell lung cancer patients from Korea and reported that HPV16 was more common in younger lung cancer patients and HPV18 was more common in patients diagnosed with advanced stage. Although we categorized HPV16 and HPV18-positive tumors into a single group, we found no associations with age or stage, neither for gender, smoking history or histology.

However, for small studies only, HPV16/18 was associated with old age (>66 years), female gender and advanced stage. It is possible that patients with metastatic disease (by definition, stage IV) could include metastases from other or mixed tumors, such as head and neck or cervical cancers [22], and the resulting misclassification could account for some of these findings. Therefore, the association with older females with stage IV tumors warrants further investigation in a single, larger and more representative study.

Although HPV-positive oropharyngeal cancers have been found to be associated with non-smokers, these tumors are also associated with patients having lower number of pack-years of cigarettes compared with heavy smokers [23].

In vitro studies also show that tobacco smoke carcinogens have been shown to increase HPV16 and 18 viral synthesis as well as interact with HPV16 E6/E7 oncoproteins to increase lung cell proliferation. Furthermore, smoking suppresses the host innate immunity including functional and structural changes in the respiratory ciliary epithelium, lung surfactant protein and immune cells in the lung [24], thus may facilitate HPV infection and persistence in the lung in a subset of tumors.

Mechanistically, it is not yet clear how HPV might promote lung carcinogenesis; however, the cooperation between HPV and tobacco smoke carcinogens for lung carcinogenesis is plausible. In this study, the association of HPV with smoking status was not conclusive. Therefore, further studies are needed to investigate the relationship between HPV and smoking status among patients with HPV-positive lung tumors [25].

Although our study cannot conclusively confirm the carcinogenetic role of HPV in lung cancer, we have shown that lung cancer tissues were almost 4-fold more likely to be HPV-positive compared with normal lung tissues.

Secondly, our preliminary investigation of HPV16/18 physical status among a subset of tumors shows that the majority of the female tumors carried integrated HPV DNA while the physical status of HPV16/18 in male tumors was inconsistent. Given the predominance of integrated HPV genomes in female lung cancer patients, it is possible that HPV may play a role in lung cancer development but is unlikely to contribute to a large proportion of lung cancer cases. Although the presence of integrated DNA would suggest that the DNA detected was not simply due to contamination, it is important to note that there are no published studies comparing E2/E6-based integration with direct integration detection methods, which are more reliable. Thus, the results must be interpreted with caution [26].

Our findings suggest an association of HPV DNA with a small fraction of lung tumors, with large geographic variations, but further comprehensive analysis is needed to assess whether this

association reflects a causal relationship. Such detailed analysis should include not only HPV DNA testing but evaluation of all criteria that were postulated to prove a causal involvement of HPV in carcinogenesis, such as p16 expression and HPV E6/E7 oncogene expression [27], and measures to exclude pulmonary metastases [28].

The survival data in this pooled analysis only included 14 HPV16/18-positive cases and 5 events, and there were no significant differences between the Kaplan–Meier curves. However, based on the 2- and 5-year survival rates between HPV16/18-positive and HPV16/18-negative patients, there was some suggestion that HPV16/18-positive lung cancer patients had improved survival.

This trend toward improved survival is consistent with HPV-related carcinogenesis in non-cervical sites, such as the head and neck and penile cancer, where HPV-positive cancer patients have an improved overall and disease-free survival compared with HPV-negative patients [29]. This observation needs to be verified in a much larger number of cases/events and adjustment for known prognostic factors including a history of HPV-related disease as well as treatment for previous HPV-related disease.

Conclusions

HPV16/18 E6 is strongly associated with lung cancer and might represent novel therapeutic targets to manage lung cancer.

Competing interests

The authors declare that they have no competing interests.

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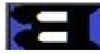
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