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Signaling and Regulation

Molecular Cancer Research

Activation of Wnt Signaling Pathway by Human Papillomavirus E6 and E7 Oncogenes in HPV16-Positive Oropharyngeal Squamous Carcinoma Cells

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Abstract

We sought to determine the role of human papillomavirus (HPV) *E6* and *E7* oncogenes in nuclear β-catenin accumulation, a hallmark of activated canonical Wnt signaling pathway. We used HPV16-positive oropharyngeal cancer cell lines 147T and 090, HPV-negative cell line 040T, and cervical cell lines SiHa (bearing integrated HPV16) and HeLa (bearing integrated HPV18) to measure the cytoplasmic and nuclear β-catenin levels and the β-catenin/Tcf transcriptional activity before and after *E6/E7* gene silencing. Repression of HPV *E6* and *E7* genes induced a substantial reduction in nuclear β-catenin levels. Luciferase assay showed that transcriptional activation of Tcf promoter by β-catenin was lower after silencing. The protein levels of *β*-catenin are tightly regulated by the ubiquitin/proteasome system. We therefore performed expression analysis of regulators of β-catenin degradation and nuclear transport and showed that seven in absentia homologue (Siah-1) mRNA and protein levels were substantially upregulated after *E6/E7* repression. Siah-1 protein promotes the degradation of *β*-catenin through the ubiquitin/proteasome system. To determine whether Siah-1 is important for the proteasomal degradation of *β*-catenin in HPV16-positive oropharyngeal cancer cells, we introduced a Siah-1 expression vector into 147T and 090 cells and found substantial reduction of endogenous *β*-catenin in these cells. Thus, *E6* and *E7* are involved in β-catenin nuclear accumulation and activation of Wnt signaling in HPV-induced cancers. In addition, we show the significance of the endogenous Siah-1–dependent ubiquitin/proteasome pathway for β-catenin degradation and its regulation by *E6/E7* viral oncoproteins in HPV16-positive oropharyngeal cancer cells. *Mol Cancer Res; 8(3); 433–43. ©2010 AACR.*

Introduction

The high-risk human papillomavirus types 16 (HPV-16) and 18 (HPV-18) have been etiologically implicated in the majority of cervical carcinomas (1). In addition, high-risk HPVs, especially type 16, have been associated with a subset of oropharyngeal cancers in individuals without a history of alcohol use or tobacco exposure (2-4). There is substantial epidemiologic and molecular pathology evidence to support the causal association between HPV16 and a subset of oropharyngeal cancers. HPV-associated oropharyngeal cancers carry a better prognosis compared with stage-matched HPV-negative counterparts and may need less aggressive treatment. Identification of biomarkers that distinguish HPV-positive versus HPV-negative oropharyngeal cancers will shed light to the pathogenesis of HPV-induced head and neck cancers and may help identify potential targets for early detection and therapy.

The molecular events in HPV-induced carcinogenesis have been extensively studied in cervical cancer, the most widely acknowledged HPV-associated malignancy. According to the molecular progression model of cervical cancer, integration of the viral DNA into the infected host cell genome often disrupts the expression of the gene that encodes for the E2 protein, a transcriptional repressor of E6 and *E7* oncogene expression (5, 6). The disruption of E2 expression results in the unconstrained expression of *E6* and *E7* viral oncogenes. The *E6* and *E7* oncogenes of the high-risk HPVs encode oncoproteins that bind and degrade p53 and retinoblastoma (Rb) tumor suppressor proteins, respectively (7, 8). $p105^{Rb}$ and the Rb family members p107 and p130 regulate the activity of E2F transcription factors, and complexes consisting of E2F and

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hypophosphorylated $p105^{Rb}$ repress the transcription of genes such as *cyclin A* that are required for cell cycle progression (9). Most cervical carcinomas contain wild-type *p53* and *Rb* tumor suppressor genes. Thus, the tumor suppressor pathways are intact but dormant in these cells due to the continuous expression of *E6* and *E7* oncogenes. We have recently shown that the retrovirus-mediated delivery of short hairpin RNAs (shRNA) targeting HPV16 *E6/E7* oncogenes in HPV16-positive oropharyngeal cancer cell lines results in apoptosis and restoration of p53 and pRb tumor suppressor pathways (10).

We used automated quantitative protein analysis on an oropharyngeal cancer tissue microarray to identify biomarkers that distinguish HPV16-positive versus HPV-negative cancers. The HPV16 DNA status was determined using quantitative real-time PCR for HPV16. We found that β-catenin protein levels were upregulated in HPV16-positive oropharyngeal cancers compared with their HPV-negative counterparts (11).

β-Catenin plays a dual role in carcinogenesis. It binds to the cytoplasmic domain of type I cadherins and functions as a component of cadherin-catenin adhesion system (12-14). β-Catenin is also the nuclear effector of Wnt signaling pathway (15). Wnt signaling determines the abundance of nuclear β-catenin. In the absence of Wnt signaling, GSK-3β is active and phosphorylates β-catenin, resulting in its degradation. Activation of Wnt pathway inhibits GSK-3β and induces cytoplasmic accumulation of cytoplasmic β-catenin. The accumulation of cytoplasmic (signaling) β-catenin leads to its nuclear localization in which it binds to the T-cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors and induces expression of target genes. In mammalian cells, β-catenin-TCF/LEF complexes regulate the expression of several proto-oncogenes, including c-*myc* and *cyclin D1*, as well as genes important for growth and tumor progression, such as *MMP7, PPARδ, gastrin, connexin 43,* and WISP proteins (16). Moreover, an additional GSK3-independent pathway of β-catenin ubiquitination and proteasomal degradation has been described. This pathway involves a distinct ubiquitin-ligase complex, seven in absentia homologue 1 (Siah-1)-Siah-interacting protein-Skp1-Ebi, which promotes the degradation of β-catenin through a mechanism independent of GSK3β-mediated phosphorylation (17-19). Siah proteins are members of an evolutionarily highly conserved family of E3 ubiquitin ligases that target other proteins for ubiquitination and proteasomal degradation (20). Siah proteins are involved in apoptosis and tumor suppression. Some studies have also indicated that Siah-1 functions as a downstream effector of p53 (21).

In the present study, we sought to determine whether the activation of Wnt signaling is involved in HPV-induced malignant conversion. We found that β-catenin is accumulated in the nucleus of HPV16-positive oropharyngeal cancer cells and HeLa cervical cancer cells. Furthermore, we show that at least a proportion of the accumulated nuclear β-catenin in HPV-positive oropharyngeal cancer cell lines is transcriptionally active. This nuclear β-catenin accumulation seems to be a direct consequence of *E6* and *E7* HPV oncogene expression in HPV16-positive oropharyngeal cancer cell lines. Nuclear accumulation of β-catenin in these cancer cell lines is not associated with constitutively activated Akt pathway. Finally, we show the significance of the endogenous Siah-1–dependent ubiquitin/proteasome pathway for β-catenin degradation in HPV16-positive oropharyngeal cancer cells and its regulation by E6 and E7 viral oncoproteins.

Materials and Methods

Cell Lines, Siah-1–Expressing Plasmids, and Transient Transfection

The human cervical carcinoma cell lines HeLa (HPV18 positive) and SiHa (HPV16 positive) and human embryonic kidney 293T cells were obtained from the American Type Culture Collection. The human oropharyngeal squamous cell cancer cell lines 147T and 40T were kind gifts of Dr. Renske Steenbergen (VU University Medical Center, Amersterday, the Netherlands; ref. 22) and the human oropharyngeal squamous 090 cell line was a kind gift of Dr. Susanne Gollin (University of Pittsburgh, Pittsburgh, PA; ref. 23). The 147T cells stably express the HPV16 E6/E7 transcript and contain one to two integrated copies of HPV16 DNA per cell genome (22). The oropharyngeal cancer cell line 090 contains several integrated copies of HPV16 DNA and HPV is transcriptionally active (23). Oropharyngeal cancer cell line 040T is devoid of HPV DNA. HeLa/16E6, HeLa/16E7, and HeLa/16E6E7 cell lines were generated as previously described (24). The Siah-1–expressing plasmid was obtained from Qiagen and encodes the full-length Siah-1 sequence downstream of the T7 promoter in pQE-TriSystem-6 vector backbone. For transient expression experiments, 2×10^5 cells per 60-mm-diameter plate were transfected with 0.5 μg Siah-1–expressing plasmid or empty vector (pQE-TriSystem-6 vector as control) using the Fugene 6 transfection kit (Roche) following the manufacturer's instructions.

Semiquantitative Reverse Transcription-PCR

Total RNA was isolated by using an RNeasy kit and RNase-free DNase (both from Qiagen) according to the manufacturer's instructions. Single-stranded complementary DNA (cDNA) was synthesized from 1 μg of total RNA by using an iScript cDNA synthesis kit (Bio-Rad). The cDNA samples were serially diluted (1:5 and 1:20) and subjected to PCR amplification with forward and reverse primers with Taq Platinum polymerase (Invitrogen). Amplified products were separated on a 1.5% agarose gel containing ethidium bromide and observed with a UV transilluminator (wavelength, 300 nm). Primers and annealing temperature are shown in Supplementary Table S1.

Immunofluorescence

Slides were deparaffinized and stained as previously described (11). A mouse monoclonal primary antibody

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specific for β-catenin (clone 14, BD Transduction Laboratories; 1:500 dilution) was used.

shRNA Oligonucleotides and Retrovirus Production

The shRNA oligo (shRNAHN4) for silencing both HPV16 *E6* and *E7* gene expression contained 21 nucleotide of the target sequence (5′-GGACAGAGCCCATTACAATAT-3′). Complementary oligonucleotides encoding shRNAHN4 were annealed and cloned into the pSIREN-RetroQ retroviral expression vector (BD Biosciences) at a site 3 to the human U6 promoter resulting in retroviral plasmids RV-shRNAHN4. The negative control vector (RV– control shRNA) expressed a scrambled sequence that did not form a hairpin. The plasmids were purified using the HiSpeed Plasmid Maxi kit (Qiagen) according to the manufacturer's instructions and the presence of the correct inserts was confirmed by DNA sequencing. Retroviral production and titration were as previously described (25).

Quantitative Real-time Reverse Transcription-PCR

HPV type 16–positive oropharyngeal cancer cell lines (147T and 090) were infected with concentrated RV-shRNAHN4 retroviral stock as described above and then cultured for 48 h. DNA-free total RNA was then extracted from the infected cells with the use of an RNeasy mini kit and RNase-Free DNase (Qiagen) according to the manufacturer's instructions. Total RNA (1 μg) was converted to cDNA by using the iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's instructions. Quantitative real-time reverse transcription-PCR (RT-PCR) was done in separate 20-μL reaction volumes to evaluate the expression of the *cyclin-dependent kinase inhibitor 1A, p21* (*CDKN1A*), *Drosophila SIAH1*, HPV16 *E7*, and *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) genes. Quantitative PCRs were done in triplicate with 40 ng of cDNA as template, the gene-specific forward and reverse primers (0.3 μmol/L each), hot-start iTaq DNA polymerase (Bio-Rad), and the iQ SYBR Green supermix (Bio-Rad) in a single color icycler iQ Real-time PCR detection system (Bio-Rad). The amplification program for all primers sets was 95°C for 3 min, followed by 40 cycles of 95°C for 10 s and 60°C for 90 s. Real-time PCR amplification data were analyzed and threshold cycle (Ct) numbers were automatically determined by iQ software (Bio-Rad). The relative expression of each mRNA was calculated by the comparative δCt method (26). Endogenous glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were used for the normalization of RNA expression. Primer sequences are available on request from the author.

Subcellular Fractionation, Western Blotting, and Immunoprecipitation

Cells were grown in 75-cm^2 tissue culture flasks. For the preparation of whole-cell protein lycates, cells were harvested by centrifugation and washed with ice-cold PBS (SIGMA). Then, cells were lysed in 200 μL of radioimmunoprecipitation assay buffer [10 mmol/L Tris-HCl (pH 8.0), 140 mmol/L NaCl, 1% Triton X-100, and 0.1% SDS], 1% deoxycholic acid, and protease and phosphatase inhibitors (SIGMA). For the preparation of cytosolic and nuclear-soluble and nuclear-insoluble protein fraction, the Qproteome Nuclear Protein kit (QIAGEN) was used according to the manufacturer's instructions.

For Western blot analysis, ~25 μg of protein were separated by 12% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (BIO RAD, Immun-Blot PVDF membrane). Monoclonal antibody DO-7 (clone DO-7, DAKO) was used to detect human p53 protein; anti-pRb (clone 1F8, LAB VISION) and anti-GAPDH (clone FL-335, Santa Cruz Biotechnology) were used to detect pRb and GAPDH proteins, respectively. The membranes were also probed with monoclonal antibodies specific for the following proteins: β-catenin (clone 14, BD Transduction Laboratories), p21 (187, Santa Cruz Biotechnology), E-cadherin (clone 34, BD Transduction Laboratories), and GSK-3β (clone 7, BD Transduction Laboratories). The polyclonal antibodies anti–Siah-1 (P-18, Santa Cruz Biotechnology), anti–phospho-GSK-3β (Ser9; Cell Signaling Technology), anti-Akt (Cell Signaling Technology), anti–phospho-Akt (Thr308; Cell Signaling Technology), anti–phospho-Akt (Ser473; Cell Signaling Technology), anti–phospho- β-catenin (Ser33/37/Thr41; Cell Signaling Technology), anti-Histone H3 (Cell Signaling Technology), anti–β-actin (Cell Signaling Technology), and anti-grp78 (Novus Biologicals) were also used. Visualization of bound antibodies was enchanced by chemiluminescence (Amersham Biosciences). Immunoprecipitation assay was done as previously described (27).

Luciferase Reporter Assay

For luciferase reporter assay, uninfected, control shRNA–infected and RVshRNAHN4-infected 147T and 090 cells were plated at a subconfluent density in six-well tissue culture plates, 48 h after retrovirus infection, and grown for 24 h. Cells were then transiently cotransfected with 0.15 μg of Tcf reporter plasmid TOPFLASH or FOPFLASH (Upstate Biotechnology) and 0.05 μg of control reporter plasmid pRL-TK (Promega Corp.) as an internal control for transfection efficiency. Transfections were done using Fugene 6 Transfection Reagent (Roche Applied Science) at 0.2 μg/1 μL DNA. After 48 h, cells were lysed in passive lysis buffer and the reporter activity was measured using the Dual-Luciferase Assay System (Promega Corp.) as described by the manufacturer. All experiments were repeated thrice and all samples were done in triplicate.

Results

***β*-Catenin Protein Levels in HPV-Positive Oropharyngeal Cell Lines**

We detected increased *β*-catenin protein levels, as determined by automated quantitative protein analysis, in HPV16-positive–associated oropharyngeal squamous cell cancers compared with their tobacco-associated counterparts (11, 28). As seen in Fig. 1A, in HPV16-negative oropharyngeal squamous carcinomas, *β*-catenin is mainly

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localized at the cell membrane. However, in HPV16-positive oropharyngeal squamous carcinomas, β-catenin is also detected in the cytoplasm and nucleus.

To determine whether there is any correlation between HPV infection and increase in β-catenin protein levels, we compared the endogenous β-catenin protein levels in different HPV-positive and HPV-negative cell lines. In our analysis, we included HPV16-positive oropharyngeal (090 and 147T) and cervical (SiHa) cell lines. Two HPV-negative cell lines, one oropharyngeal (040T) cell line, and the human embryonic kidney cell line 293T were also included as controls. Western blot analysis revealed an increase in total β-catenin protein levels in all HPV-positive cells, whereas the control cell lines 040T and 293T had low or intermediate levels (Fig. 1B). To individually assess the levels of cytoplasmic versus nuclear β-catenin, all cell lines were fractionated into cytoplasmic, nuclear-soluble, and nuclear-insoluble fractions and β-catenin levels were subsequently determined in each fraction by Western blotting. Interestingly, protein expression analysis showed accumulation of β-catenin in cytosolic and nuclear-insoluble fraction of HPV-positive cells (Fig. 1D). To the contrary, the total nuclear protein levels of β-catenin in HPV-negative cell lines (040T, 293T) were substantially lower compared with the HPV-positive ones. Incubation of the nuclear-insoluble fraction of HPV-positive cells with DNase I resulted in the solubilization of a substantial amount of β-catenin, suggesting its association with chromatin. These data provide evidence that HPV infection is associated with a strong nuclear accumulation of β-catenin in HPV16-positive oropharyngeal and cervical cell lines.

Repression of *E6* and *E7* Oncogene Expression Leads to Downregulation of Nuclear β-Catenin

To determine whether *E6* and *E7* oncogenes are responsible for the increased β-catenin protein levels, the status of β-catenin in HPV16-positive oropharyngeal cancer cell lines (147T and 090) after E6/E7 repression was investigated. E6/E7 repression in HPV16-positive oropharyngeal cancer cell lines was achieved by the retrovirus-mediated expression of a shRNA oligo (shRNAHN4) specific for the silencing of both HPV16 E6 and E7 gene expression. As shown in Fig. 2A, immunoblotting with an antibody specific for β-catenin revealed a marked decrease in the level of β-catenin protein in whole protein lysates of 147T and 090 cell lines infected by retrovirus expressing shRNAHN4 (RVshRNAHN4). To the contrary, the β-catenin levels in the HPV-negative cell line (040T) remained unaffected by retrovirus infection. To determine whether the *E6* and *E7* viral oncogenes upregulate β-catenin by increasing the transcription of *β-catenin* gene, the β-catenin mRNA levels were analyzed by RT-PCR before and after *E6/E7* repression in 147T and 090 cells. As levels of *β-catenin* transcript were barely affected in RVshRNAHN4-infected 147T and 090 cells compared with uninfected or control-infected (RVcontrol shRNA) cells (data not shown), it is unlikely that *E6* and *E7* oncogenes upregulate β-catenin expression at the transcriptional level. Our next step was to determine whether the levels of nuclear signaling β-catenin are affected by *E6/E7* expression. HPV16-positive oropharyngeal 147T cancer cells, infected with RVshRNAHN4, were lysed and fractionated into soluble cytoplasmic and nuclear fraction and subjected to immunoblotting with antibodies recognizing β-catenin, β-actin, histone H3, and GRP78, 48 hours after retrovirus infection. Actin levels were evaluated to determine equivalent loading, whereas the detection of histone H3, a nuclear protein, and GRP78, an endoplasmic reticulum protein, were used to indicate nuclear purity. As seen in Fig. 2B, both cytoplasmic and nuclear β-catenin levels in 147T cells were substantially decreased after infection with retrovirus expressing shRNAHN4. Western blot analysis of total lysates for E-cadherin in whole protein lysates of shRNAHN4-treated 147T and 090 cell lines 48 hours after infection did not reveal any alteration in E-cadherin protein levels after *E6/E7* repression (data not shown). Several studies have described β-catenin relocalization from the cell membrane to the nucleus (29, 30). To investigate a possible dissociation of the cadherin-catenin complex in our HPV-positive squamous carcinoma cell lines, we compared the β-catenin and E-cadherin protein levels of the immunoprecipitated catenin-cadherin protein complexes and the levels of free cytoplasmic E-cadherin in the supernatants before and after *E6/E7* repression in 090 and 147 cell lines. β-Catenin immunoprecipitations revealed that the amount of coimmunoprecipitated E-cadherin and β-catenin was stable between nontreated and shRNA-treated cells (Supplementary Fig. S1A and C). In parallel, the fraction of free cytoplasmic E-cadherin in supernatants remained unchanged after *E6/E7* repression (Supplementary Fig. S1B and D). These findings suggest that the accumulated cytoplasmic and nuclear β-catenin in HPV-positive squamous carcinoma cell lines 090 and 147T does not derive from β-catenin relocalization. Taken together, these results indicate that E6/E7 viral oncogene expression is associated with the accumulation of cytoplasmic and nuclear β-catenin in HPV16-positive oropharyngeal cancer cell lines and this accumulation is not a consequence of β-catenin relocalization from cell membrane to the nucleus.

E6/E7 Expression Enhances Activation of a TCF-Sensitive Reporter by β-Catenin

In the cell nucleus, β-catenin interacts with members of the TCF/LEF transcription factor family to activate expression of target genes such as *c-myc* and *cyclin D1* (16). To determine whether the elevated nuclear β-catenin in HPV16-positive oropharyngeal cancer cell lines is transcriptionally active, a reporter plasmid that contains four TCF4-responsive sites upstream of the luciferase gene (TOPFLASH) and a reporter plasmid that contains mutated TCF/LEF sites (FOPFLASH) were used for measuring β-catenin reporter activation by luciferase assays in 147T and 090 cell lines. Uninfected, control shRNA–infected, and RVshRNAHN4-infected 147T and 090 cells were

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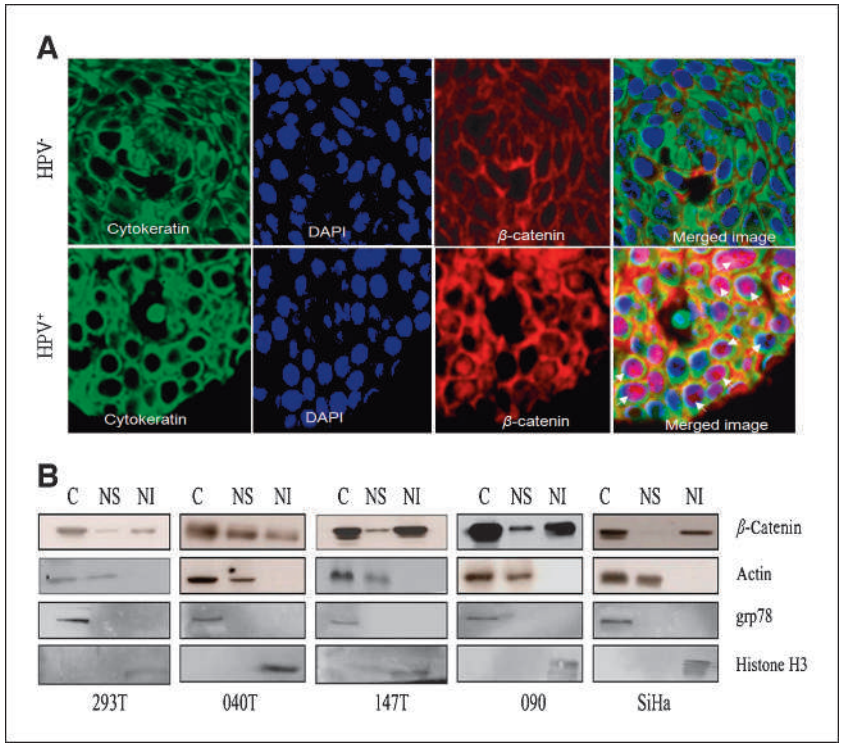

FIGURE 1. Detection of β-catenin expression in head and neck squamous cell carcinomas. A, false-color immunofluoresence images of β-catenin expression in HPV-negative (HPV^-) or HPV-positive (HPV^+) head and neck squamous cell carcinomas. Green, antibody to pan-cytokeratin; red, antibody to β-catenin; blue, 4′,6-diamidino-2-phenylindole (DAPI) nuclear counterstain. Arrows, nuclear staining of endogenous β-catenin. B, 2.0×10^6 cells from 293T, 040T, 147T, 090, and SiHa cancer cell lines were used for protein fractionation and immunodetection of β-catenin on cytosolic, nuclear-soluble, and nuclear-insoluble fractions (C, NS, and NI, respectively).

transfected with TOPFLASH reporter plasmid, 2 days after retroviral infection. To control for transfection efficiency, the reporter plasmid pRL-TK, which contains a herpes simplex virus thymidine kinase promoter driving a *Renilla* luciferase gene, was also cotransfected. The β-catenin-TCF/LEF–mediated transcription was determined by luciferase activity. Reporter activities were normalized to the internal control *Renilla* luciferase activity. As shown in Fig. 2C and D, TOPFLASH luciferase activity in RVshRNAHN4-infected 147T and 090 cells was substantially decreased compared with the activity in uninfected or control-infected (control shRNA) 147T and 090 cells. Because there no specific effects of the shRNA silencing on FOPFLASH activity, these data suggest that upregulation of nuclear β-catenin levels by *E6* and *E7* viral oncogene expression lead to an enhanced activation of TCF-mediated transcription.

Both *E6* and *E7* Contribute to Nuclear β-Catenin Accumulation

To determine the consequences of individually repressing the HPV oncogenes *E6* and *E7* on *β*-catenin protein levels, we used a system to separately extinguish the expression of the E6 or the E7 protein in HeLa cells. In this system, recombinant retroviruses were used to introduce constitutively expressed copies of the high-risk HPV16 *E6* or HPV16 *E7* gene in HeLa cells (24). Briefly, retroviruses composed of the empty vector (LXSN) and a vector containing the HPV16 *E6* or *E7* gene were used to infect a cloned strain of HeLa cells, and individual G418-resistant colonies were expanded to generate HeLa/LXSN, HeLa/16E6, and HeLa/16E7 cell lines, respectively. We then used a recombinant SV40-based viral vector to introduce the BPV *E2* gene into these cells. The E2 protein specifically represses transcription of the endogenous HPV18 *E6* and *E7* genes in HeLa cells, whereas the retroviral long terminal repeat driving expression of the HPV16 *E6* or *E7* gene is not affected by the E2 protein. This approach efficiently delivers the *E2* gene so that the acute biochemical and physiologic response of the entire population of cells can be determined. Thus, following E2 expression, the HPV16 E6 protein is the only HPV protein expressed in HeLa/E6 cells and the HPV16 E7 protein is the only HPV protein expressed in the HeLa/16E7 cells. In our analysis, we also used a cell line (HeLa/16E6-18E7) in which both *E6* and *E7* were constitutively expressed. To generate this cell line, HeLa/16E6 cells were infected with a retrovirus carrying the hygromycin resistance gene and the HPV18 E7 gene. HeLa/16E6-18E7 cells were established from colonies resistant to both G418 and hygromycin. The E2 protein repressed the expression of the endogenous HPV18 *E6* and *E7* genes in these cells, but expression of the exogenous *E6* and *E7* genes persisted. We used the latter cell line to confirm that the observed effects are due to *E6/E7* repression and not to some E2 function unrelated to *E6/E7* repression. Protein extracts were prepared from E2-uninfected and E2-infected HeLa/LXSN, HeLa/16E6, HeLa/16E7, and HeLa/16E6-18E7 cell lines 2 d after infection and were analyzed by Western blotting to determine the status of *β*-catenin expression. As shown in Fig. 3, *β*-catenin levels were markedly decreased in HeLa/LXSN cells after E2 infection in

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which both *E6* and *E7* were repressed. Decrease in β-catenin levels to a lesser degree was observed in HeLa/16E6 and in HeLa/16E7 cells following E2 infection, whereas E2-uninfected and E2-infected HeLa/16E6-18E7 cells displayed β-catenin expression levels similar to E2-uninfected HeLa/LXSN cells. Taken together, these results indicate that both *E6* and *E7* viral oncogenes contribute to the up-regulation of β-catenin protein levels.

β-Catenin Accumulation Occurs through an AKT-Independent Pathway in HPV16-Positive Oropharyngeal Squamous Cancer Cell Lines 147T and 090

It is well known that activated (phosphorylated) Akt targets GSK3β for phosphorylation, which leads to GSK3β inactivation and β-catenin stabilization and nuclear translocation. Therefore, it was of interest to determine whether nuclear accumulation of β-catenin is associated with constitutively activated Akt pathway in HPV-positive oropharyngeal cancer cell lines 147T and 090. To investigate if the activation status of Akt or GSK-3β changes after E6/E7 repression, HPV-positive oropharyngeal (090 and 147T) cells were grown to 90% to 95% confluency and then infected with retrovirus expressing either shRNAHN4 or control shRNA (147T, 090). Western blot analysis using a phosphospecific antibody against Ser^{473} was done to detect the activated form of Akt. Another phospho-specific antibody was also used to detect the inactive form of GSK-3β, phosphorylated at Ser^{9}. In this protein expression analysis, phosphorylated Akt and GSK-3β were not detected in the HPV-positive cell lines analyzed, regardless of *E6*/*E7* expression status (Supplementary Fig. S2). In

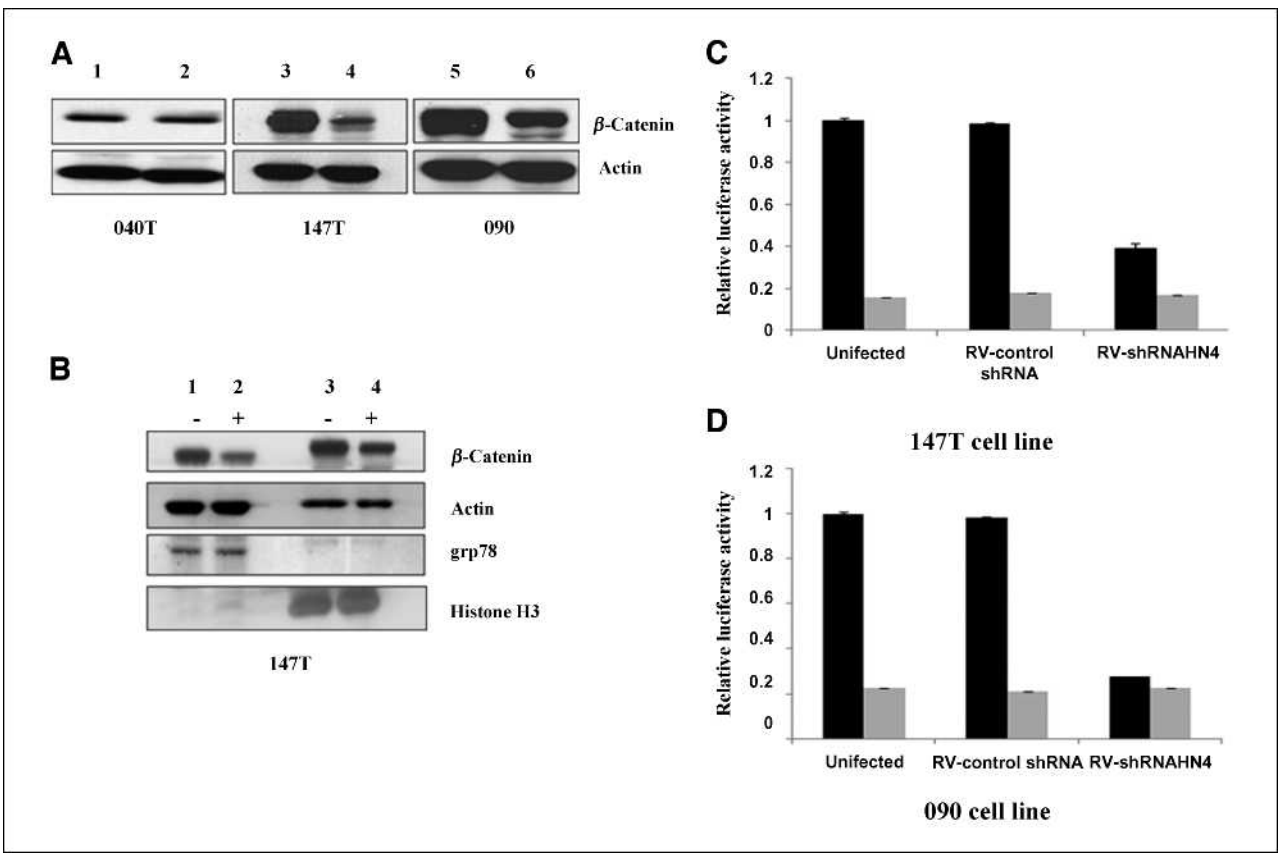

FIGURE 2. Downregulation of β-catenin in 147T and 090 cell lines after repression of HPV16 *E6* and *E7* genes. A, E6/E7 repression decreases the total β-catenin protein levels in HPV-positive 147T and 090 cell lines. The oropharyngeal cancer cell lines 040T, 147T, and 090 were infected with retrovirus expressing control shRNA (lanes 1 and 3) or shRNAHN4, which is specific for silencing of both HPV16 E6 and E7 gene expression (lanes 2 and 4). Total protein lysates from infected cell lines were separated by SDS-PAGE and immunoblotted with antibodies specific for β-catenin and β-actin. B, E6/E7 repression decreases the β-catenin protein levels in cytosolic and nuclear-insoluble fraction in 147 T-cell line. 147T cells, infected with retrovirus expressing control shRNA (–) or shRNAHN4 (+) were fractionated into cytoplasmic (lanes 1 and 2) and nuclear (lanes 3 and 4) fraction 48 h after retrovirus infection. Fractions were separated by SDS-PAGE and immunoblotted with antibodies specific for β-catenin, β-actin, histone H3, and grp78. C, luciferase activity of lysates of 090 cells. Uninfected and infected with retrovirus expressing shRNAHN4 (RVshRNAHN4) or control shRNA (RVcontrol shRNA) 090 cells were cotransfected with a reporter construct (TOPFLASH or FOPFLASH) and a pRL-TK reporter plasmid as internal control. D, luciferase activity of lysates of 147T cells. Uninfected and infected with retrovirus expressing shRNAHN4 (RVshRNAHN4) or control shRNA (RVcontrol shRNA) 147T cells were cotransfected with a β-catenin/TCF responsive reporter construct (TOPFLASH) or a reporter plasmid that contains mutated TCF/LEF sites (FOPFLASH) and a pRL-TK reporter plasmid as internal control. Black columns, relative TOPFLASH activities. Gray columns, relative FOPFLASH activities. For the relative luciferase values, luciferase/*Renilla* ratios were calculated. The relative luciferase value of uninfected cells is set at 1 on the vertical axis. Data represent results from triplicate dishes in two separate experiments.

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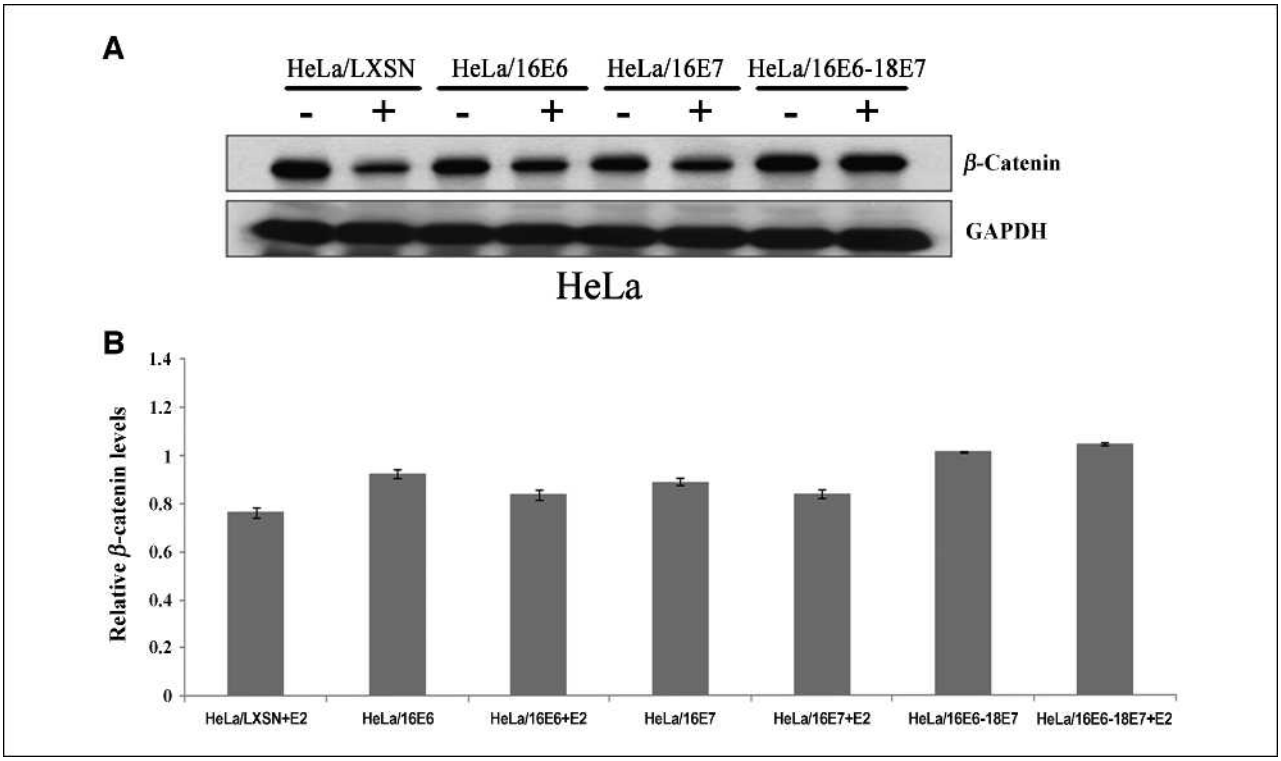

FIGURE 3. Effects of specific repression of HPV E6 and E7 genes on β-catenin levels in HeLa cells. A, protein was extracted from HeLa/LXSN, HeLa/16E6, HeLa/16E7, and HeLa/16E-18E7 cell lines 2 d after infection with the E2 virus (+) or mock infection (–). A 15 μg amount of each sample was resolved by gel electrophoresis, transferred to a membrane, and probed with antibodies specific for β-catenin and GAPDH. B, relative quantitation of β-catenin protein levels through Western blot analysis, was done using the ImageJ software (http://rsb.info.nih.gov/ij/). The value for the band intensity corresponding to HeLa/LXSN after mock infection (–) was set as 1 and other conditions were recalculated correspondingly to allow ratio comparisons.

addition, in all HPV-positive cell lines analyzed the protein levels of nonphosphorylated forms of AKT and GSK-3β did not change after *E6/E7* repression. These results imply that nuclear accumulation of *β*-catenin occurs through an AKT-independent pathway in the HPV-positive cervical and oropharyngeal squamous cancer cell lines.

E6 and *E7* Viral Oncogenes Downregulate the Siah-1 Protein

Because nuclear accumulation of *β*-catenin in the HPV-positive oropharyngeal squamous cancer cell lines 090 and 147T occurs through an AKT-independent pathway, it was of interest to determine whether reduced Siah-1 level is altered following *E6/E7* repression. In this direction, we performed real-time PCR and Western blot analysis, respectively, to measure Siah-1 mRNA and protein levels at baseline and 48 hours after *E6/E7* repression in 090 and 147T cancer cell lines. Previous studies have provided evidence that Siah-1 is a direct transcriptional target of p53 (21). Because repression of *E6/E7* expression in HPV16-positive oropharyngeal cell lines is associated with a rapid restoration of p53 and pRb protein levels (10), it is possible that upregulation of p53 increases Siah-1 transcription. To investigate whether repression of *E6/E7* expression can lead to a p53-driven transcriptional activation, in our real-time PCR, we included *p21* as a direct *p53* transcriptional target gene. As shown in Fig. 4B and C), Siah-1 protein levels in 147T and 090 cells were substantially upregulated after *E6/E7* gene repression. Real-time PCR analysis showed only a slight induction (1.3-fold) of Siah-1 expression after *E6/E7* repression in both cell lines. Notably, p21 mRNA levels were induced ~2-fold in 090 cells and 1.6-fold in 147T cells, 48 hours after *E6/E7* repression (Fig. 4A). These results show that E6/E7 expression is associated with the downregulation in Siah-1 protein levels but it is still inconclusive whether this upregulation occurs at transcriptional or posttranscriptional level. Therefore, restoration of p53 function after *E6* repression may account for the upregulation of Siah-1 levels and subsequent degradation of β-catenin.

Siah-1 Promotes the Proteasomal Degradation of *β*-Catenin in HPV16-Positive Oropharyngeal Cell Lines 147T and 090

We next examined whether the degradation of β-catenin through Siah-1 ubiquitin ligase is functional in HPV16-positive oropharyngeal cell lines 147T and 090. In this direction, we introduced a Siah-1 expression vector into the HPV16-positive 147T and 090 cells. Consequently, we observed that overexpression of exogenous Siah-1 in 147T and 090 markedly reduced the amounts of endogenous *β*-catenin in these cells (Fig. 5A). Moreover, reduction

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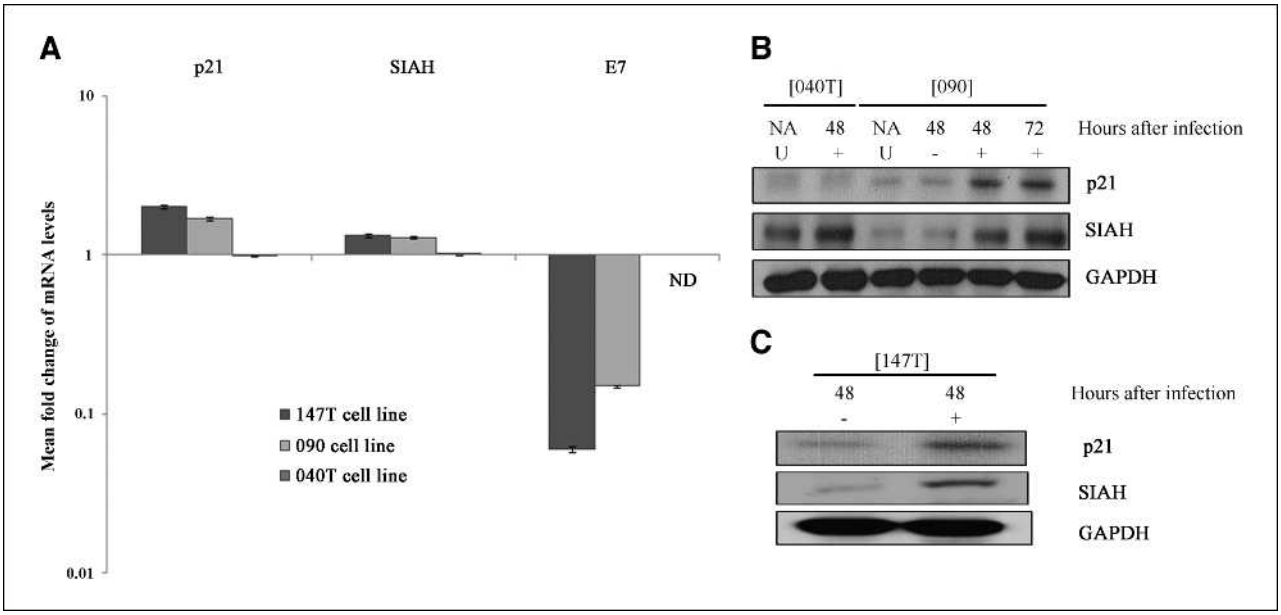

FIGURE 4. Transcriptional and protein expression analysis of Siah-1 in 090 and 147 T-cell lines after repression of HPV16 *E6* and *E7* genes. A, gene expression of p21, Siah-1, and E7 was examined with qRT-PCR analysis of RNA harvested 3 d after RV-shRNAHN4 infection of 147T (black) and 090 (gray) cells compared with control (RV-control shRNA infection) and normalized to glyceraldehydes-3-phosphate dehydrogenase (GAPDH) expression. B, repression of HPV16 E6/E7 expression by shRNA silencing upregulates the levels of Siah-1 in 090 cell line. Total protein lysates were made from uninfected (U), infected with retrovirus expressing shRNAHN4 (+), or control shRNA (–) 090 and 040 T-cell lines. Twenty-five micrograms of extracted protein of each sample were subjected to electrophoresis and immunoblotted with an antibody specific for Siah-1 as indicated. GAPDH expression was used as loading control. C, repression of HPV16 E6/E7 expression by shRNA silencing upregulates the levels of Siah-1 in 147 T-cell line. Total protein lysates were extracted from infected with retrovirus expressing shRNAHN4 (+) or control shRNA (–) 147T cells and was subjected to Western blot analysis as described in B.

in endogenous β-catenin protein levels through the expression of exogenous Siah-1 in 147T and 090 cell lines was not due to a decrease in β-catenin mRNA levels as determined by semiquantitative RT-PCR analysis (Fig. 5C), indicating that the downregulation of β-catenin in these cells was caused through the activation of its Siah-1–mediated proteasomal degradation. The decline of endogenous β-catenin protein levels through the overexpression of exogenous Siah-1 in 147T and 090 cells was suppressed by the addition of proteasome inhibitor MG132 (Fig. 5B), providing further evidence of the regulation of β-catenin levels through Siah-1–mediated proteasomal degradation.

Discussion

In the present article, we show that the Wnt signaling pathway is active in HPV-positive oropharyngeal cancer cell lines. Moreover, nuclear β-catenin accumulation seems to be a direct consequence of E6 and E7 oncoprotein expression. Furthermore, we show that at least a proportion of the accumulated nuclear β-catenin in HPV-positive oropharyngeal cancer cell lines is transcriptionally active. Finally, we show the significance of the endogenous Siah-1–dependent ubiquitin/proteasome pathway for β-catenin degradation in HPV16-positive oropharyngeal cancer cell lines and its regulation by the E6 and E7 viral oncoproteins.

Deregulated Wnt signaling is associated with several human cancers. Aberrant Wnt signaling was first described in colon cancer in which mutations in adenomatous polyposis coli, mutations in the GSK-3β phosphorylation sites of β-catenin, and mutations in axin 2 are prevalent (31). Activation of Wnt signaling pathway by viral oncoproteins has been previously reported for Epstein Barr virus (EBV) and Kaposi's sarcoma–associated herpesvirus (32). In the case of Kaposi's sarcoma–associated herpesvirus, it is the encoded latency-associated nuclear antigen protein that regulates the nuclear accumulation of GSK-3β (33). Depletion of cytoplasmic GSK-3β leads to consequent stabilization and accumulation of β-catenin, which subsequently enters the nucleus and activates responsive cellular genes. In EBV-associated malignancies, activation of Wnt signaling pathway seems to occur through different mechanisms, depending on the cancer type. In EBV-associated nasopharyngeal carcinoma, nuclear accumulation of β-catenin has been proposed to occur through the activation of the phosphoinositide 3-kinase/AKT pathway by LMP1 and LMP2A EBV proteins. In these EBV-infected cancer cells, activated (phosphorylated) Akt targets GSK3β for phosphorylation and inactivation, which leads to the nuclear accumulation of β-catenin (34). In EBV-infected B lymphocytes, increased Ser^{9}-phosphorylated GSK-3β, the inactive form of the enzyme, was detected, but β-catenin deregulation in these cells did not derive from phosphoinositide 3-kinase activation (35). In the case of EBV-infected B cells, LMP1 was found to downregulate transcription of the human homologue of *Drosophila* Siah-1 (17). Siah-1 is an E3

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ubiquitin ligase that acts in a complex with Skp1, Eb1, Siah-interacting protein, and adenomatous polyposis coli protein to facilitate, in a p53-dependent manner, the degradation of β-catenin (19). In the present article, the HPV-positive oropharyngeal cancer cell lines investigated lack the phosphorylation of AKT and GSK3β, indicating that the AKT/GSK3b pathway is not activated and therefore does not seem to play an important role in β-catenin accumulation. The data reported here provide evidence that *E6/E7* repression in HPV-positive oropharyngeal cell lines leads to the upregulation of Siah-1 mRNA and protein levels. In addition, we show that *E6/E7* expression inhibits the Siah-1–mediated degradation of β-catenin. It seems that HPV type 16 viral oncoproteins E6 and E7 inhibit Siah-1 in a way similar to LMP1 in EBV-infected B cells.

Uren et al. (36) generated an *in vitro* experimental cervical carcinoma model using primary human keratinocytes immortalized by HPV and transformed by SV40 small-t (smt) antigen. They hypothesized that in a multistep carcinogenesis model, HPV provides the initial hit and activation of canonical Wnt pathway may serve as the second hit. The authors found that smt-transformed cells had high cytoplasmic β-catenin levels, a hallmark of activated canonical Wnt pathway, and that activation of this pathway by smt was mediated through its interaction with protein phosphatase-2A. Protein phosphatase-2A is involved in the degradation complex of β-catenin. It inhibits Wnt signaling through its direct interactions with adenomatous polyposis coli and axin. Therefore, in this experimental cervical carcinoma model, the activation of Wnt pathway

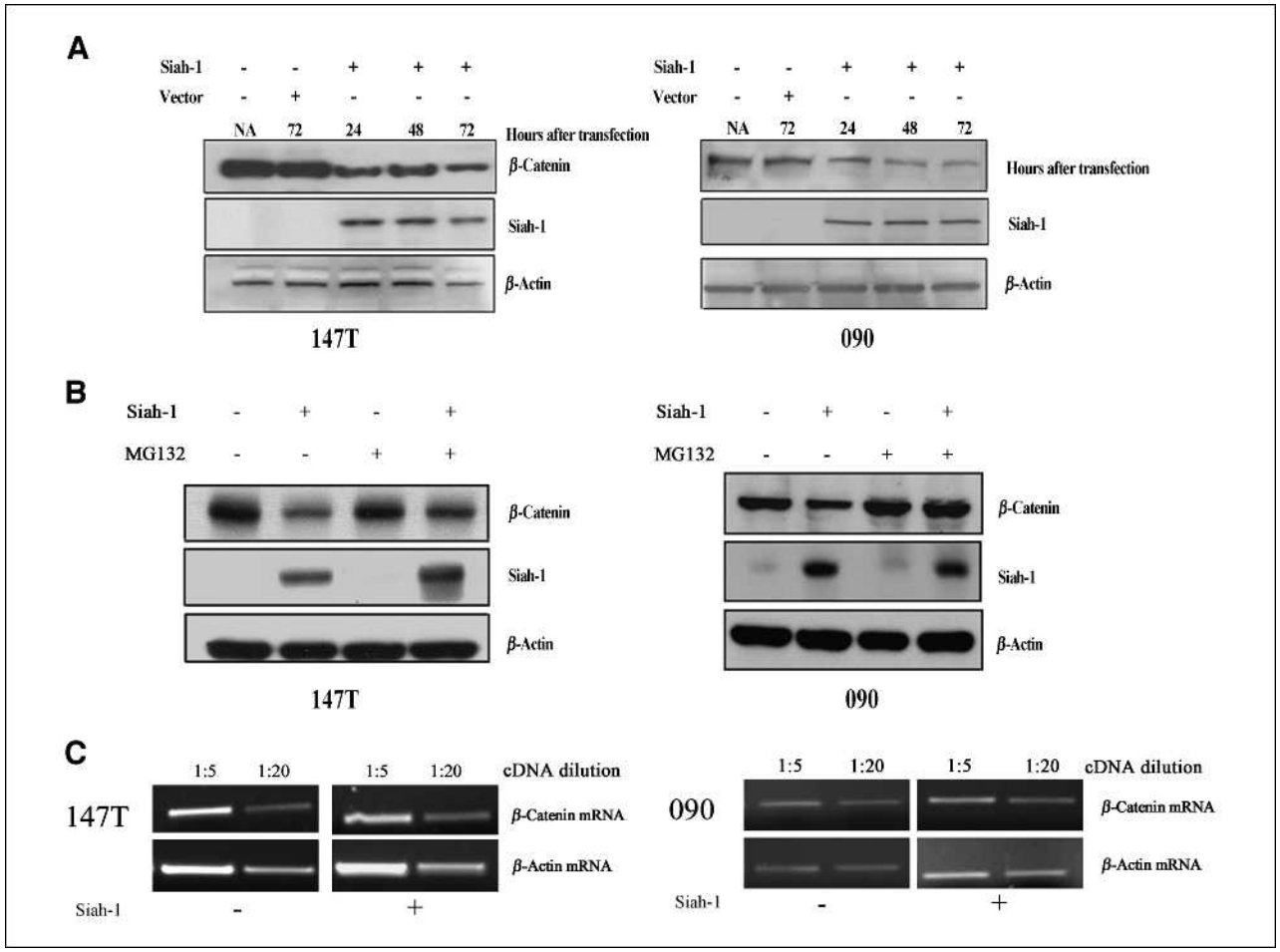

FIGURE 5. Downregulation of β-catenin through Siah-1 ubiquitin ligase overexpression in HPV16-positive oropharyngeal cancer cell lines 147T and 090. A, 147T and 090 cells were transfected with Siah-1–expressing plasmid or empty vector as control. At indicated times (24, 48, or 72 h posttransfection), cell extracts from transfected and untransfected cells were collected and the protein levels of β-catenin and Siah-1 were compared by Western blot analysis using monoclonal antibodies specific for β-catenin or Siah-1. Endogenous actin was used as loading control. B, 147T and 090 cells were transiently transfected with Siah-1–expressing plasmid and either mock treated or treated with 10 μmol/L MG132 during the last 4 h of culture. Whole-cell lysates were prepared, normalized for protein concentration (40 μg per lane), and analyzed by Western blotting using antibodies specific for β-catenin or Siah-1. Endogenous actin was used as loading control. C, endogenous expression of β-catenin at the transcriptional level. 147T and 090 cells were transiently transfected with a Siah-1–expressing plasmid. After 72 h, total RNA was extracted from transfected and untransfected 147T and 090 cells and reverse transcribed to generate cDNA, which was diluted 1:5 and 1:20 and subjected to PCR amplification of β-catenin. Expression of β-actin was used as internal control.

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is linked with the expression of SV40 smt antigen. The authors also detected increased cytoplasmic and nuclear staining of β-catenin in invasive cervical carcinoma samples from 48 patients and weak cytoplasmic or no nuclear staining of β-catenin in 18 cases of cervical dysplasia. In our study, we show that viral *E6* and *E7* gene expression activates the canonical Wnt pathway and therefore high-risk HPV infection is per se sufficient to promote carcinogenesis. Our findings contradict the results of Uren et al. (36) because the activation of Wnt signaling seems not to be a second hit in cervical carcinogenesis but rather a direct consequence of viral *E6/E7* expression.

To our knowledge, the present report is the first to show that the E6 and E7 oncoproteins of HPVs type 16 and 18 upregulate nuclear β-catenin, the effector of Wnt signaling pathway. In addition, this is the first analysis of β-catenin signaling in HPV16-positive oropharyngeal squamous cancer cells. Thus, high-risk HPV types 16 and 18 represent another example of a virus that can activate the Wnt signaling pathway with its viral oncoproteins. The fact that individual repression of *E6* and *E7* oncogenes leads to a substantial downregulation of β-catenin seems to imply that nuclear accumulation of β-catenin can be achieved through different cellular pathways. In addition, the reproducibility of this finding in both oropharyngeal and cervical cancer cell lines implicates a common mechanism of regulation of β-catenin levels by E6 and E7 oncoproteins in these two HPV-associated malignancies.

It is worth to note that repression of *E6* gene is also associated with a rapid restoration of p53 tumor suppressor pathway. Interplay between β-catenin and p53 has been described in the past. β-Catenin induces an ADP ribosylation factor–dependent accumulation of p53 (37). In addition, the proteasomal degradation of β-catenin by p53 is either mediated by GSK-3β (38) or by a GSK-3β–independent mechanism that involves Siah-1 (18, 19). Siah-1 is a *p53* target gene. Thus, upregulation of p53 leads to a direct upregulation of Siah-1 mRNA levels. However, we observed that both *E6* and *E7* contribute to nuclear β-catenin accumulation. Therefore, there is possibly a second mechanism of β-catenin accumulation, one that is related to E7 function.

Wilding et al. (39) generated an immortalized keratinocyte cell line by cotransfection with HPV-16 *E6* and *E7*, which displayed decreased membrane E-cadherin expression and redistribution of α-, β-, and γ-catenin from the undercoat membrane to the cytoplasm. The authors subsequently selected an immortalized keratinocyte cell line with resistance to differentiation and generated a more transformed cell line with an invasive phenotype, downregulated E-cadherin and α-catenin, and upregulated epidermal growth factor receptor. Subsequent transfection of an E-cadherin expression construct into the differentiation-resistant cell line induced restoration of membrane-bound E-cadherin and catenin expression, induced downregulation of epidermal growth factor receptor, and reversed the invasive phenotype. The authors showed that overexpression of the epidermal growth factor receptor correlates with perturbation of the E-cadherin/catenin complex seen in the HPV-16 *E6*- and *E7*-transfected keratinocytes, which may indicate a functional interaction between growth regulatory factors and adhesion molecules. The authors did not study potential activation of Wnt signaling by *E6* and *E7*. Our study shows that HPV-positive oropharyngeal squamous carcinomas display increased cytoplasmic and nuclear β-catenin levels. However, we found no dissociation of β-catenin from E-cadherin by coimmunoprecipitation studies and no change in E-cadherin protein levels. Because E-cadherin/β-catenin dissociation results in E-cadherin internalization and degradation (40), our data suggest that the cytoplasmic and nuclear accumulation of β-catenin is not a consequence of β-catenin relocalization from membrane to the nucleus.

Al Moustafa et al. (41) developed normal HN epithelial cells of oral origin expressing ErbB-2 and/or *E6/E7*. The authors found that overexpression of ErbB-2 receptor or expression of *E6/E7* of HPV alone does not affect the expression patterns of E-cadherin/catenin or induce malignant transformation of normal HN epithelial cells. To the contrary, both processes are deregulated by E6/E7 and ErbB-2 in normal epithelial cells of the HN. However, this *in vitro* model does not seem to mimic HPV-induced malignant transformation in oropharynx. There is lack of strong evidence supporting the contribution of ErbB-2 activation in head and neck carcinogenesis.

In summary, in the present study, we show nuclear accumulation of transcriptionally active β-catenin in HPV-positive cervical and oropharyngeal cancer cell lines that seems to be a direct consequence of *E6/E7* oncogene expression. This β-catenin nuclear accumulation may serve as a screening tool in HPV-positive populations to detect malignant progression. Nuclear β-catenin protein detection may serve as a biomarker to either distinguish individuals infected with HPV who are at high risk of developing cancer or diagnose early stages of cancer in infected individuals. HPV16-positive oral lesions are common and biomarkers that determine the malignant potential of cells are needed. Early detection will eventually reduce mortality in HPV-associated cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Activation of Wnt Signaling Pathway by Human Papillomavirus E6 and E7 Oncogenes in HPV16-Positive Oropharyngeal Squamous Carcinoma Cells

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