Published in final edited form as:

Head Neck. 2008 February ; 30(2): 139–147. doi:10.1002/hed.20673.

DELETION OF THE PDZ MOTIF OF HPV16 E6 PREVENTING IMMORTALIZATION AND ANCHORAGE-INDEPENDENT GROWTH IN HUMAN TONSIL EPITHELIAL CELLS

William C. Spanos, MD¹, Jeremy Geiger, BS¹, Mary E. Anderson, BS¹, George F. Harris, MD¹, Aaron D. Bossler, MDPhD², Russell B. Smith, MD¹, Aloysius J. Klingelhutz, PhD³, and John H. Lee, MD¹

¹ Department of Otolaryngology – Head and Neck Surgery, University of Iowa, Roy J. and Lucille A. Carver College of Medicine, Iowa City, Iowa. E-mail: john-h-lee@uiowa.edu

² Department of Pathology, University of Iowa, Roy J. and Lucille A. Carver College of Medicine, Iowa City, Iowa

³ Department of Microbiology, University of Iowa, Roy J. and Lucille A. Carver College of Medicine, Iowa City, Iowa

Abstract

Background—Human papillomavirus 16 (HPV16) has been associated with head and neck squamous cell carcinoma (HNSCC) in up to 60% of sampled specimens.

Methods—To understand better the viral genes required to transform human tonsil epithelial cells (HTEC), we isolated HTECs and transduced them with retroviral vectors containing HPV16 *E6* and *E7*.

Results—Immortalization and anchorage-independent growth of HTECs only occurred with expression of *E6* and *E7* with resultant degradation of p53. However, cells expressing *E6* lacking the PSD-95/disc-large/Zo-1 (PDZ) motif did not immortalize or grow anchorage independent. Telomerase activity and degradation of p53 were similar for wild-type and mutant *E6*.

Conclusion—The mechanism of oncogenic transformation by *E6* in HTECs is dependent on the PDZ binding motif. Identification of pathways affected by the interaction of *E6* and PDZ domain containing proteins will further our understanding of how HPV causes HNSCC and will provide potential therapeutic targets.

Keywords

HPV; squamous cell cancer; tonsil epithelium; PDZ motif; immortalization; invasion

Head and neck squamous cell carcinoma (HNSCC) affects approximately 30,000 individuals annually and results in death in approximately 40% of these cases.¹

Recently, human papillomavirus type 16 (HPV 16) DNA has been found to be associated with approximately 25% of HNSCCs.^{2–6} Interestingly, the oropharyngeal subsite (tonsil, base of tongue, soft palate, posterior pharyngeal wall) has a significantly higher percentage of HPV-positive (HPV+) cancers, 50% to 60%.³ Unlike cervical cancers, in which almost all tumors are HPV+, the variable presence of HPV in HNSCC allows a unique opportunity to compare

HPV+ and HPV-negative (HPV-) tumors for distinct cell characteristics associated with the presence of HPV. For instance, it has been noted that HPV+ head and neck tumors that express the viral oncogenes *E6* and *E7* rarely have p53 mutations and have less loss of heterozygosity compared with HPV- tumors.⁷⁻⁹ Although these associative studies strongly implicate HPV as a causative agent for HNSCC, they do not provide mechanism for transformation.⁷⁻⁹

When a normal cell transforms to a malignant cell, a myriad of potential combinations of altered cellular mechanisms are involved in this multistep process.¹⁰ Current understanding of malignant cell transformation suggests that although a vast number of potential cellular changes can occur, these changes ultimately result in 6 common traits (Figure 1) that are seen in all malignancies.¹⁰⁻¹² In HPV-related cancers, many of the disrupted pathways will be predictable because at least some of the required changes are performed by the viral oncogenes. Because an HPV+ cell would not need to acquire as many genetic changes, one would predict that HPV+ HNSCC would have fewer cellular mutations than HPV- HNSCC. Recent publications support this hypothesis.^{13,14}

The effect a viral oncogene has on a cell could vary because of gene expression pattern differences between cells of different types. Therefore, it is necessary to systematically define the mechanisms for transformation of each cell type and in each species.¹⁵ The role HPV16 plays in malignant transformation has predominantly been investigated in anogenital keratinocytes.¹⁶ Although some of the mechanisms resulting in transformation of anogenital keratinocytes will likely be similar in head and neck epithelial cells, there will also be differences. For instance, multiple high-risk HPVs cause cervical cancer,¹⁷ but HNSCC is associated almost exclusively with HPV16.^{3-5,18} Although this HPV16 predilection in the HNSCC could be because of viral tropism, it could also be related to how specific viral oncogenes, such as *E6* and *E7*, affect tonsil epithelium. Therefore, to best understand the mechanisms of HPV-related transformation in HNSCC, it will be necessary to complete studies in keratinocytes from the most affected area (tonsil).

Some of the specific mechanisms that result in the 6 common traits of epithelial cancer formation (represented above the dashed line in Figure 1) can be studied in vitro because they result in cell immortality.¹⁹ HPV16 *E6* and *E7* are both multi-functional oncoproteins, and coexpression results in efficient immortalization of anogenital epithelial cells. The best-known function of the *E6* protein is targeting of the p53 protein for ubiquitin-mediated degradation.²⁰ Inactivation of the p53 pathway has been shown to be important for cellular immortalization and abrogation of normal responses to DNA damage.^{21,22} It has also been shown that *E6* has numerous other functions,^{23,24} including a role in activation of telomerase, the ribonucleoprotein complex that adds telomeric repeats to the ends of eukaryotic chromosomes.^{25,26} The best known function of *E7* is the binding and inactivation of the retinoblastoma tumor suppressor protein, pRb.²⁷ The pRb protein is a key player in the transit of cells from G1 to S. *E7* also has many other reported functions,²³ including blocking the effects of cyclin-dependent kinase inhibitors p21 and p27.^{28,29} Inactivation of the p53 and pRb pathways, as well as activation of telomerase, are common events that occur in many cancers, regardless of HPV status. Past reports using anogenital keratinocytes have shown that these events are orchestrated by overexpression of viral oncoproteins *E6* and *E7*. It is unknown whether these same cellular mechanisms reported for anogenital keratinocytes also result in immortalization of tonsil epithelial cells.

Although these known functions of *E6* and *E7* have been shown to be directly responsible for immortality, other *E6* functions apparently are necessary for complete malignant progression. Although the exact mechanism remains elusive, substantial evidence suggests that the *E6* PSD-95/disc-large/Zo-1 (PDZ) binding motif plays a major role in malignant progression. Protein-protein interactions based on PDZ binding are as follows: (1) PDZ domains named

after internal sequences about 90 amino acids long and form a 3-dimension binding pocket. These sequences were initially discovered in PSD-95, DLG and Zo-1, hence PDZ. (2) PDZ binding motifs (ligands) are short amino acid sequences that bind in these PDZ domains. The interactions between the motif and the domain proteins play a role in many cellular functions, especially functions localized immediately adjacent to the cell membrane.^{30,31} Clinical evidence shows that in high-risk cancer-causing HPV subtypes, the E6 proteins contain a PDZ binding motif, whereas low-risk HPV types, which rarely cause cancer, lack this motif. Laboratory analysis of immortal human cell lines transfected with E6 also suggests that the PDZ motif function is required for a change in cell shape and distribution of cell architectural proteins, which are similar to changes seen in cells that grow malignantly in vivo.³² Whether the PDZ binding motif plays a role in immortalization in tonsil cells has not been evaluated.

In this study, we use primary human tonsil epithelial cells (HTECs) and retroviral transduction with key viral oncogenes *E6* and *E7* to determine which viral genes are required for immortalization. These results were compared with previous work with anogenital keratinocytes to determine if the viral oncogenes alter the same cellular pathways in HTECs. Finally, an E6 mutant lacking the PDZ binding motif (*E6*^{Δ146–151}) was utilized to assess the role of this motif in tonsil cell immortalization.

MATERIALS AND METHODS

Isolation and Passage of Human Tonsil Cells

Normal HTECs were isolated from routine bilateral tonsillectomy specimens. Prior to use, the absence of HPV DNA was confirmed by polymerase chain reaction (PCR) (data not shown). The most superficial epithelial sheet was separated from the underlying stroma by an overnight dispase treatment, manual tissue separation, brief trypsinization with 0.25% trypsin (Invitrogen, Carlsbad, CA), and subsequent outgrowth in keratinocyte serum free media (K-SFM) (Invitrogen) containing 0.2 ng/mL epidermal growth factor (EGF) and 25 µg/mL bovine pituitary extract, 1% pen/strep (Invitrogen), and 25 µg/mL Fungizone (Invitrogen). After the first passages, the cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% fetal bovine serum (Invitrogen), 22% Ham's F12 (Invitrogen), 1% pen/strep, 25 µg/mL hydrocortisone (Sigma, St. Louis, MO), 8.4 ng/mL cholera toxin (Sigma), 5 µg/mL transferrin (Sigma), 5 µg/mL insulin (Sigma), 1.36 ng/mL tri-iodo-thyronine (Sigma), and 5 ng/mL EGF (Invitrogen). Cells in serum containing media required the addition of irradiated murine J2-3T3 feeder cells for maximal growth and viability.

Retroviral Production, Infection, and Generation of Stable Lines

Replication incompetent retrovirus, produced by plasmid transfection in packaging cell lines using previously reported techniques, was a gift from the Galloway lab (University of Washington, Seattle).^{33,34} To ensure that the vectors would express the correct transgenes, all plasmids were sequenced prior to viral production. Early passage HTECs from 5 different individuals were infected at early passage no. 2. Retroviruses expressing E6, *E6*^{Δ146–151}, or E7 and a control vector with the antibiotic resistance cassette (LXSN) were used to infect approximately 40% confluent normal human tonsillar cells for 12 hours at 37°C, 5% CO₂. Following media change, cells were cultured for 16 hours, split 1:4, and subjected to initial antibiotic selection. Transduced cells were selected by the addition of 200 µg/mL G418 (Invitrogen) or 8 µg/mL hygromycin B (Invitrogen) respectively, beginning 24 hours post retroviral infection. Cells were maintained at this high antibiotic concentration until 100% uninfected control cells had died, approximately 14 days. Stably expressing E6 or E7 cell lines were maintained in 50 µg/mL G418 or 8 µg/mL hygromycin, respectively. The number of clones resistant to antibiotic selection was similar (approximately 40%) for all vectors.

After the initial selection period, clonal cell lines from these pooled infections were generated in 2 of the 5 different samples. To derive these clones, after transduction and selection the cells were split 1:1000 onto 10-cm tissue culture plates and allowed to proliferate until small colonies of cells were present. Similar numbers of colonies were visible for all vectors. These small clonal populations were ring cloned from the tissue culture plate with 8-mm × 8-mm cloning rings (Fisher Scientific), and allowed to proliferate as independent clonal populations. More than 20 clones were initially isolated from each transduction condition. During in vitro culture, the cells were split 1:4, which represents 2 population doubling events. Growth rate was calculated by determining the number of days required for 2 population doublings while in culture over 10 passages.

Cell Lysis and Immunoblot

Whole cell lysates were harvested at 4°C with protein lysis buffer consisting of 50 mM Tris-HCl pH 7.5 (Sigma), 150 mM NaCl (Sigma), 5 mM EDTA (Sigma), 2 mM NaVO₄ (Sigma), 10 mM NaPP₆ (Sigma), 100 mM NaF (Sigma), 10% glycerol (Fisher, Fairlawn, NJ), 1% Triton X-100 (Pierce Biotechnology, Rockford, IL), 10 µg/mL pepstatin (Sigma), 20 µg/mL leupeptin (Sigma), and 20 µg/mL aprotinin (Sigma). Lysates were drawn through a 25-gauge needle 10 times to ensure complete protein dissociation from other cellular components. Cell lysates were snap-frozen at -80°C until use. Expression of human p53 and pRb was measured by immunoblotting 25 µg total cellular proteins with the p53 antibody (OP-43, CalBiochem, San Diego, CA) at 1:1000 dilution and the pRb antibody (pRb 780, Cell Signaling, Danvers, MA) using a standard Western blot technique. Blots were developed by ECL chemiluminescent detection (Pierce Biotechnology).

Real-Time Reverse Transcriptase PCR Quantitation of Oncogene Expression

RNA isolation, reverse transcriptase-PCR (RT-PCR) quantitation of *E6* and *E7* oncogenes were analyzed as previously described.^{35–37} The message levels shown in Figure 5 are standardized to Caski cells, which are an HPV16-positive cancer cell line known to express *E6*, as well as to *18S* ribosomal RNA levels using the primer probe kit from Applied Biosystems (Foster City, CA).

Soft Agar Assay

Anchorage-independent growth assays were performed following previously published methods.³⁸ Polyester membranes of trans-well inserts (12-mm diameter, 0.4-µm pore; Costar, Corning, NY) were coated with 100 µL of 1% noble agar. After counting cells using a hemacytometer, 1 part of 3.3% noble agar at 45°C was diluted with 10 parts cell suspension for a final concentration of 1×10^4 cells per 400 µL. Triplicate wells for each cell line were seeded. After agar solidification, media were added to the outer wells. After 3 weeks, colonies approximately 2 mm in diameter or greater were counted.

Telomerase Activity Assay

In triplicate, telomerase activity was measured by using quantitative PCR assay as described previously.³⁹ Telomerase levels were compared with cell equivalents of an HPV16 E6/E7 immortalized foreskin keratinocyte cell line.

RESULTS

Isolation and Growth of HTEC

To establish a relevant cell culture model, a method was developed to isolate and culture tonsil epithelial cells from routine tonsillectomy specimens. After the first passage in serum free media to eliminate contamination with human fibroblasts, the cells were plated with irradiated

feeders and the media was switched to a basal media with serum. Under these conditions, the cells grow in epithelial colonies that gradually displace the irradiated feeder fibroblasts as they proliferate (Figure 2). The cells under these conditions can be propagated for up to approximately 18 to 22 population doublings. Spontaneous immortalized epithelial cells were never observed in the absence of HPV16 viral oncogenes.

Viral Gene Requirements for Tonsil Cell Immortalization

To determine which HPV16 viral genes were required for immortalization, early passage HTECs were infected with retroviral constructs that overexpressed either *E6* or *E7* and a gene conferring neomycin resistance. Vector controls (LXSN) underwent senescent cell death within several population doublings after selection (Figure 3). Clones expressing *E7* did have a slightly extended life span in culture, but did not result in the outgrowth of immortalized cells (Figure 3). In comparison, *E6* alone did not extend growth in culture. These data demonstrate that neither a retroviral insertion event nor a single HPV viral oncogene is sufficient to cause an immortal cellular phenotype in primary HTECs. *E6* and *E7* dual infected HTECs expressed from separate retroviral vectors resulted in immortalization of all (5/5) primary tonsil epithelial cell lines tested. Furthermore, the efficiency of immortalization in the *E6/E7* clones from transductions was very high, 70% (Figure 3). The consistency of immortalization between cell lines derived from multiple individuals and the high efficiency of transformed clones in an individual transduction strongly suggest that *E6* and *E7* are directly responsible for the changes resulting in immortalization. These findings are consistent with findings in primary cervical and foreskin keratinocytes.

The PDZ Binding C-Terminus of E6 Is Required for Immortalization of Tonsil Cells

Since the PDZ binding motif of *E6* has been associated with malignant transformation, we wanted to examine if this motif plays a role in immortalization by using an *E6* mutant that lacks the PDZ motif (*E6*^{Δ146–151}). As with wild-type *E6* (wt-*E6*), when expressed alone, *E6*^{Δ146–151} did not help further prolong cell life (Figure 3). When *E6*^{Δ146–151} was expressed with *E7*, the in vitro lifespan of cells was prolonged significantly (Figure 3). However, immortality was a very rare event. Infections with *E6*^{Δ146–151}/*E7* in primary cells from different individuals resulted in immortal cells in only 1 patient. Clones were isolated from the 1 successful transduction and the efficiency of immortalization in this 1 instance was less than 5% (1 clone of 22).

Although we used identical retroviral production and infection protocols, it is possible the differences in immortalization efficiency could be related to transgene expression level differences. A reliable antibody is not available for *E6* or *E7*; therefore, we evaluated mRNA levels of the viral oncogenes and levels of the cell proteins that *E6* and *E7* target for degradation. Wt-*E6* and *E6*^{Δ146–151} are known to degrade p53.²⁵ Both the wt-*E6/E7* and *E6*^{Δ146–151}/*E7* expressing cells displayed loss of p53 and pRb compared with the control, LXSN (Figure 4). All cell lines expressed viral oncogenes at a similar level with a maximum of 3 times difference in *E6* or *E7* expression (Figure 5). The expression levels did not correlate with survival or immortality (not shown). In addition to improved immortalization of tonsil cells, *E6/E7* cells also grew faster in culture compared with *E6*^{Δ146–151}/*E7* cells (Table 1). Taken together, these findings suggest that the PDZ motif likely is required for immortalization and enhanced growth rate by a mechanism not related to *E6*'s ability to degrade p53.

HPV16 E6-Mediated Anchorage-Independent Growth Requires the E6 PDZ Motif

During the progression to grow in an invasive manner, an immortal cell must acquire the ability to grow in the absence of regulatory messages from the extracellular environment, such as the basement membrane. Nontransformed cells undergo induced cell death when detached from their surrounding structure (anoikis). Overcoming anoikis is thought to be a critical step for

invasive and metastatic growth because it allows cells to function in an environment outside their normal stroma. A method to assay for this aspect of transformation is testing growth in semisolid media or anchorage-independent growth (AIG). To determine if the PDZ binding motif plays a role in overcoming anoikis, we examined growth of cells expressing E6 and E6 Δ 146–151 in an AIG assay.

Multiple clones were assayed in triplicate. Colony forming efficiency (CFE) was averaged between clones for the indicated cell type (Table 2). Only cells containing E6 with an intact PDZ motif have the ability to grow anchorage independent in appreciable quantities. These data strongly suggest that a mechanism associated with the PDZ motif of E6 is required to permit invasive growth.

Activation of Telomerase

To determine if the lack of immortality was related to the inability of the E6 Δ 146–151 transgene to activate telomerase, we completed telomeric repeat amplification protocol assays to evaluate telomerase activity. Figure 6 shows that wt-E6, E6 Δ 146–151/E7, and E6/E7 expression resulted in similar telomerase activity. This result further suggests that the PDZ binding region enhances immortalization by a mechanism that is separate from E6-mediated telomerase activation.

DISCUSSION

Conversion of a normal cell to a cancer cell requires multiple cellular changes. To understand better the role HPV plays in this multistep process, we have used tonsil cells isolated from normal human specimens and expressed the HPV viral oncogenes in them to understand what viral genes are required for immortalization, a key component of carcinogenic transformation. These studies have increased our understanding regarding HPV-related HNSCC by providing the following evidence: (1) although E7 slightly extends growth in culture, both E6 and E7 are required for tonsillar epithelial immortalization; (2) E6 efficiently degrades the cellular protein p53 and activates telomerase; (3) the PDZ c-terminal binding motif is required for AIG and efficient immortalization of primary tonsillar cells. Each of these findings will be discussed separately.

Our findings that E6 and E7 immortalize tonsil epithelial cells are consistent with past studies investigating anogenital and oral epithelial cell immortalization with HPV viral oncogenes.⁴⁰ In these past studies, oral epithelial cells were immortalized in culture using transfection of HPV-16 DNA or a retroviral vector expressing both open reading frames for E6 and E7. Our findings add to these previous studies by investigating the individual contributions of each of the viral proteins. Certain epithelial cells (for instance, mammary epithelial cells) do not require both oncogenes for immortalization⁴¹; therefore, it was important for us to investigate the contribution of both viral proteins in our cell of interest. The finding that both viral proteins are required for tonsillar immortalization may have important clinical implication because it is likely that strategies aimed at reversing the cellular fate will not need to target the function of both of these viral proteins.

Interestingly, the requirement of the PDZ binding motif portion of the E6 protein for in vitro cellular immortalization has not been previously reported as an essential component contributing to epithelial cell immortality. Except for 1 rare clone that appears immortal, the E6 Δ 146–151/E7 cells all have substantially prolonged growth in culture but undergo slow senescent death at approximately 30 to 40 population doublings despite activation of telomerase and degradation of p53. The fact that only 1 clone became immortal suggests that the immortality observed in this clone is related to an E6-independent change that occurred as a result of the retroviral insertion or potentially a second mutation that occurred in culture.

The suggestion that the PDZ motif is required for AIG may give insight into how E6 induces invasive growth. The PDZ motif on E6 has been postulated to bind and degrade several cellular proteins.^{42–44} The physiologic significance of these E6 interactions with PDZ domain containing proteins has not been determined. However, recent work with our colleagues has shown HPV16 E6 to activate nuclear factor kappa B (NF- κ B) in human airway epithelial cells with subsequent inhibition of tumor necrosis factor–stimulated apoptosis in a PDZ dependent manner.⁴⁵ In planned experiments, we will evaluate whether activation of NF- κ B also depends on the E6 PDZ motif in tonsil epithelial cells. In addition, we will screen PDZ domain proteins to identify the PDZ-containing protein that is targeted by the E6 PDZ motif. The E6-mediated degradation of such protein(s) may play a role in the increased growth rate, immortalization, and AIG we observed in these tonsil cells.

In summary, the findings from these studies indicate that both HPV16 *E6* and *E7* are required for the immortalization of tonsil epithelial cells. They also strongly suggest that a mechanism related to the E6 PDZ motif plays a significant role in transformation of tonsil cells. Identification of the specific pathways affected by E6's interaction with PDZ containing proteins will further our understanding of how HPV16 causes HNSCC and provide potential targets for therapeutic interaction.

Acknowledgements

The authors thank Andrea Van Gelder for clerical assistance with editing.

Contract grant sponsor: Veteran Administration Merit Award; Contract grant sponsor: NIH; Contract grant numbers: KO8, T32.

References

1. SEER (Surveillance, Epidemiology and end Results). Program: 12 Regs Public-Use Data, November 2002 Submission for expanded races (1992–2000), SEER Stat Database. National Cancer Institute; 2003.
2. Gillison M, Shah KV. Human papillomavirus-associated head and neck squamous cell carcinoma: mounting evidence for an etiologic role for human papillomavirus in a subset of head and neck cancers. *Curr Opin Oncol* 2001;13:183–186. [PubMed: 11307062]
3. Smith EM, Ritchie JM, Summersgill KF, et al. Age, sexual behavior and human papillomavirus infection in oral cavity and oropharyngeal cancers. *Int J Cancer* 2004;108:766–772. [PubMed: 14696105]
4. Kreimer AR, Clifford GM, Boyle P, Franceschi S. Human papillomavirus types in head and neck squamous cell carcinomas worldwide: a systematic review. *Cancer Epidemiol Biomarkers Prev* 2005;14:467–475. [PubMed: 15734974]
5. Begum S, Cao D, Gillison M, Zahurak M, Westra WH. Tissue distribution of human papillomavirus 16 DNA integration in patients with tonsillar carcinoma. *Clin Cancer Res* 2005;11:5694–5699. [PubMed: 16115905]
6. Pfister DG, Su YB, Kraus DH, et al. Concurrent cetuximab, cisplatin, and concomitant boost radiotherapy for locoregionally advanced, squamous cell head and neck cancer: a pilot phase II study of a new combined-modality paradigm. *J Clin Oncol* 2006;24:1072–1078. [PubMed: 16505426]
7. Dai M, Clifford GM, le Calvez F, et al. Human papillomavirus type 16 and TP53 mutation in oral cancer: matched analysis of the IARC multicenter study. *Cancer Res* 2004;64:468–471. [PubMed: 14744758]
8. Braakhuis BJ, Snijders PJ, Keune WJ, et al. Genetic patterns in head and neck cancers that contain or lack transcriptionally active human papillomavirus. *J Natl Cancer Inst* 2004;96:998–1006. [PubMed: 15240783]
9. Gillison ML, Koch WM, Capone RB, et al. Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. *J Natl Cancer Inst* 2000;92:709–720. [PubMed: 10793107]

10. Hahn WC, Weinberg RA. Modelling the molecular circuitry of cancer. *Nat Rev Cancer* 2002;2:331–341. [PubMed: 12044009]
11. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000;100:57–70. [PubMed: 10647931]
12. Hahn WC, Weinberg RA. Rules for making human tumor cells. *N Engl J Med* 2002;347:1593–1603. [PubMed: 12432047]
13. Braakhuis BJM, Snijders PJF, Kuene W-JH, et al. Genetic patterns in head and neck cancers that contain or lack transcriptionally active human papillomavirus. *J Natl Cancer Inst* 2004;96:998–1006. [PubMed: 15240783]
14. Dahlgren L, Mellin H, Wangsa D, et al. Comparative genomic hybridization analysis of tonsillar cancer reveals a different pattern of genomic imbalances in human papillomavirus-positive and -negative tumors. *Int J Cancer* 2003;107:244–249. [PubMed: 12949801]
15. Rangarajan A, Hong SJ, Gifford A, Weinberg RA. Species- and cell type-specific requirements for cellular transformation. *Cancer Cell* 2004;6:171–183. [PubMed: 15324700]
16. Steenbergen RD, de Wilde J, Wilting SM, Brink AA, Snijders PJ, Meijer CJ. HPV-mediated transformation of the anogenital tract. *J Clin Virol* 2005;32(Suppl 1):S25–S33. [PubMed: 15753009]
17. Hiller T, Poppelreuther S, Stubenrauch F, Iftner T. Comparative analysis of 19 genital human papillomavirus types with regard to p53 degradation, immortalization, phylogeny, and epidemiologic risk classification. *Cancer Epidemiol Biomarkers Prev* 2006;15:1262–1267. [PubMed: 16835321]
18. Ioka A, Tsukuma H, Ajiki W, Oshima A. Trends in head and neck cancer incidence in Japan during 1965–1999. *Jpn J Clin Oncol* 2005;35:45–47. [PubMed: 15681605]
19. Hahn WC. Immortalization and transformation of human cells. *Mol Cells* 2002;13:351–361. [PubMed: 12132573]
20. Scheffner M, Werness BA, Huibregtse JM, Levine AJ, Howley PM. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell* 1990;63:1129–1136. [PubMed: 2175676]
21. Lechner MS, Laimins LA. Inhibition of p53 DNA binding by human papillomavirus E6 proteins. *J Virol* 1994;68:4262–4273. [PubMed: 8207801]
22. Vousden KH, Lu X. Live or let die: the cell's response to p53. *Nat Rev Cancer* 2002;2:594–604. [PubMed: 12154352]
23. Munger K, Baldwin A, Edwards KM, et al. Mechanisms of human papillomavirus-induced oncogenesis. *J Virol* 2004;78:11451–11460. [PubMed: 15479788]
24. Gao Q, Singh L, Kumar A, Srinivasan S, Wazer DE, Band V. Human papillomavirus type 16 E6-induced degradation of E6TP1 correlates with its ability to immortalize human mammary epithelial cells. *J Virol* 2001;75:4459–4466. [PubMed: 11287601]
25. Liu Y, Chen JJ, Gao Q, et al. Multiple functions of human papillomavirus type 16 E6 contribute to the immortalization of mammary epithelial cells. *J Virol* 1999;73:7297–7307. [PubMed: 10438818]
26. Klingelhutz AJ, Foster SA, McDougall JK. Telomerase activation by the E6 gene product of human papillomavirus type 16. *Nature* 1996;380:79–82. [PubMed: 8598912]
27. Dyson N, Howley PM, Munger K, Harlow E. The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. *Science* 1989;243:934–937. [PubMed: 2537532]
28. Helt AM, Funk JO, Galloway DA. Inactivation of both the retinoblastoma tumor suppressor and p21 by the human papillomavirus type 16 E7 oncoprotein is necessary to inhibit cell cycle arrest in human epithelial cells. *J Virol* 2002;76:10559–10568. [PubMed: 12239337]
29. Zerfass-Thome K, Zwerschke W, Mannhardt B, Tindle R, Botz JW, Jansen-Durr P. Inactivation of the cdk inhibitor p27KIP1 by the human papillomavirus type 16 E7 oncoprotein. *Oncogene* 1996;13:2323–2330. [PubMed: 8957073]
30. Harris BZ, Lim WA. Mechanism and role of PDZ domains in signaling complex assembly. *J Cell Sci* 2001;114(Part 18):3219–3231. [PubMed: 11591811]
31. Fan JS, Zhang M. Signaling complex organization by PDZ domain proteins. *Neurosignals* 2002;11:315–321. [PubMed: 12566920]
32. Watson RA, Thomas M, Banks L, Roberts S. Activity of the human papillomavirus E6 PDZ-binding motif correlates with an enhanced morphological transformation of immortalized human keratinocytes. *J Cell Sci* 2003;116(Part 24):4925–4934. [PubMed: 14625386]

33. Halbert CL, Demers GW, Galloway DA. The E7 gene of human papillomavirus type 16 is sufficient for immortalization of human epithelial cells. *J Virol* 1991;65:473–478. [PubMed: 1845902]
34. Foster SA, Demers GW, Etscheid BG, Galloway DA. The ability of human papillomavirus E6 proteins to target p53 for degradation in vivo correlates with their ability to abrogate actinomycin D-induced growth arrest. *J Virol* 1994;68:5698–5705. [PubMed: 8057451]
35. Spanos WC, El-Deiry M, Lee JH. Cidofovir incorporation into human keratinocytes with episomal HPV 16 results in nonselective cytotoxicity. *Ann Otol Rhinol Laryngol* 2005;114:840–846. [PubMed: 16363057]
36. Lee JH, Yi SM, Anderson ME, et al. Propagation of infectious human papillomavirus type 16 by using an adenovirus and Cre/LoxP mechanism. *Proc Natl Acad Sci U S A* 2004;101:2094–2099. [PubMed: 14769917]
37. Lanham S, Herbert A, Watt P. HPV detection and measurement of HPV-16, telomerase, and survivin transcripts in colposcopy clinic patients. *J Clin Pathol* 2001;54:304–308. [PubMed: 11304848]
38. Berger KL, Barriga F, Lace MJ, et al. Cervical keratinocytes containing stably replicating extrachromosomal HPV-16 are refractory to transformation by oncogenic H-Ras. *Virology* 2006;356:68–78. [PubMed: 16945398]
39. Cawthon RM. Telomere measurement by quantitative PCR. *Nucleic Acids Res* 2002;30:e47. [PubMed: 12000852]
40. Li SL, Kim MS, Cherrick HM, Park NH. Low p53 level in immortal, non-tumorigenic oral keratinocytes harboring HPV-16 DNA. *Eur J Cancer B Oral Oncol* 1992;28:129–134. [PubMed: 1339128]
41. Band V, De Caprio JA, Delmolino L, Kulesa V, Sager R. Loss of p53 protein in human papillomavirus type 16 E6-immortalized human mammary epithelial cells. *J Virol* 1991;65:6671–6676. [PubMed: 1658367]
42. Kiyono T, Hiraiwa A, Fujita M, Hayashi Y, Akiyama T, Ishibashi M. Binding of high-risk human papillomavirus E6 oncoproteins to the human homologue of the *Drosophila* discs large tumor suppressor protein. *Proc Natl Acad Sci U S A* 1997;94:11612–11616. [PubMed: 9326658]
43. Nakagawa S, Huibregtse JM. Human scribble (Vartul) is targeted for ubiquitin-mediated degradation by the high-risk papillomavirus E6 proteins and the E6AP ubiquitin-protein ligase. *Mol Cell Biol* 2000;20:8244–8253. [PubMed: 11027293]
44. Thomas M, Laura R, Hepner K, et al. Oncogenic human papillomavirus E6 proteins target the MAGI-2 and MAGI-3 proteins for degradation. *Oncogene* 2002;21:5088–5096. [PubMed: 12140759]
45. James MA, Lee JH, Klingelutz AJ. Human papillomavirus type 16 E6 activates NF-kappaB, induces cIAP-2 expression, and protects against apoptosis in a PDZ binding motif-dependent manner. *J Virol* 2006;80:5301–5307. [PubMed: 16699010]

FIGURE 1.

Common acquired traits for epithelial cancers. The changes associated with a cell during carcinogenesis involve common cellular events, albeit by different mechanisms. For carcinogenesis all components of this circle are usually present in vivo.¹¹ The traits superior to the dotted line represent changes that must occur for immortal growth in vitro.^{12,15} HPV16 E6/E7 provides the cellular changes for limitless replication, apoptosis evasion, and insensitivity to antigrowth signal.

FIGURE 2.

Cell morphology of human tonsil epithelial cells in culture grown with irradiated murine J2-3T3 feeders. (1) HPV16 E6/E7 cells (late passage pool). (2) HPV16 E6^{Δ146-151}/E7 cells (late passage pool). (3) Uninfected control tonsil cells (early passage pool). (4) Control LXS vector alone cells (early passage pool). Light microscopy at $\times 10$ magnification epithelial islands of keratinocytes growing among the spindle shaped feeder fibroblasts (indicated by *). Cell morphology remains similar between conditions. [Color can be viewed in the online issue, which is available at www.interscience.wiley.com.]

FIGURE 3.

Kaplan–Meier survival curve for human tonsil epithelial cells (HTECs) expressing HPV16 viral genes. HTECs were transduced with retroviral constructs expressing the indicated viral genes. Stable transduction with E6/E7 demonstrates a significantly higher percent survival (70% at 180 days) of clonal populations. Cells expressing E6^{Δ146–151} + E7 show extended life span (40% at 90 days) compared with E7, E6^{Δ146–151}, E6, and LXS transduced primary tonsil cells. Senescence was defined as no growth in culture after 30 days.

FIGURE 4.

Loss of p53 and pRb in cells containing HPV16 oncogenes. Human tonsil epithelial cells (HTECs) were transduced with the indicated retroviral constructs expressing the control vector, HPV16 E6 and E7, or HPV16 E6^{Δ146–151} and E7. The Western blot shows decreased p53 and pRb in HTECs with E6/E7 or E6^{Δ146–151}/E7 compared with empty vector control cells.

FIGURE 5.

Expression of E6 and E7 in clones. Levels of E6 and E7 mRNA for all clones were determined by quantitative real-time PCR and expressed relative to the message level of Caski cells, a HPV16 cancer cell line known to overexpress these viral oncogenes (approximately 500 copies HPV16 genome per cell). The signal was normalized to 18S ribosomal RNA levels.

FIGURE 6.

Telomerase activation by HPV16 E6. DNA isolated from early passage (passage 7) human tonsil epithelial cells infected with the various HPV16 genes or control vector (LXS) was analyzed with quantitative PCR to determine the activity of telomerase per cell equivalent.

Table 1

Human tonsil cell clone growth rates.

Cell line	Growth rate (d/population doubling)
Primary	1.75 ± 0.5
LXSN	1.7 ± 0.07
E6Δ	1.65 ± 0.11
E6	1.6 ± 0.21
E6Δ/E7	1.6 ± 0.1 *
E6/E7	1.35 ± 0.03 *

Note: The growth rate of the clones was calculated after selection and prior to senescence. The average and standard deviation between clones for each condition is shown as the days required for population doubling. The growth rate of HPV16 E6/E7 was significantly faster than HPV16 E6^{Δ146–151}/E7 as compared by t-test statistical analysis.

*
(p value < .01).

Table 2

Colony forming efficiency of human tonsil epithelial cells.

	AIG CFE% (SD)
Primary HTEC	0
HTEC + LXS	0
HTEC + E6Δ/E7	0.04 (0.02)
HTEC + E6/E7	5.9 (1.6)

HPV16 E6-mediated anchorage-independent growth (AIG) requires the E6 PDZ motif. Human tonsil epithelial cells (HTECs) were transduced with HPV16 E6 +E7, HPV16 E6 Δ 146–151 + E7, or empty vector (LXS). The data represent the average and standard deviation of 3 clones plated in triplicate. Colony forming efficiency (CFE) is reported as a percent of colonies/number of cells plated. It was not possible to test the effect of E6 or E6 Δ 146–151 alone without the addition of E7 because the clones did not survive long enough in culture (Figure 3).