THE EXPRESSION OF KEY CELL CYCLE MARKERS AND PRESENCE OF HUMAN PAPILLOMAVIRUS IN SQUAMOUS CELL CARCINOMA OF THE TONSIL

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Abstract: Background. Chemical carcinogens induce squamous cell carcinoma (SCC) of the head and neck by targeting the p53 and the retinoblastoma (pRb) pathways. Human papillomavirus (HPV) might have an etiologic role in these cancers at particular sites. Few studies have compared cell cycle protein expression in HPV-positive and HPV-negative tumors in this region.

Methods. Fifty tonsil SCCs were analyzed for HPV by PCR and for expression of cell cycle proteins (p53, pRb, p16INK4A, p21CIP1/WAF1, p27KIP1, and cyclinD1) by immunohistochemistry.

Results. HPV was present in 42%; almost all were type 16. There were statistical associations between HPV positivity and reduced expression of pRb and cyclinD1, overexpression of p16, and younger patient age. Tumor with down-regulated p27 tended to have down-regulated pRb and p21.

Conclusions. HPV-positive tonsil SCCs have distinct molecular pathways. Their association with younger patient age suggests that they are biologically distinct from HPV-negative tumors. © 2004 Wiley Periodicals, Inc. Head Neck 26: 1–9, 2004

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Squamous cell carcinoma (SCC) of the head and neck is a common malignancy in most parts of the world. In developed countries like Australia, there is a strong epidemiologic association with heavy smoking and alcohol consumption, which is believed to disrupt the two major pathways controlling cell proliferation—that of the tumor suppressor protein p53 and of the product of the retinoblastoma gene (pRb).1,2

The p53 protein has potent cell cycle arrest and apoptotic functions. Under normal circumstances, p53 is dormant until activated by DNA damage or other genomic aberrations. p53 then activates the p21CIP1/WAF1 (p21) protein to arrest the cell cycle in G1 for repair or to induce apoptosis (programmed cell death) if the damage is too severe (Figure 1). Disruption to the p53 pathway through gene mutation, protein inactivation,
or modification is known to occur in more than 50% of cases of SCC of the head and neck. The lack of functional p53 allows continued replication of abnormal cells, leading to an accumulation of genetic changes that contribute to malignant progression.

The pRb protein also has a central role in controlling the passage of cells through G1/S transition with feedback loops involving the cyclin D1 and cyclin-dependent kinases (cdks). On phosphorylation, pRb is released from its complex with a protein called E2F. Free E2F “switches on” proteins needed for DNA replication. Cyclin-dependent kinase inhibitors, including p16, p21, and p27, negatively regulate this process.

The presence of oncogenic human papillomaviruses (HPVs), notably type 16, in a subset of head and neck cancers suggests that they might play an etiologic role in these tumors, as has been well documented in the anogenital tract. The association is strongest with tonsil cancers in which HPV DNA positivity rates have frequently exceeded 50%, with type 16 predominating. There is growing evidence suggesting that the HPV-positive subset might represent head and neck cancers displaying distinct clinical, morphologic, and molecular features. In HPV-induced anogenital cancers, the viral oncoproteins E6 and E7 disrupt the p53 and pRb pathways by interacting with p53 and pRb, respectively. E6 and E7 have also been shown to interact with p27KIP1 [p27] and p21. There have been relatively few studies of the expression of cell cycle proteins in HPV-positive and HPV-negative cancers of the head and neck. Data relating to p53 have been conflicting; in some studies the incidence of p53 mutations has been reported to be inversely proportional to the presence of HPV, whereas in others, p53 mutations and HPV have coexisted. This confusion might, in part, reflect cohort diversities in risk factors, culture, or ethnicity and anatomic site. A strong association between the presence of HPV and down-regulation of pRb and cyclin D1 expression and up-regulation of p16 has been recently reported in a small series of tonsil cancers. These findings support the contention that HPV has a causal association in a proportion of head and neck cancers, but further data are needed for confirmation.

The major aim of this study was to investigate the relationship between HPV and the expression of six key cell cycle proteins in 50 tonsil cancers. The proteins chosen for analysis were identified as potential targets of HPV E6 and E7: p53 and p21 in the p53 pathway; and pRb, p16, p21, p27, and cyclin D1 in the pRb pathway. Tumors were analyzed for the presence or absence of HPV DNA by direct sequence analysis of PCR products, and findings were related to the immunohistochemically quantified expression of the markers. An additional aim was to determine whether any of the targeted molecular markers had a significant association with clinical stage, tumor grade, patient age, or gender.

**MATERIALS AND METHODS**

**Study Population.** Investigations were carried out on 50 patients treated surgically for primary tonsil SCC at Royal Prince Alfred Hospital (Sydney, Australia) between 1987 and 1998. None of these had previously undergone tonsillectomy. Selection was on the basis of availability of fixed tumor tissue, clinicopathologic data, and the presence of amplifiable DNA in the tumor as determined by PCR of the β-globin gene. Eleven patients had early stage (I, II) and 39 had advanced stage (III, IV) disease. The median age of the
group was 56.7 years (range, 35–79), and the male–female ratio was 38:12.

Detection of HPV DNA and Analysis of p53 Mutations by PCR. PCR was carried out on crude extracts of 5-μm sections of paraffin-embedded tumor tissues prepared according to our routine published protocols and with precautions taken to minimize contamination. Microtome blades were thoroughly cleaned between paraffin blocks. Malignant cells were confirmed in hematoxylin–eosin–stained sections cut sequentially from the same block. Between two and five 5-μm paraffin sections were deparaffinized with xylene and absolute alcohol, and desiccated pellets were digested in 100 μL lysis buffer (50 mM Tris-HCl, pH 8.5, 1 mM EDTA, 0.5% Tween-20) containing 200 μg/mL proteinase K. All lysates were tested using two HPV 16 type-specific PCRs that amplified overlapping 109-bp and 332-bp regions of the HPV 16 E6 gene; all samples were also tested by a general primer mediated PCR, GP5+/6+, targeting the L1 region of papillomaviruses and capable of detecting a broad spectrum of mucosal HPVs. Tumors testing HPV-negative were subsequently analyzed by nested PCRs using degenerate L1 primers A10/A5-A6/A8 and CP62/70CT-CP65/69 designed to detect mucosal HPVs and group B1/cutaneous epidermodysplasia verruciformis (EV) HPVs, respectively. All results were confirmed by repeat PCR testing on at least one occasion.

Sequence Analysis of HPV. Approximately 20% of amplified products were electrophoresed in 2% LE agarose gels. The gels were stained with ethidium-bromide, and the bands visualized and photographed under UV transillumination. Pooled PCR products were purified by polyethylene glycol precipitation. Sequence analysis was carried out using ABI prism Dye DeoxyTerminator cycle sequencing chemistry (Applied Biosystems, Foster City, CA). After resolution of data on an ABI PRISM 377 sequencer, the type of HPV was determined on the basis of >90% homology with sequences deposited in Genbank using BLAST software.

Immunohistochemistry. Analysis of the expression of cell cycle markers was carried out by performing immunohistochemistry on 5-μm sections of paraffin-embedded tumor on silane (Sigma, St. Louis, Missouri)-coated slides. Single representative tumor blocks were selected for examination from each patient. If multiple blocks were available, selection was on the basis of substantial amounts of viable tumor as well as some histologically normal epithelium for comparison; otherwise, a block of normal tonsillar epithelium from the corresponding patient was used. In the very few cases in which only tumor tissue was available, normal tissues from multiple patients having tonsillectomy for reasons unrelated to malignancy were used as reference.

The following monoclonal antibodies were used: p53, p21, p27, pRb, and cyclin D1 (DO7/ M7001, Sx×118, Sx×53G8, Rb1, and DCS-6: all from DAKO, Carpinteria, California) and two p16 antibodies (F-12 Santa Cruz Biotechnology, Santa Cruz, CA, and Ab-4, Clone 16p04 Neomarkers, Fremont, CA). Antigen retrieval was performed by heating the slides at 90°C for 12 minutes in 1×10 buffer: 13.4 mM EDTA, 20.6 mM Tris, 10.9 mM trisodium citrate, pH 8.0 (pRb, p16 Santa Cruz, p53, p21, and p27), 10 mM citric acid pH 6.0 (p16 Neomarkers), or Target Retrieval Solution (high pH DAKO Corporation, Carpinteria, CA) (cyclin D1). After extensive trials to optimize the protocols, all subsequent steps were carried out at room temperature. Endogenous peroxidase was blocked using 3% hydrogen peroxide in PBS for 5 minutes. The sections were incubated with unlabeled primary antibodies for 30 minutes (p21, p27, pRb, cyclin D1, p53, p16 [Santa Cruz] or 60 minutes (p16 [Neomarkers]) at the following dilutions: 1:50 (p21, pRb, and p16 Neomarkers), 1:100 (p27), 1:200 (p53, cyclin D1), 1:400 (p16 Santa Cruz). Processing was then carried out using the DAKO LSAB and chromogen kits. Biotinylated anti-mouse, anti-rabbit, and anti-goat immunoglobulins were applied for 15 minutes. After
washed in PBS, streptavidin-peroxidase was added for 15 minutes, then 3,3′-diaminobenzidine and hydrogen peroxide for 5 minutes. The sections were counterstained with Mayer’s hematoxylin. Negative controls included omitting the primary antibody and substitution of the primary antibody with normal serum. A positive control comprising a section of tonsil cancer previously shown to be positive for the particular marker was incorporated in each run.

Immunostaining was evaluated semiquantitatively by at least three of the four independent observers (WL, RS, BR, CT) without knowledge of the clinical data or HPV status. At least 1000 tumor cells were evaluated for each section. For all cell cycle markers except p16 (Neomarkers), the evaluation method was similar to that described by Erber et al.,21 taking account of the intensity of staining (graded + to ++++) and the proportion of stained cells (graded + to ++++) using normal epithelium as reference. Tumor scored as “down-regulated” showed >20% reduction in numbers of cells staining and/or a reduced intensity of staining compared with normal epithelium; those scored as “unchanged” had up to a 20% difference from normal; whereas those designated as “up-regulated” showed >20% increase in numbers and/or intensity of staining. The p53 and p16 (Neomarkers) antibodies rarely stained normal epithelium; tumor cells were graded according to the proportion of cells stained as used by Sano et al.22 Tumors with >10% staining were regarded as up-regulated for p53 and those with <10% staining as down-regulated. For p16, tumors with >5% staining were taken as up-regulated and <5% as down-regulated. Results were validated by repeat staining of sequential sections cut from the same block on at least one occasion. Interobserver variations occurred infrequently and were reconciled over a double-headed microscope.

**Histologic grading** was performed by two observers in a blinded manner according to the World Health Organization criteria for SCC of aerodigestive tract mucosa, which divides the tumor into well-differentiated, moderately well-differentiated, and poorly differentiated.23

**Statistical Analysis.** Tests of association between the HPV status of the tumor and expression of the individual cell cycle proteins (p53, p21, pRb, p16, p27, cyclin D1) and gender, clinical stage, and tumor grade were obtained using the Mantel-Haenszel method. P values of <.05 were considered significant. Odds ratios and 95% confidence intervals were used to determine the strength of association between HPV and tumor markers. The comparison between age and HPV-positive/HPV-negative tumor was carried out using Student’s t test.

**RESULTS**

**Detection and Type Delineation of HPV.** Twenty-one (42%) of the 50 tumors were positive for HPV DNA. Twenty tumors were positive for both HPV 16 E6-specific PCRs, and all 20 were confirmed as containing type 16 by sequence analysis. One tumor yielded a band of predicted size using the GP5+/6+ primers; sequencing of this PCR product showed less than 90% homology with known HPVs, suggesting the presence of multiple types.

**Immunohistochemistry.** The percentages of tumor showing up-regulated expression for p16 (Santa Cruz and Neomarkers), p21, p27, pRb, cyclin D1, and p53 were 54%, 50%, 48%, 14%, 28%, 28%, and 68%, respectively, whereas down-regulated expression was noted in 20%, 50%, 18%, 42%, 30%, 24%, and 32%, respectively. The percentages of tumor showing up-regulated or down-regulated expression in relation to HPV positivity are presented in Figure 2. Evaluation of the immunostaining using the proportion of tu-

**FIGURE 3.** Illustrations of immunohistochemical staining for (1) p53, (2 a,b) pRb, (3) p16, (4) p21, (5) p27, and (6 a,b) cyclin D1. Staining for all markers was nuclear. (1) Positive p53: strong nuclear staining is seen throughout this poorly differentiated SCC; original magnification ×600. (2) Down-regulated pRb: staining is confined to a few malignant cells in this moderately differentiated SCC (2a). In contrast, staining is evident throughout the entire normal epithelium, with most intense signal in the lower stratified layers (2b); original magnification ×200. (3) Up-regulated p16 (Santa Cruz): strong staining is evident in much of this moderately differentiated SCC; in contrast, the staining of the adjacent normal epithelium is weak and confined to the lower layers; original magnification ×100. (4) Up-regulated p21: strong staining can be seen in most of the tumor cells of this well-differentiated tonsillar SCC; in contrast, staining of the adjacent normal epithelium is weak and confined to the lower layers; original magnification ×100. (5) Down-regulated p27: only a few tumor cells are stained in this moderately differentiated SCC; original magnification ×400. (6a) Down-regulated cyclin D1: weak staining is seen in a few malignant cells of this moderately well-differentiated tonsillar SCC; original magnification ×200. (6b) In contrast, strong staining is seen in the basal and suprabasal cells of the normal epithelium; original magnification ×200.
mor cells showing positive staining as the sole criterion of up-regulation or down-regulation according to other published criteria for head and neck cancers22,24 yielded almost identical results (data not shown).

Representative staining of the markers in normal epithelium and tumor tissues is shown in Figure 3. Only nuclear staining was considered positive in all instances, except for p16 (Neomarkers), which stained both nucleus and cytoplasm.

p53. Staining was often of high intensity and largely confined to tumor cells. In a small number of cases, weak staining was noted in normal cells of tumor-containing sections. In almost all of these cases, the tumor cells were p53 negative. In rare instances in which normal and tumor cells both showed staining, the p53 result for the tumor was recorded as negative.

pRb. In normal tonsil, staining was moderately strong and evident throughout the entire epithelium. Most intense staining was evident in the lower stratified layers. Staining in the tumor cells was variable in intensity and distribution.

p16 (Santa Cruz). In normal tonsil epithelium there was moderate to strong staining in the lower to mid-layers and weak staining of the upper differentiating layers. Staining of tumor cells was variable in intensity and distribution. p16 (Neomarkers)-stained tumor cells strongly but rarely stained normal cells.

cyclinD1. Staining in normal tonsillar epithelium was strongest in the basal and parabasal layers. Staining of malignant cells was diffuse and variable in distribution and intensity.

p21. Expression of p21 in normal tonsillar epithelium was uniformly low and confined to basal cells and the first cell layers undergoing differentiation. The level and distribution of expression in tumor was variable.

p27. In normal tonsillar epithelium, staining was mainly confined to the basal two thirds of the epithelium. In tumor cells, intensity and distribution of staining were variable. Lymphocytes stained strongly and served as an internal positive control.

Histologic Grade. Seventeen of the 50 (34%) tumors were classified as poorly differentiated, 29 (58%) as moderately well differentiated, and 4 (8%) as well differentiated.

Associations Between Presence/Absence HPV and Expression of Cell Cycle Markers. Among the 50 tumors, there were strong associations between HPV positivity by PCR and reduced pRb and cyclin D1 expression (OR = 7.9, CI = 5.9,23; OR = 7.4, CI = 5.7,25. Tumors that were HPV negative were more likely to have up-regulated pRb and cyclin D1 and down-regulated p16 (OR undefined in all cases). All 21 tumors that were HPV positive had up-regulated p16 expression (when the Neomarkers antibody was used), but only 4 of the 29 (14%) tumors that were HPV negative overexpressed p16 (OR undefined). There was also an association between HPV positivity and p53 overexpression (OR = 4.3, CI = 0.2, 8.5). However, this relationship is probably not statistically significant, because the confidence intervals overlap 1. There was no association between the presence/absence of HPV and p16 (Santa Cruz), p21, and p27 expression. Across all tumors, those with down-regulated p27 tended to have down-regulated pRb (OR = 11.6, CI = 2.6,50) and down-regulated p21 (OR = 17.2, CI = 1.9,152). Tumors with overexpressed cyclin D1 tended to overexpress pRb (OR = 11.2, CI = 2.6,47), but this association was no longer evident once HPV status was taken into account.

Association Between HPV Status, Expression of Cell Cycle Markers, and Clinicopathologic Variables. There was a highly significant association between HPV-positive tumors and younger patient age (p < .001). The mean age of those with HPV-positive tumors was 50 years compared with 61 years for those with HPV-negative tumors. There was no relationship between HPV status or cell cycle protein expression and patient gender, clinical stage, or tumor grade.

DISCUSSION

This study is one of the first to focus on the expression of a range of key cell cycle markers in cancers occurring at a single site within the head and neck where oncogenic HPVs are frequently detectable. Although the associations between HPV types 6 and 11 and benign juvenile laryngeal papillomatosis have long been known,25 the putative link between HPV 16 and tonsil cancer has only been made comparatively recently.8,9,26 Papillomaviruses are renowned for their strict tropism, and the spectrum of HPVs infecting muco-
sal surfaces of the anogenital tract and head and neck varies markedly from that found in common skin warts. However, the mechanisms underlying the clear predilection of papillomaviruses with different oncogenic capabilities for specific sites within the head and neck are unknown. The HPV positivity rate of 42% and the predominance of HPV type 16 in this study were consistent with data reported in previous studies. Interestingly, none of our cancers contained EV-related HPVs, in contrast with a positivity rate of 14% in a recent report using a similar detection system. 

The overall rate of detection of p53 of more than 50% was consistent with other studies of head and neck cancers. Further studies will be needed to establish whether aberrant p53 expression was due to mutations in p53 or in other genes in the p53 pathway. Alternately, the overexpression of p53 could have resulted from increased activation and stability of the protein after stress arising from DNA damage, oncogene activation, or hypoxia. The possibility that the regulation of p53 expression in tonsil cancers might differ from that at other sites within the head and neck warrants investigation, because tonsil cancers have represented a relatively small proportion of the total sample in most previous studies. Previous reports concerning the relationship between p53 status and HPV positivity in head and neck cancer have generated conflicting results. Although some workers have described overexpression of p53 and/or p53 mutations almost exclusively in HPV-negative tumors, others have found that HPV and aberrant p53 can coexist. Sample difference might well account for the conflicting results reported in studies in which HPV status and p53 expression have been compared.

Analyses of upper aerodigestive tract cancers have indicated that disruption to the pRb pathway might preferentially occur along two distinct pathways. Overexpression of cyclin D1 and loss of the p16 protein in the context of preserved pRb have been frequently reported. Conversely, reduced expression of pRb and cyclin D1 in conjunction with high levels of p16 has been reported in cell lines from various sources and in small series of HPV-positive tumors, including tonsil cancers. This has indicated the existence of a feedback loop between pRb, cyclin D1, and p16. Because the HPV E7 protein binds to the same site as cyclin D1 on pRb, it has been suggested that E7 might overcome the need for cyclin D1 in the G1 phase of the cycle. The strong association between HPV positivity and down-regulation of cyclin D1 and pRb in our study supports this model of papillomavirus-induced carcinogenesis. Interestingly, however, the association between HPV positivity and p16 expression varied markedly according to the particular antibody used. Although there was a strong association between HPV positivity and up-regulation of p16 with the Neomarkers antibody, this relationship was not observed with the Santa Cruz antibody. In fact, in the latter case, up-regulation of p16 was slightly more common in HPV-negative than HPV-positive cancers (16 versus 11), and three HPV-positive tumors actually showed reduced p16 expression. This discrepancy was reproducible and is likely to reflect the specificity of the particular antibodies.

It is noteworthy that in a recent study of HPV-positive cervical cancers, p16 was almost invariably strongly expressed in tumor with preserved
pRb. Further investigations will be needed to determine whether the differences between head and neck and cervical tumor reflect biologic differences, sample size, or specificities of the antibodies used.

There have been few published studies of p21 or p27 expression in the tonsil. Our findings suggest that p21 expression patterns in normal tonsil epithelium are similar to those at other locations in the head and neck, being confined to cells arresting proliferation and initiating differentiation. Overall, up-regulation of p21 was noted in approximately half of the cancers, a finding consistent with that of another recent series. p21 is known to be transactivated by p53, and loss of p53 function is believed to influence the pRb cell cycle regulatory cascade indirectly by altering p21 function. However, there was no relationship between p53 and p21 expression in this study, indicating that overexpression occurred by way of p53 independent pathways. Further investigations will be needed to determine the mechanisms that allow cell proliferation in the presence of accumulated p21. The lack of an association between the presence of HPV and p21 expression was noteworthy, because both of the HPV oncogenes E6 and E7 have been shown to bind p21, and loss of p21 protein has been reported in HPV E6 expressing normal human fibroblasts.

Our observation that p27 was expressed at higher levels in quiescent and differentiating cells compared with proliferating normal cells is consistent with a recent study of oropharyngeal tissues. Although p27 mutations are reportedly rare in human cancer, deregulated expression is common, particularly reduced expression. Although one third of our tumors showed down-regulation of p27, there was no association with HPV status. This contrasts with a study of cervical cancers in which low levels of p27 were attributed to interactions between p27 and the viral oncogene E7. The association between down-regulation of p21, pRb, and p27 provides further evidence of interactions between the p53 and pRb pathways and to our knowledge has not been previously reported.

The strong association between young patient age and HPV status was of particular interest. Mucosal SCC of the head and neck occurring in the younger age groups has long been thought to constitute an etiologically distinct group, and HPV has come under scrutiny as a possible risk factor. Nonetheless, the relationship between HPV and age is controversial, because some previous studies have linked HPV positivity with older age, and others have found no association at all. Further investigations will be needed to determine whether the links between HPV positivity and younger age reflect sexual practices (i.e., orogenital sexual contact or genetic predisposition).

Overall, our findings suggest that HPV-positive tonsil cancers might be biologically and etiologically distinct from HPV-negative tumors. Proposed pathways of HPV-induced carcinogenesis have been presented in Figure 4. However, the practical implications of this categorization for head and neck oncologists are as yet uncertain. Prospective studies are needed to ascertain the natural histories of the two groups.

REFERENCES