Published in final edited form as:

Cancer. 2009 April 25; 117(2): 108–119. doi:10.1002/cncy.20001.

p16^{INK4A} Immunohistochemical Staining May Be Helpful in Distinguishing Branchial Cleft Cysts From Cystic Squamous Cell Carcinomas Originating in the Oropharynx

Reetesh K. Pai, MD¹, Julianna Erickson², Nader Pourmand, PhD^{2,3}, and Christina S. Kong, MD¹

¹Department of Pathology, Stanford University, Stanford, California

²Stanford Genome Center, Stanford University, Stanford, California

³Department of Biomolecular Engineering, University of California Santa Cruz, Santa Cruz, California

Abstract

BACKGROUND—We investigated p16^{INK4A} expression in branchial cleft cysts and its utility in distinguishing branchial cleft cysts from metastatic head and neck squamous cell carcinomas (SCCs) in fine-needle aspiration biopsies (FNABs).

METHODS—A study set comprising 41 resections (15 SCC and 26 branchial cleft cysts) and a test set of 15 FNABs (11 SCC and 4 branchial cleft cysts) were analyzed with p16^{INK4A} immunohistochemistry and human papillomavirus (HPV) polymerase chain reaction (PCR)/pyrosequencing. Cases with discrepant p16^{INK4A} and PCR/pyrosequencing results were further evaluated with HPV in situ hybridization (ISH). SCCs were divided into keratinizing SCC and nonkeratinizing SCC groups and site of origin.

RESULTS—Metastatic oropharyngeal nonkeratinizing SCC in the study set exhibited diffuse, strong p16^{INK4A} (7 of 7) and HPV16 DNA positivity (6 of 6), while keratinizing SCC from the larynx and oral cavity was negative for p16^{INK4A}. p16^{INK4A} reactivity in the branchial cleft cyst study set was characterized by focal, strong staining (6 of 21) involving the superficial squamous epithelium. HPV DNA was identified in 7 of 19 branchial cleft cyst study set cases by PCR/pyrosequencing, but these cases were negative by HPV ISH. In the test set, oropharyngeal nonkeratinizing SCC exhibited diffuse, strong p16^{INK4A} (3 of 3) and HPV16 DNA (2 of 2), while metastatic keratinizing SCC was negative for p16^{INK4A} and HPV DNA. All 4 FNABs of branchial cleft cysts were negative for p16^{INK4A}. Diffuse, strong p16^{INK4A} correlated with oropharyngeal origin ($P = .001$) and nonkeratinizing morphology ($P = .0001$).

CONCLUSIONS—Branchial cleft cysts can exhibit focal strong reactivity limited to the superficial squamous epithelium and glandular epithelium. Although p16^{INK4A} immunohistochemistry may be helpful in distinguishing oropharyngeal nonkeratinizing SCC from branchial cleft cysts in FNAB specimens, it is not helpful in cases of keratinizing SCC because these cases are typically negative for p16^{INK4A}.

© 2009 American Cancer Society

Corresponding author: Reetesh K. Pai, MD, Stanford University, Department of Pathology, 300 Pasteur Drive, Room H-2110, Stanford, CA 94305; Fax (650) 725-7409; reeteshp@stanford.edu.

Conflict of Interest Disclosures

The authors made no disclosures.

Keywords

p16^{INK4A}; head and neck squamous cell carcinoma; human papillomavirus; oropharynx; fine-needle aspiration

A subset of metastatic squamous cell carcinoma (SCC) of the head and neck presents as cystic masses in the neck. Often, distinguishing between these cystic metastases of SCC from benign cystic neck lesions, such as branchial cleft cyst, can be very challenging. Squamous lining cells in benign cystic lesions may, at times, exhibit significant cytologic atypia, raising concern for SCC. Alternatively, metastases from SCC may occasionally be cytologically bland and lack overt features of malignancy. The challenge in distinguishing benign from malignant cystic squamous lesions of the neck is made more difficult when evaluating fine-needle aspiration biopsies (FNABs) given the limited sample size.¹

Recent studies have suggested an etiologic relationship between high-risk oncogenic human papillomavirus (HPV) type 16 and some head and neck SCC.²⁻⁴ A subset of primary oropharyngeal SCC, including carcinomas of the palatine tonsils and base of tongue, demonstrates a high prevalence of HPV type 16 DNA.^{2,5-7} This subset of HPV-related SCC typically occurs in younger patients, rather than non-HPV-related SCC of the head and neck, and it usually has a nonkeratinizing “basaloid” morphology.⁸⁻¹¹ In addition, HPV-related oropharyngeal SCC has been reported to have a more favorable prognosis compared with HPV-negative tumors.^{3,4} As with uterine cervical SCC, HPV-mediated transformation to oropharyngeal SCC is thought to result from integration of the viral genome into the host genome, leading to increased expression of the E6 and E7 oncogenes.¹² The E7 gene product of HPV inactivates the retinoblastoma (RB) protein, resulting in increased production of the *CDNK2A* gene product, p16^{INK4A}, a tumor-suppressor gene involved in cell cycle control.¹³ Thus, increased protein expression of p16^{INK4A} can act as a surrogate marker for high-risk HPV infection in such squamous cell lesions.

A readily available marker that can reliably distinguish between benign and malignant cystic squamous lesions would have tremendous diagnostic utility. In Begum et al’s study of 77 cases with metastatic head and neck SCC diagnosed by FNAB, p16^{INK4A} overexpression was identified in 17 of them (13 oropharyngeal, 1 oral cavity, 3 unknown primary).¹⁴ Of note, 2 of the 77 cases represented false-positive diagnoses where branchial cleft cysts were found on resection and were noted to have weak p16^{INK4A} staining, which was interpreted as negative. In situ hybridization (ISH) for HPV16 was also performed, and fewer cases of SCC were positive (13 of 75). The advantage of HPV ISH is direct observation of the infected cell and high specificity. However, p16^{INK4A} is more sensitive, albeit less specific, and more laboratories have the ability to perform immunohistochemistry than ISH.¹⁵

Although several studies have looked at p16^{INK4A} expression in head and neck SCC, there is little published on the pattern of p16^{INK4A} expression in branchial cleft cysts. In this study, we further evaluate the diagnostic utility of p16^{INK4A} in distinguishing benign from malignant cystic head and neck squamous lesions by examining p16^{INK4A} expression in branchial cleft cysts and metastatic SCC both in surgical excision specimens and in FNAB cell block samples.

MATERIALS AND METHODS

Case Selection

The Pathology Laboratory archives of Stanford University Hospital and Clinics were searched from January 1, 2004, to May 1, 2007, for patients with cystic SCC involving the neck. All cases of SCC were either macroscopically described as cystic on surgical excision or yielded cyst fluid on FNAB. First, a study set comprising 41 surgical resections, including cystic metastatic SCC (15 cases) and branchial cleft cysts (26 cases), was constructed. All branchial cleft cysts cases were required to exhibit, at least, focal lining by ciliated respiratory epithelium to ensure accurate diagnosis. Cases of SCC that were not cystic were excluded from this study, as these lesions in FNAB specimens are not diagnostically difficult and are not often confused with branchial cleft cysts. The SCC cases were divided into keratinizing and nonkeratinizing groups and further subdivided on the basis of the primary site of origin into the following categories, oropharynx (7 nonkeratinizing SCC, 2 keratinizing SCC), oral cavity (1 keratinizing SCC), larynx (3 keratinizing SCC), and unknown (2 keratinizing SCC).

A test set containing 15 FNABs, including cystic metastatic SCC (11 cases) and branchial cleft cysts (4 cases) with adequate cell block material, was included in this study. All FNAB diagnoses were confirmed by subsequent surgical excision. The SCC cases in the test set were divided into keratinizing SCC and nonkeratinizing SCC and further subdivided based on primary site of origin into the following categories, oropharynx (3 nonkeratinizing SCC, 3 keratinizing SCC), oral cavity (3 keratinizing SCC), larynx (1 keratinizing SCC), and unknown (1 keratinizing SCC). Age and sex distribution of the cystic squamous lesions are listed in Table 1. Institutional review board approval was obtained for this study.

p16^{INK4A} Immunohistochemistry

Immunohistochemical staining for p16^{INK4A} was performed with the Dako Autostainer on 4 µm-thick sections of paraffin-embedded tissue from study and test set cases by using a primary mouse antihuman monoclonal antibody (clone E6H4, Dako, Glostrup, Denmark). Antigen retrieval was performed with a pressure cooker (30 minutes at 115°C). The slides were incubated with peroxidase-blocking reagent, followed by the primary antibody (dilution 1:50), and finally the visualization reagent (secondary goat-antimouse immunoglobulin and horseradish peroxidase linked to a dextran polymer backbone). After rinsing with distilled water, the slides were incubated with 3,3'-diaminobenzidine substrate-chromagen solution, and Mayer hematoxylin counterstain was applied before coverslipping. A paraffin-embedded section of invasive uterine cervical SCC was included as a positive control for each run. In addition, negative control serum was applied to a section of invasive uterine cervical SCC as a negative control.

Staining was scored as strong, weak, or negative on the basis of nuclear or cytoplasmic staining. Weak cytoplasmic staining or strong reactivity in <5% of cells was interpreted as negative. Diffuse (>80%) and focal (5% to 80%) strong reactivity was scored as positive.

HPV ISH

A subset of 16 study set cases (13 branchial cleft cysts, 3 SCC) with discordant results between HPV detection by pyrosequencing and p16^{INK4A} immunohistochemistry were sent to the Johns Hopkins Hospital Immunopathology Laboratory for HPV16 or HPV18 ISH, which used the Dako GenPoint system. Punctate nuclear staining was scored as positive.

HPV Polymerase Chain Reaction and Pyrosequencing

Formalin-fixed, paraffin-embedded material was available for 41 cases for HPV polymerase chain reaction (PCR) analysis and pyrosequencing according to a previously described method.¹⁶ Briefly, PCR designed to target the L1 region of HPV was performed by using the forward primer GP5+(5'-TTTGTACTGTTGTTGATACTAC-3') and the biotinylated reverse primer GP6+(5'-GAAAAATAAAGTGTAAATCATATTC-3'). Amplification using a touchdown thermoprofile comprised initial denaturation at 95°C for 3 minutes, 7 cycles of 95°C for 45 minutes, and 56°C for 45 minutes with a 1°C decrease in annealing temperature per cycle, 33 cycles of 95°C for 45 minutes, and 48°C for 45 minutes, and a final extension step of 72°C for 5 minutes.

Pyrosequencing was performed by first immobilizing the biotinylated PCR product (5 ng/μL) onto 2.5 μL streptavidin-coated high performance sepharose beads (Amersham Biosciences, Piscataway, NJ) by incubation at 43°C with agitation at 1050 rpm. A Vacuum Prep Tool and Vacuum Prep Worktable (Biotage, Uppsala, Sweden) were then used to subject the samples attached to the beads to the following treatments, excess primers in the solution were removed by washing with 70% EtOH, followed by DNA denaturation with 0.1 M NaOH and a final washing step with TE-Buffer (0.1M Tris-Acetate, pH 7.6) to remove nonimmobilized complementary strands. The beads were then suspended in 12 μL annealing buffer (10mM Tris-acetate pH 7.75, 5 mM Mg-acetate) containing 0.4 pmol sequencing primer. Sequencing primers were then annealed to single-stranded DNA by incubation at 95°C for 2 minutes, 50°C for 5 minutes, and 25°C for 5 minutes.

For each sample, 4 reaction pools were prepared, 1 for each of 4 sequencing primers or primer pools; as described by Gharizadeh et al.¹⁷ GP5+ is a general primer designed to provide sequence signals for any HPV type. Three primer pools containing 4 type-specific primers each were also used. Multiple sequencing primer pool 1 contains primers specific to HPV16, 31, 59, and 39. Multiple sequencing primer pool 2 contains primers for HPV18, 33, 52, and 56. Multiple sequencing primer pool 3 contains primers for HPV35, 45, 51, and 58. The primers included in the 3 pools are designed to target the most common high-risk HPV types.

After primer hybridization, samples were sequenced by using the PSQ HS96A system (Biotage, Uppsala, Sweden). HPV type was determined by comparing sequence signals for each of the primers or pools to known HPV type-specific sequence data. The qualitative signal strength compared with positive control was also noted for each case. Samples that gave no signals when sequenced with the general primer were classified as HPV-negative.

Statistical Analysis

The Fisher exact test (2-tail) was used to evaluate the correlation of diffuse, strong p16^{INK4A} immunohistochemical labeling with SCC site of origin (oropharyngeal vs nonoropharyngeal), cytomorphology (nonkeratinizing SCC vs keratinizing SCC), and the presence of HPV16 or HPV18 DNA by PCR. The presence of HPV DNA by pyrosequencing was also correlated with pathologic diagnosis (SCC vs branchial cleft cysts). Data from both the study set and the test set were combined for statistical analysis. The significance level was set at $P < .05$.

RESULTS

Pathologic Features

The histologic and cytomorphologic features of all carcinomas were classified into keratinizing SCC and nonkeratinizing SCC groups (Table 1). In the study set of surgical

excisions, keratinizing SCCs were defined either by the presence of keratin pearl formation or by easily identified intercellular bridges between the neoplastic cells (Fig. 1). Keratinizing SCC more often originated from the larynx and oral cavity (Table 1). In contrast, nonkeratinizing SCC almost exclusively originated from the oropharynx (Table 1). Histologically, the nonkeratinizing SCC comprised monomorphic ovoid cells with indistinct cell borders forming nests, often with central comedo-like necrosis (Fig. 2). The neoplastic cells exhibited enlarged, oval, hyperchromatic nuclei without nucleoli and indistinct basophilic cytoplasm, imparting a “basaloid” appearance. Mitotic figures were typically numerous. Areas of squamous maturation characterized by cytoplasmic eosinophilia and intercellular bridge formation could be identified in all cases (Fig. 2); however, these areas of squamous differentiation were very focal, and no obvious keratin pearl formation was identified in any case. Surgical excisional biopsies of branchial cleft cysts were characterized by cysts lined both by cytologically bland squamous epithelium and by respiratory-type glandular epithelium associated with lymphoid tissue (Fig. 3). Keratinous debris comprising anucleate squamous cells often filled the cystic cavity.

In the test set of FNAB samples, keratinizing SCC was characterized by malignant cells having abundant dense, glassy orangeophilic cytoplasm on Papanicolaou-stained smears (Fig. 1) and blue-gray dense cytoplasm on Giemsa stained smears. The background typically had numerous degenerating cells. Intact neoplastic cells were more often dispersed singly and were differentiated from benign cystic squamous lesions by the presence of nuclear enlargement, nuclear hyperchromasia, and irregular nuclear outlines. However, these features of malignancy were often subtle and focal.

FNAB samples of nonkeratinizing SCC exhibited distinctive 3-dimensional hyperchromatic, crowded clusters of neoplastic cells with significant nuclear overlap and indistinct cell borders imparting a syncytia-like morphology (Fig. 2). The nuclei of nonkeratinizing SCC were also characteristically monomorphic and hyperchromatic with absent nucleoli and only focal irregular nuclear outlines. The background in these cases contained proteinaceous material associated with numerous degenerating neoplastic cells. Unlike keratinizing SCC, single intact neoplastic cells were difficult to find given the extensive cellular degeneration. In fact, the smear preparations mostly comprised degenerating cellular debris.

FNAB of branchial cleft cysts showed cytologically bland squamous epithelial cells with abundant cytoplasm dispersed singly in a background anucleate squames. The smear preparations also showed an abundant background of degenerating cellular debris (Fig. 3). Occasional clusters of squamous cells were noted in most cases, often raising concern for the possibility of well-differentiated keratinizing SCC. The cell block material from branchial cleft cysts predominantly comprised keratinous debris with only rare nucleated squamous cells.

p16^{INK4A} Immunohistochemical Staining in Surgical Excisions (Study Set)

Of the 15 SCC cases in the study set, 9 exhibited diffuse, strong nuclear and cytoplasmic reactivity with p16^{INK4A} (Table 2). Each positive case exhibited >95% strong full-thickness staining of the squamous lining cells (Fig. 2). Cases exhibiting p16^{INK4A} overexpression originated from either an oropharyngeal primary (8 cases: 7 nonkeratinizing SCC and 1 keratinizing SCC) or an unknown primary (1 keratinizing SCC case; Table 2). In addition, scattered p16^{INK4A}-positive single neoplastic cells were identified within the keratinous cyst contents of 6 cases with diffuse, strong p16^{INK4A} (5 nonkeratinizing SCC and 1 keratinizing SCC). Many of the cells within the center of the cyst did not label with p16^{INK4A}. Metastatic cystic SCC from the larynx (3 cases) and oral cavity (1 case) did not label with p16^{INK4A} (Fig. 1, Table 2).

Focal nuclear and cytoplasmic reactivity was identified in 6 of 21 cases of excised branchial cleft cysts in the study set (Table 2). In these 6 cases, the focal, strong staining predominantly involved the superficial cyst-lining squamous cells (Fig. 3). No cases exhibited diffuse staining of the basal layer or full-thickness staining of the squamous lining cells. In 21 of 21 cases, the keratinous cyst contents of branchial cleft cysts did not label with p16^{INK4A}. Interestingly, 2 of 21 cases had strong diffuse p16^{INK4A} staining within the glandular epithelium. In both cases, the adjacent squamous epithelium did not label with p16^{INK4A}.

p16^{INK4A} Immunohistochemical Staining in FNAB Samples (Test Set)

Four of 11 SCC cases in the test set exhibited diffuse, strong staining with p16^{INK4A} (Table 3). The p16^{INK4A}-positive cases arose from primary oropharyngeal nonkeratinizing SCC (3 cases) and a nonkeratinizing SCC of unknown primary site of origin (1 case). In all positive FNAB cases, diffuse, strong p16^{INK4A} was identified in neoplastic cell clusters and also in scattered nucleated malignant cells dispersed singly (Fig. 2). All cases of keratinizing SCC from the oropharynx (3 cases), oral cavity (3 cases), and larynx (1 case) did not label with p16^{INK4A} (Fig. 1). All 4 branchial cleft cyst FNABs were negative for p16^{INK4A}, although the stained cell block material predominantly comprised anucleate keratinous debris with only rare nucleated squamous cells (Fig. 3).

p16^{INK4A} Immunolabeling Correlates With Identification of HPV DNA in SCC

In the study set of surgical excisions, 9 of 13 SCCs were positive for HPV DNA by HPV PCR/pyrosequencing, with each case showing a strong signal on PCR analysis (Table 2). Of the 9 HPV-positive cases, 7 exhibited diffuse, strong labeling with p16^{INK4A} (6 nonkeratinizing SCC oropharyngeal, 1 keratinizing SCC unknown primary). Two keratinizing SCC cases (1 oral cavity and 1 unknown primary) were positive for HPV DNA by PCR/pyrosequencing but lacked p16^{INK4A} immunohistochemical labeling (Table 3). HPV16 and HPV18 ISH performed in both of these keratinizing SCC cases were negative (Table 4). One nonkeratinizing SCC case in the study set exhibited diffuse, strong p16^{INK4A} staining but HPV PCR was not performed. HPV16 ISH in this case was positive (Table 4).

Seven of 19 branchial cleft cyst cases in the study set showed a strong signal on HPV PCR/pyrosequencing analysis (4 HPV18, 3 HPV16, 18). Four branchial cleft cyst cases had HPV18 infection alone and 3 branchial cleft cyst cases exhibited both HPV18 and HPV16 infection. All 7 branchial cleft cyst cases with positive HPV DNA by PCR/pyrosequencing were negative for HPV16 or HPV18 by ISH (Table 4). Of the 6 branchial cleft cyst cases with focal, strong p16^{INK4A} staining, only 2 were HPV positive by PCR/pyrosequencing. All 6 branchial cleft cyst cases exhibiting p16^{INK4A} staining were negative for HPV16 or HPV18 by ISH (Table 4). In 2 branchial cleft cyst cases, the glandular epithelial component exhibited p16^{INK4A} staining; however, each of these cases were negative for HPV16 and HPV18 by ISH (Table 4).

In the test set of FNAB samples, 3 of 8 SCCs were positive for HPV DNA with each case showing a strong signal on PCR analysis by PCR (Table 3). The 3 HPV-positive cases included 2 oropharyngeal nonkeratinizing SCC and 1 nonkeratinizing SCC of unknown origin. All 3 HPV-positive cases exhibited diffuse, strong labeling with p16^{INK4A}. Five keratinizing SCC cases, including cases from the oropharynx (1), larynx (1), and oral cavity (3), were negative for HPV DNA. Only 1 FNAB branchial cleft cyst case from the test set had sufficient DNA for HPV analysis and was negative.

Statistical Summary

Diffuse, strong p16^{INK4A} immunolabeling significantly correlated with oropharyngeal primary site of origin ($P = .001$), nonkeratinizing morphology ($P = .0001$), and the presence of HPV16 DNA by PCR/pyrosequencing ($P = .0001$). Detection of HPV DNA by PCR/pyrosequencing did not correlate with a diagnosis of SCC ($P = .36$).

DISCUSSION

Although the diagnosis of metastatic SCC involving neck lymph nodes usually poses little diagnostic difficulty, the subgroup of cystic metastases is often challenging for pathologists, especially in those patients without a prior diagnosis of malignancy, where distinguishing cystic SCC from branchial cleft cysts is critical. Recent studies have reported p16^{INK4A} overexpression in a subset of head and neck squamous cell carcinomas that are HPV-associated and arise primarily from the oropharynx.^{8,10,14,15} In addition, this subset of SCC appears to have a predilection for causing cystic lymph node metastases.^{18,19} On the basis of these findings, it would appear that p16^{INK4A} overexpression can be exploited as an aid in distinguishing cystic SCCs from branchial cleft cysts. However, no studies have specifically evaluated p16^{INK4A} expression in branchial cleft cysts. In this study, we compared p16^{INK4A} expression in cystic SCCs and branchial cleft cysts, evaluating both excision specimens and FNAB samples.

Head and neck SCC is a heterogeneous group of malignancies. The recent identification of HPV type 16-related SCC has yielded new insight into the tumorigenesis of SCC and has allowed the characterization of a distinct subset of head and neck SCC. Previous investigations have shown that oropharyngeal primary tumors, including the palatine tonsil and base of tongue, are the most common site of HPV-related SCC.^{8–10,20} Perturbation of the retinoblastoma pathway via inactivation of the *p16INK4A* gene is one of the most common abnormalities in head and neck SCC, with most carcinomas exhibiting loss of p16^{INK4A} expression.²¹ In contrast, like their uterine cervical counterparts, HPV-related head and neck SCC often exhibits increased expression of p16^{INK4A}. We and others have shown that diffuse, strong staining with p16^{INK4A} correlates with infection with high-risk HPV-subtypes in uterine cervical specimens and that immunohistochemical assay for p16^{INK4A} is more sensitive but less specific than HPV in ISH when compared with HPV PCR as the gold standard.^{15,22} In a similar study examining head and neck SCC cases, p16^{INK4A} showed 100% sensitivity and 79% specificity, while fluorescent ISH had a sensitivity of 83% and specificity of 100%.¹⁵ Standard GP5 + /6 + PCR also resulted in false-positive results in a few cases, corresponding to 89% specificity and 100% sensitivity. HPV16 E6 RT-PCR was used as the gold standard. These results high-light the limitations of using a single assay for detecting HPV. Each assay has its limitations, including the gold standard of E6/E7 expression. The p16^{INK4A} immunohistochemical assay appears to represent the best surrogate marker for high-risk HPV infection in squamous lesion given its wide availability, ease of interpretation, and high sensitivity.^{23,24}

The utility of the immunohistochemical assay for p16^{INK4A} in metastatic SCC to cervical lymph nodes has been previously studied and has been shown to localize the origin of tumor from the oropharynx.²⁴ Our study is complementary to work from other groups and enhances previous data by incorporating both surgical and FNAB of benign and malignant neck masses in our analysis. We found that diffuse, strong p16^{INK4A} expression was primarily restricted to those cystic metastatic SCC of oropharyngeal origin with a nonkeratinizing morphology. In contrast, laryngeal and oral cavity SCCs were negative for p16^{INK4A} and exhibited a keratinizing morphology. In addition, the presence of p16^{INK4A} in SCC correlated with the detection of HPV DNA, providing further support for the use of p16^{INK4A} as a surrogate marker for HPV infection in neoplastic squamous epithelium.

Relatively few efforts have evaluated the potential of using p16^{INK4A} in distinguishing benign from neoplastic squamous lesions of the head and neck.²⁵ In our series, diffuse, strong p16^{INK4A} expression was restricted to SCC and was not identified in any case of branchial cleft cysts. A subset of surgically resected branchial cleft cysts exhibited focal staining with p16^{INK4A} that was primarily localized to the superficial squamous cells or benign glandular epithelium lining the cyst. No diffuse basal layer or full-thickness staining was identified. Often, FNAB of cystic squamous lesions of the neck yields only the keratinous cyst contents with variable numbers of nucleated cyst-lining cells. In our series, the anucleate keratinous cells in FNAB samples failed to label with p16^{INK4A} in both cystic SCC and in branchial cleft cysts. p16^{INK4A} labeling was seen only in those cases in which hyperchromatic crowded groups were present in cell-block material. These cases are usually identifiable as malignant on routine cytologic preparations. Although branchial cleft cysts do not typically enter into the differential diagnosis, the cytologic diagnosis can be challenging in cases where only rare clusters are present or where the possibility of skin adnexal structures cannot be excluded. In contrast, FNAB samples from keratinizing SCC in which the neoplastic cells are dispersed singly and may not form cohesive aggregates did not label with p16^{INK4A}. These keratinizing SCC cases are the cases that are often difficult to distinguish from branchial cleft cysts, and in these instances, the p16^{INK4A} immunohistochemical assay was not helpful in resolving the differential diagnosis between SCC and branchial cleft cysts.

Similar to prior studies, we found that cystic metastases from oropharyngeal primary sites vastly outnumbered metastases from other head and neck sites.¹⁸⁻¹⁹⁻²⁶ In addition, it is this subgroup of nonkeratinizing metastatic SCC that is associated with HPV and labels strongly with the p16^{INK4A} immunohistochemical assay. Subsequently, in this subset, HPV detection and evaluation of p16^{INK4A} expression may be exploited as an aid in the diagnosis of malignancy or in identifying the primary site of origin.

Our results confirm that diffuse, strong p16^{INK4A} staining correlates with the presence of HPV DNA. However, focal strong reactivity is less specific. A subset of branchial cleft cyst cases exhibited focal, strong p16^{INK4A} immunolabeling but lacked HPV DNA. In a study by Nielsen et al, p16^{INK4A} was found to be strongly expressed in a wide variety of normal human tissues and did not correlate with cellular proliferation or maturation.²⁷ Their study further suggested that increased p16^{INK4A} expression in normal tissues may play a role in cellular senescence. In our series, p16^{INK4A} labeling in branchial cleft cysts was focal and predominantly involved the superficial cyst-lining squamous cells. One can hypothesize that these superficial cyst-lining cells gain p16^{INK4A} expression in the process of cellular senescence before being shed into the cyst lumen. However, further studies on the presence and function of p16^{INK4A} in branchial cleft cysts would be necessary to test this hypothesis.

Although HPV16 or HPV18 DNA was detected in a subset of branchial cleft cyst cases by PCR/pyrosequencing, ISH for HPV DNA failed to identify HPV DNA in these cases. Although we cannot entirely exclude the possibility of carry-over DNA contamination resulting in false-positive detection of HPV DNA by PCR/pyrosequencing analysis, another possibility is differing analytic sensitivities between the various assays. The HPV PCR/pyrosequencing technique may have a higher analytical sensitivity compared with HPV ISH. The lack of associated diffuse, strong p16^{INK4A} staining in the branchial cleft cyst cases with HPV DNA identified by the PCR/pyrosequencing assay would suggest that the virus is not integrated into the host genome. Further studies evaluating HPV in branchial cleft cysts using a variety of HPV PCR detection methods are needed to verify if the HPV detection by PCR/pyrosequencing in our study is a true positive or if it represents contamination. In addition, semiquantitative methods for HPV detection, which yield information on viral load in clinical samples, would be helpful in determining the relative analytical sensitivities and

clinical specificities of the different HPV detection methods that are currently in use.²⁸ A test with high analytic sensitivity may lead to detection of HPV DNA, which is clinically irrelevant resulting in decreased clinical specificity.²⁹

In conclusion, our findings demonstrate that focal strong p16^{INK4A} immunostaining of the superficial lining cells may be seen with branchial cleft cysts in surgical excision specimens, but the cyst contents obtained by FNAB are typically negative. Strong p16^{INK4A} staining on FNAB cell block material supports a diagnosis of metastatic SCC and is most consistent with an oropharyngeal primary. However, negative p16^{INK4A} results do not completely exclude the possibility of malignancy, as cystic keratinizing SCC is typically negative for p16^{INK4A}. Given the relatively few number of FNAB cases in this study, a larger cohort of FNAB cases with p16^{INK4A} immunohistochemistry, HPV ISH and PCR, and correlation with subsequent surgical excisions would be helpful in further evaluating the utility of p16^{INK4A} immunohistochemistry in distinguishing SCC from branchial cleft cysts in FNAB samples.

References

- Engzell UZJ. Aspiration biopsy of tumors of the neck. I. Aspiration biopsy and cytologic findings in 100 cases of congenital cysts. *Acta Cytologica*. 1970; 14:51–57. [PubMed: 5264434]
- 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]
- Licitra L, Perrone F, Bossi P, et al. High-risk human papillomavirus affects prognosis in patients with surgically treated oropharyngeal squamous cell carcinoma. *J Clin Oncol*. 2006; 24:5630–5636. [PubMed: 17179101]
- Weinberger PM, Yu Z, Haffty BG, et al. Molecular classification identifies a subset of human papillomavirus-associated oropharyngeal cancers with favorable prognosis. *J Clin Oncol*. 2006; 24:736–747. [PubMed: 16401683]
- Helbig M, Andl T, Kahn T, et al. The role of oncogenic human papillomaviruses in tonsillar squamous cell carcinomas with functional inactivation of the retinoblastoma protein. *HNO*. 1999; 47:796–803. [PubMed: 10525608]
- Paz IB, Cook N, Odom-Maryon T, Xie Y, Wilczynski SP. Human papillomavirus (HPV) in head and neck cancer. An association of HPV 16 with squamous cell carcinoma of Waldeyer's tonsillar ring. *Cancer*. 1997; 79:595–604. [PubMed: 9028373]
- Andl T, Kahn T, Pfuhl A, et al. Etiological involvement of oncogenic human papillomavirus in tonsillar squamous cell carcinomas lacking retinoblastoma cell cycle control. *Cancer Res*. 1998; 58:5–13. [PubMed: 9426048]
- El-Mofty SK, Lu DW. Prevalence of human papillomavirus type 16 DNA in squamous cell carcinoma of the palatine tonsil, and not the oral cavity, in young patients: a distinct clinicopathologic and molecular disease entity. *Am J Surg Pathol*. 2003; 27:1463–1470. [PubMed: 14576481]
- El-Mofty SK, Patil S. Human papillomavirus (HPV)-related oropharyngeal nonkeratinizing squamous cell carcinoma: characterization of a distinct phenotype. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*. 2006; 101:339–345. [PubMed: 16504868]
- Klussmann JP, Gultekin E, Weissenborn SJ, et al. Expression of p16 protein identifies a distinct entity of tonsillar carcinomas associated with human papillomavirus. *Am J Pathol*. 2003; 162:747–753. [PubMed: 12598309]
- Shiboski CH, Schmidt BL, Jordan RC. Tongue and tonsil carcinoma: increasing trends in the U.S. population ages 20–44 years. *Cancer*. 2005; 103:1843–1849. [PubMed: 15772957]
- Wiest T, Schwarz E, Enders C, Flechtenmacher C, Bosch FX. Involvement of intact HPV16 E6/E7 gene expression in head and neck cancers with unaltered p53 status and perturbed pRb cell cycle control. *Oncogene*. 2002; 21:1510–1517. [PubMed: 11896579]

13. Alani RM, Munger K. Human papillomaviruses and associated malignancies. *J Clin Oncol.* 1998; 16:330–337. [PubMed: 9440761]
14. Begum S, Gillison ML, Nicol TL, Westra WH. Detection of human papillomavirus-16 in fine-needle aspirates to determine tumor origin in patients with metastatic squamous cell carcinoma of the head and neck. *Clin Cancer Res.* 2007; 13:1186–1191. [PubMed: 17317828]
15. Smeets SJ, Hesselink AT, Speel EJ, et al. A novel algorithm for reliable detection of human papillomavirus in paraffin embedded head and neck cancer specimen. *Int J Cancer.* 2007; 121:2465–2472. [PubMed: 17680565]
16. Gharizadeh B, Kalantari M, Garcia CA, Johansson B, Nyren P. Typing of human papillomavirus by pyrosequencing. *Lab Invest.* 2001; 81:673–679. [PubMed: 11351039]
17. Gharizadeh B, Zheng B, Akhras M, et al. Sentinel-base DNA genotyping using multiple sequencing primers for high-risk human papillomaviruses. *Mol Cell Probes.* 2006; 20:230–238. [PubMed: 16516439]
18. Goldenberg D, Begum S, Westra WH, et al. Cystic lymph node metastasis in patients with head and neck cancer: An HPV-associated phenomenon. *Head Neck.* 2008; 30:898–903. [PubMed: 18383529]
19. Thompson LD, Heffner DK. The clinical importance of cystic squamous cell carcinomas in the neck: a study of 136 cases. *Cancer.* 1998; 82:944–956. [PubMed: 9486586]
20. 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]
21. Reed AL, Califano J, Cairns P, et al. High frequency of p16 (CDKN2/MTS-1/INK4A) inactivation in head and neck squamous cell carcinoma. *Cancer Res.* 1996; 56:3630–3633. [PubMed: 8705996]
22. Kong CS, Balzer BL, Troxell ML, Patterson BK, Longacre TA. p16INK4A immunohistochemistry is superior to HPV in situ hybridization for the detection of high-risk HPV in atypical squamous metaplasia. *Am J Surg Pathol.* 2007; 31:33–43. [PubMed: 17197917]
23. Umudum H, Rezanko T, Dag F, Dogruluk T. Human papillomavirus genome detection by in situ hybridization in fine-needle aspirates of metastatic lesions from head and neck squamous cell carcinomas. *Cancer.* 2005; 105:171–177. [PubMed: 15822131]
24. Begum S, Gillison ML, Ansari-Lari MA, Shah K, Westra WH. Detection of human papillomavirus in cervical lymph nodes: a highly effective strategy for localizing site of tumor origin. *Clin Cancer Res.* 2003; 9:6469–6475. [PubMed: 14695150]
25. Gologan O, Barnes EL, Hunt JL. Potential diagnostic use of p16INK4A, a new marker that correlates with dysplasia in oral squamoproliferative lesions. *Am J Surg Pathol.* 2005; 29:792–796. [PubMed: 15897745]
26. Goldenberg D, Sciubba J, Koch WM. Cystic metastasis from head and neck squamous cell cancer: a distinct disease variant? *Head Neck.* 2006; 28:633–638. [PubMed: 16477605]
27. Nielsen GP, Stemmer-Rachamimov AO, Shaw J, Roy JE, Koh J, Louis DN. Immunohistochemical survey of p16INK4A expression in normal human adult and infant tissues. *Lab Invest.* 1999; 79:1137–1143. [PubMed: 10496532]
28. Gillison ML. Human papillomavirus and prognosis of oropharyngeal squamous cell carcinoma: implications for clinical research in head and neck cancers. *J Clin Oncol.* 2006; 24:5623–5625. [PubMed: 17179099]
29. Snijders PJ, van den Brule AJ, Meijer CJ. The clinical relevance of human papillomavirus testing: relationship between analytical and clinical sensitivity. *J Pathol.* 2003; 201:1–6. [PubMed: 12950011]

FIGURE 1.

(A) Metastatic cystic squamous cell carcinoma (SCC) from the larynx with a keratinizing morphology exhibiting keratin pearl formation and intercellular bridges between tumor cells. The cyst contains extensively keratinized tumors cells and debris. (B) Metastatic keratinizing SCC, seen in Figure 1A, showing negative staining with p16^{INK4A} immunohistochemistry; (C) fine-needle aspirate smear from a metastatic cystic SCC from the larynx showing extensive cytoplasmic keratinization, nuclear enlargement and hyperchromasia, and occasional single intact tumor cells in a background of histiocytes and degenerating cells; (D) cell block section from a metastatic keratinizing SCC from the larynx with (E) accompanying negative p16^{INK4A} immunohistochemical stain. (A: H&E stain, original magnification ×100; B: original magnification ×100; C: Papanicolaou stain, original magnification ×200; D: H&E stain, original magnification ×200; E: original magnification ×200).

FIGURE 2.

(A) Metastatic cystic squamous cell carcinoma (SCC) from the oropharynx with a nonkeratinizing morphology exhibiting a monomorphic population of ovoid cells with indistinct cell borders and an extensive cystic component with degenerating cells. (B) Focal areas of keratinization in an otherwise nonkeratinizing SCC showing predominantly rounded nests of basaloid tumor cells with central comedo-like necrosis; (C) nonkeratinizing SCC, seen in Figures 2A–B, showing diffuse, strong full thickness p16^{INK4A} positive staining as well as staining of scattered tumor cells within the cyst contents; (D) fine-needle smear from a metastatic cystic SCC from the oropharynx showing a background of extensive degenerating cells with a rare crowded 3-dimensional cluster of tumor cells; (E) 3-dimensional clusters of tumor cells forming syncytia without keratinization from a metastatic cystic SCC from the oropharynx; (F) cell block section from a metastatic nonkeratinizing oropharyngeal SCC with (G) accompanying p16^{INK4A} immunohistochemical stain showing diffuse, strong staining in the neoplastic cell clusters as well as scattered positive tumor cells dispersed singly (A,B: H&E stain, original magnification $\times 100$; C: original magnification $\times 100$; D: Diff-Quik, original magnification $\times 200$; E: Papanicolaou stain, original magnification $\times 400$; F: H&E, original magnification $\times 200$; G: original magnification $\times 200$).

FIGURE 3.

(A) Branchial cleft cyst characterized by cytologically bland squamous lining cells without nuclear enlargement or mitotic activity; (B) branchial cleft cyst, seen in Figure 3A, showing focal, strong p16^{INK4A} immunohistochemical staining involving the superficial squamous cells lining the cyst without full-thickness staining; (C) fine-needle smear of branchial cleft cyst exhibiting bland squamous cells and anucleate squamous forming occasional clusters; (D) cell block section from the branchial cleft cyst with (E) accompanying negative p16^{INK4A} immunohistochemical stain (A: H&E, original magnification $\times 200$; B: original magnification $\times 200$; C: Papanicolaou stain, original magnification $\times 200$; D: H&E, original magnification $\times 200$; E: original magnification $\times 200$).

Table 1

Demographic Parameters and Pathologic Features of Cystic Squamous Lesions of the Neck (Combined Study Set and Test Set)

Diagnosis (No. of Cases)	Mean Age, y	M:F	Keratinizing SCC	Nonkeratinizing SCC
Cystic SCC (26)	65	20:6	14	12
Oropharynx (15)	64	11:4	5	10
Laryngeal (4)	78	4:0	4	0
Oral cavity (4)	65	3:1	4	0
Unknown (3)	68	2:1	2	1
Branchial cleft cyst (25)	28	13:12	–	–

SCC indicates squamous cell carcinoma; M:F, male-to-female ratio.

Table 2

Summary of p16^{INK4A} and Human Papillomavirus Polymerase Chain Reaction/Pyrosequencing in Surgically Resected Cystic Squamous Cell Lesions (Study Set)

Anatomic Site and Morphology (No. Cases)	Diffuse, Strong p16 ^{INK4A} (%)	HPV PCR/Pyrosequencing (%)
Oropharyngeal nonkeratinizing SCC (7)	7/7 (100)	6/6 HPV 16 (100)
Oropharyngeal keratinizing SCC (2)	1/2 (50)	0/2 (0)
Laryngeal keratinizing SCC (3)	0/3 (0)	0/2 (0)
Oral cavity keratinizing SCC (1)	0/1 (0)	1/1 HPV 16 and 18 (100)
Unknown keratinizing SCC (2)	1/2 (50)*	2/2 (100)*
Branchial cleft cyst (21)	6 focal/21 [†]	7/19 (37) [‡]

HPV indicates human papillomavirus; PCR, polymerase chain reaction; SCC, squamous cell carcinoma.

* The case of unknown keratinizing SCC, which exhibited diffuse strong p16^{INK4A} immunolabeling, also was positive for HPV 16 DNA. The case of unknown keratinizing SCC lacking p16^{INK4A} immunolabeling was positive for HPV DNA of an unknown type.

[†] Six cases exhibited focal, strong p16^{INK4A} involving the superficial squamous cells lining the cyst.

[‡] Four cases were HPV 18 positive, and 3 cases were positive for both HPV 16 and HPV 18.

Table 3

Summary of p16^{INK4A} and Human Papillomavirus Polymerase Chain Reaction/Pyrosequencing in Fine-Needle Aspiration Biopsy of Cystic Squamous Cell Lesions (Test Set)

Anatomic Site and Morphology (No. Cases)	Diffuse, Strong p16 ^{INK4A} (%)	HPV PCR/Pyrosequencing (%)
Oropharyngeal nonkeratinizing SCC (3)	3/3 (100)	2/2 HPV 16 (100)
Oropharyngeal keratinizing SCC (3)	0/3 (0)	0/1 (0)
Laryngeal keratinizing SCC (1)	0/1 (0)	0/1 (0)
Oral cavity keratinizing SCC (3)	0/3 (0)	0/3 (0)
Unknown nonkeratinizing SCC (1)	1/1 (100)	1/1 HPV 16 (100)
Branchial cleft cyst (4)	0/4 (0)	0/1 (0)

HPV indicates human papillomavirus; PCR, polymerase chain reaction; SCC, squamous cell carcinoma.

Table 4

Summary of Human Papillomavirus in situ Hybridization Results in Branchial Cleft Cyst and Squamous Cell Carcinoma Study Set Cases With Discordant p16^{INK4A}-Human Papillomavirus Pyrosequencing Cases

Diagnosis	p16 ^{INK4A}	HPV Pyrosequencing	HPV 16 ISH	HPV 18 ISH
Keratinizing SCC, oral cavity primary	Negative	HPV 16 and 18	Negative	Negative
Keratinizing SCC, unknown primary	Negative	HPV unknown type	Negative	Negative
Nonkeratinizing SCC, oropharyngeal primary	Diffuse, strong	Not done	Positive	Not done
BCC	Focal, strong	HPV 16 and 18	Negative	Negative
BCC	Focal, strong	HPV 16 and 18	Negative	Negative
BCC	Focal, strong	Negative	Negative	Negative
BCC	Focal, strong	Negative	Negative	Negative
BCC	Focal, strong	Negative	Negative	Negative
BCC	Focal, strong	Not done	Negative	Negative
BCC	Glandular epithelium	HPV 16 and 18	Negative	Negative
BCC	Glandular epithelium	Negative	Negative	Negative
BCC	Negative	HPV 18	Negative	Negative
BCC	Negative	HPV 18	Negative	Negative
BCC	Negative	HPV 18	Negative	Negative
BCC	Negative	HPV 18	Negative	Negative
BCC	Negative	Not done	Negative	Not done

HPV indicates human papillomavirus; ISH, in situ hybridization; SCC, squamous cell carcinoma; BCC, branchial cleft cyst.