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Authors
Huho, Albert N
Yadak, Nour
Bocklage, Thèrése J
et al.

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Evaluation of Diagnostic Utility of a High-Risk Human Papillomavirus PCR Test on Formalin-Fixed, Paraffin-Embedded Head and Neck Tumor Tissues

Albert N. Huho,*† Nour Yadak,* Thérése J. Bocklage,* and Shangxin Yang*†

From the Department of Pathology, University of New Mexico, Albuquerque; and the TriCore Reference Laboratories,† Albuquerque, New Mexico*

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Address correspondence to Shangxin Yang, Ph.D., TriCore Reference Laboratories, 1001 Woodward Pl NE, Albuquerque, NM 87102. E-mail: shyang@salud.unm.edu.

The increasing prevalence of high-risk human papillomavirus (HR-HPV)—associated head and neck squamous cell carcinoma (HNSCC) has prompted strong clinical demands for detecting HR-HPV directly in the tumor. Although p16 immunohistochemistry (IHC) has been the standard testing method, it has limitations including false positivity, lack of sensitivity in low tumor cell samples such as fine-needle aspirate (FNA), and its subjectivity. We developed a modified method based on a commercial automated HR-HPV PCR assay and evaluated the performance characteristics and the diagnostic utility of this assay for direct HR-HPV detection in the HNSCC samples. HNSCC formalin-fixed, paraffin-embedded blocks were retrieved from archives including 44 excisions, 63 biopsies, and 16 FNAs. Tissue slices were trimmed from the blocks, deparaffinized, lysed, and loaded on the commercial automated platform for HR-HPV PCR. All specimens had a concurrent p16 IHC performed. The PCR assay showed high concordance with the p16 IHC (96%; 99/103) and excellent positive agreement (91.5%) and negative agreement (100%). In addition, the PCR assay provided more conclusive results in samples with equivocal p16 IHC results. The modified commercial automated HR-HPV PCR test is a labor-efficient, quick, reliable, sensitive, and specific method for detecting HR-HPV in formalin-fixed, paraffin-embedded samples. This assay also showed excellent diagnostic utility in samples with equivocal p16 IHC results, including FNA cell blocks. *(J Mol Diagn 2018, 20: 232—239; https://doi.org/10.1016/j.jmoldx.2017.11.008)*

High-risk human papillomavirus (HR-HPV) is a well-established etiologic agent in a subset of head and neck squamous cell carcinomas (HNSCC), particularly in the subset of oropharyngeal squamous cell carcinomas (OPSCC), in which transcriptionally active HR-HPV were found in up to 80% of the cases.[1,2] HPV-related HNSCCs are molecularly, morphologically, and prognostically different from smoking- and alcohol-related HNSCCs. Prognostically, the HPV-related HNSCCs have a much better overall and progression-free survival, and less likelihood for local recurrence than smoking-related tumors.[2] Numerous clinical trials have shown the benefit for de-escalation therapy to decrease treatment-related morbidity in HPV-positive OPSCCs, typically seen in younger patients, and allow use of immunotherapy (some targeting HPV directly).[3,4] Alarmingly, HPV-related HNSCCs continue to increase at a rapid rate, earning the label of epidemic.[5] This is linked epidemiologically to an increase in

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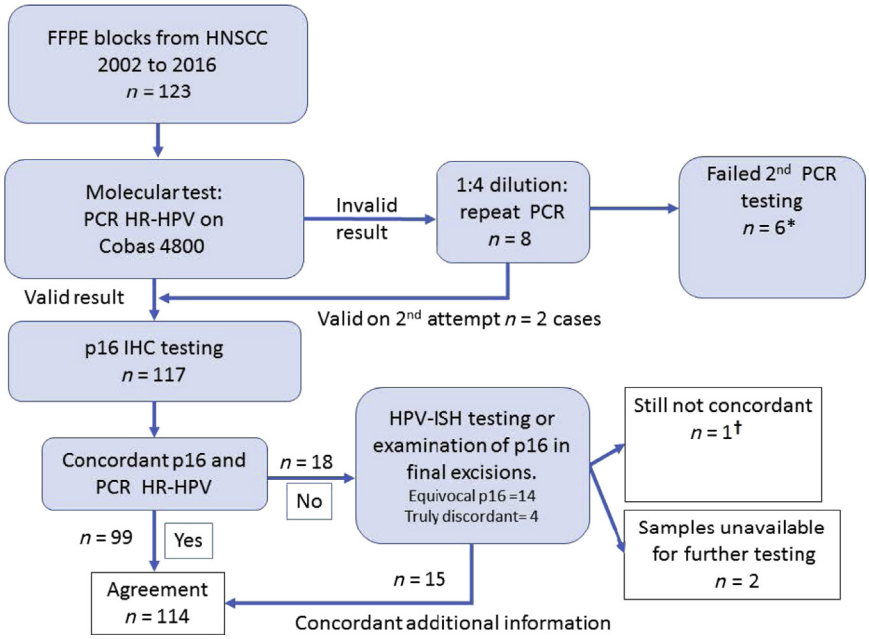

Figure 1 Flow chart of study design and result summary. An invalid result was defined as failed amplification of human papillomavirus (HPV) targets and failed β-globulin internal control. Samples with initial invalid results were retested by the high-risk HPV (HR-HPV) PCR after a 1:4 dilution of the tissue lysate. *Four cases >12 years old. FFPE, formalin-fixed, paraffin-embedded; HNSCC, head and neck squamous cell carcinoma; IHC, immunohistochemistry. †p16 IHC+, DNA-ISH+, and HR-HPV PCR− (tested twice).

oral sex practice.[6] It is predicted that HPV-related HNSCCs will exceed the number of cervical carcinomas in the United States by the year 2020.[7]

Routine testing for HPV in OPSCC and other head and neck lymph nodes harboring squamous elements from an unknown primary site is recommended by the College of American Pathologists and the American Joint Committee on Cancer.[8] Many different methods are available with variable sensitivity, specificity, technical complexity, and cost. The available tests include immunohistochemistry (IHC), *in situ* hybridization (ISH) for DNA or RNA, liquid-phase assays for cytopathology specimens, and PCR-based methods.[9]

Currently, the most widely used methods for HR-HPV detection are IHC for p16 as a surrogate marker of viral oncogenesis, and DNA-ISH for direct detection of the viral genome. IHC for p16 is shown to have a high sensitivity and specificity in the oropharynx, but limited specificity outside the oropharynx.[10–12] In addition, the interpretation of p16 IHC may be difficult in small biopsy specimens and fine-needle aspirate (FNA) materials with scant tumor cells.[13] Focal or weak staining usually needs to be supported by other forms of confirmatory HPV testing. HPV DNA-ISH is a slightly more expensive testing method with a higher specificity, but may have lower sensitivity and also can be subjective.[13,14] Because of its higher technical complexity, most clinical laboratories send out this test, incurring additional cost and delayed turnaround time. RNA ISH has become increasingly popular owing to its good sensitivity and interpretability, with the added advantage of detecting transcriptionally active HR-HPV.[15] However, the higher cost and technical complexity prohibit most clinical laboratories from performing this test in house.

Recently, several study groups have shown that detection of HR-HPV DNA by PCR in the HNSCC tumor samples may provide the benefits of higher sensitivity and specificity, less subjectivity, ease of use, lower costs, and quicker turnaround times.[16,17] In this study, we developed and validated a modified PCR method based on the Cobas 4800 (Roche Molecular Diagnostics, Pleasanton, CA) to detect HR-HPV in formalin-fixed, paraffin-embedded (FFPE) HNSCC tissues.

Materials and Methods

A total of 123 HNSCC FFPE blocks, dating from 2002 to 2016, were collected randomly from the pathology archives of the University of New Mexico Hospital, including 63 incisional biopsy specimens, 44 excisions, and 16 FNA cell blocks. Six cases that failed the HR-HPV PCR twice were excluded. Concurrent p16 IHC was performed on all specimens by using the CINtec p16 Histology assay (E6H4 clone; Ventana Medical Systems, Tucson, AZ) and they were interpreted as positive, negative, or equivocal by a pathologist. Interpretation of p16 IHC was based on previous cut-off values used in other studies.[18] Seventy percent or greater diffuse nuclear and cytoplasmic positivity was considered positive, whereas 50% to 69% diffuse or focal patchy nuclear and cytoplasmic positivity was considered equivocal. Any staining less than 50% was considered negative. Samples with discrepant results between p16 IHC and HR-HPV PCR were tested further by HR-HPV DNA ISH using Inform HPV III Family 16, Probe (B) for HR-HPV (Ventana Medical Systems), or, alternately, had

follow-up review of p16 IHC results in subsequent excision specimens (Figure 1).

Most (82.9%; 97/117) of the HNSCCs were primary tumors, whereas 17.1% (20/117) were metastatic tumors involving lymph nodes or secondary sites. Primary OPSCC accounted for 43.6% (51/117) of the cases. The remaining sample sites included larynx (4.3%; 5/117), oral cavity and anterior tongue (23.9%; 28/117), mandible and floor of mouth (6.0%; 7/117), neck (14.5%; 17/117), hypopharynx (1.7%; 2/117), and nasopharynx (6.0%; 7/117). Paraffin-embedded FNA cell blocks used in the study had been collected as needle rinsing in 10% formaldehyde, followed by centrifugation, formalin fixation, and paraffin embedment of resulting cell pellets. All retrieved blocks were cut using a strict cleaning protocol, including one-time blade use, to limit cross-contamination. Four tissue sections (5 μm per section) were collected and combined from each block irrespective of the tissue size. The tissues were deparaffinized using Citrasolv (Fisher Scientific, Pittsburgh, PA), lysed for 1 hour using lysis reagent freshly prepared by mixing buffer ATL and the proteinase K at a 9:1 ratio (Qiagen, Valencia, CA), and diluted in 50% ethanol before loading on a Roche Cobas 4800 using the HR-HPV PCR assay (detects HPV31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) and following the manufacturer's protocol. All cases with initial invalid results (failed amplification of HPV targets and β-globulin internal control) were diluted 1:4 in 50% ethanol to reduce the concentration of potential PCR inhibitory substances and retested. The limit of detection was assessed through serial dilutions of the lysate from a high p16 IHC-positive sample. The interassay and intra-assay precisions were assessed by running lysate aliquots over several different runs on different days.

Detailed Methodology for the Roche Cobas HPV PCR Test on FFPE Tissue

Cutting Tissue Blocks and DNA Extraction

All retrieved blocks were cut using a strict protocol aimed at limiting cross-contamination between specimens. Before cutting each block, the microtome was wiped down with sodium hypochlorite 0.65% (virucidal), dried, and further cleaned using 100% ethanol to remove any potential remaining material. The microtome blade then was replaced with a fresh blade between each block. The block was then lightly trimmed to remove superficial layers before four tissue sections were made, each 5-μm thick, for a total of 20 μm trimmings. Fresh, clean, 1000-μL pipette tips were used to press into the resulting scrolls gently and lift the tissue scrolls into labeled 1.5-mL Safe-Lock (Sigma-Aldrich, St Louis, MO) microcentrifuge tubes.

Deparaffinization and Tissue Lysis

The microcentrifuge tubes containing paraffin tissue scrolls were filled with 1 mL of CitriSolv (Fisherbrand, Pittsburgh, PA) solution and vortexed for approximately 10 seconds. The microcentrifuge tubes then were incubated at room temperature (15°C to 30°C) for 10 minutes followed by a second vortexing step for 10 seconds and centrifugation at 15,000 × *g* for 5 minutes. The supernatant was removed using transfer pipettes and discarded. To ensure complete deparaffinization, another 1 mL of CitriSolv was added to the pellet, and the process of deparaffinization was repeated as described earlier. The pellet then was suspended by adding 1 mL of absolute ethanol 100% (Fisherbrand) and vortexed for 10 seconds. After 5 minutes of incubation at room temperature, the samples were centrifuged at 15,000 × *g* for 5 minutes. The supernatant was decanted and the pellets were air dried for 10 minutes on a 70°C heating block with lids open.

The pellets were resuspended in 200 μL of lysis reagent freshly prepared by mixing buffer ATL and the proteinase K at a 9:1 ratio (Qiagen). The tube was vortexed gently for 30 seconds, ensuring that all tissues were submerged in lysis reagents before incubation in a 55°C heat block for 1 hour. The specimens were removed from the heat block and vortexed for 10 seconds and examined for a homogenous appearance. If the homogenization was not sufficient, an extra amount of 20 μL lysis proteinase K was added and the specimen was re-incubated for an additional 1 hour. The lysed samples were diluted into 2 mL of 50% ethanol solution and vortexed. One milliliter of the dilution was transferred into a 13-mL round-bottom secondary tube with a barcode (Sarstedt AG & Company, Sarstedt, Germany) for testing on the Roche Cobas 4800. The remaining 1 mL of diluted lysate was frozen at −20°C as a back-up for possible repeat testing.

Automated Cobas Platform

The lysed samples suspended in 50% ethanol were loaded on a Cobas 4800 following the manufacturer's protocol (Cobas HPV Test US IVD Method sheet/package insert v13; Roche Molecular Diagnostics) for HPV testing.

Test results from the Cobas system were either negative, HR-HPV16 positive, HR-HPV18 positive, or other high-risk HPV positive. Cycle threshold (Ct) values for β-globulin in all cases tested and viral target Ct values for the HR-HPV−positive case were recorded.

p16 IHC

p16 IHC was performed on a BOND-III stainer from Leica Biosystems (Buffalo Grove, IL). The antibody used was a mouse monoclonal antibody (E6H4 clone, CINtec; Ventana Medical Systems) diluted 1:4 with detection via the Polymer Refine Kit (Leica Biosystems) on a Leica Bond autostainer.

p16 IHC-stained slides were evaluated and grouped into three main categories using known criteria for tissue sections: i) positive: ≥70% diffuse nuclear and cytoplasmic staining in neoplastic cells; ii) equivocal: 50% to 69% diffuse or focal patchy nuclear and cytoplasmic staining in neoplastic cells, or cases with only cytoplasmic/nuclear

Table 1 Demography of HNSCC Cases

Variable	Total, *n*	PCR HR-HPV$^+$, *n* (%)	PCR HR-HPV$^-$, *n* (%)	Odds ratio	95% CI	*P* value*
Sex	117	54 (46.1)	63 (53.8)			
Male	83	46 (55.4)	37 (44.6)			
Female	34	8 (23.5)	26 (76.5)	4.04	1.6–9.9	0.02
Median age	55	54	58			
<62 years	80	40 (50)	40 (50)			
≥62 years	37	14 (37.8)	23 (62.1)	1.32	0.828–2.109	0.222
Primary cancer site						
OPSCC	68	50 (73.5)	18 (26.4)			
Non-OPSCC	49	4 (8.2)	45 (91.8)	9	3.4–23.88	<0.0001

*Cochran–Mantel–Haenszel statistics.
HR-HPV, high-risk human papillomavirus; OPSCC, oropharyngeal squamous cell carcinoma.

staining; and iii) negative: less than 50% of cells staining for p16.

DNA *in Situ* Hybridization

DNA ISH was performed at an outside laboratory using the Inform HPV III Family 16, Probe (B) for HR-HPV (Ventana Medical Systems).

Limit of Detection, Precision, and Stability

To estimate the limit of detection, a highly p16-positive tumor sample was macrodissected to enrich for p16-positive tumor cells (>95% p16-positive tumor) and processed into a lysate as described earlier. The lysate was serially diluted in duplicate, using 50% ethanol and tested on the Cobas 4800. A series of six dilutions at an increasing 1:5 ratio each, were made from 1:1 to 1:15,625.

The DNA from the initial lysate (before any serial dilutions) was extracted by COBAS-z and the concentration was measured fluorometrically using Quantus 2.0 (Promega-Maxwell, Fitchburg, WI). The number of cells corresponding to the extracted DNA was estimated assuming a DNA concentration of 6.6 pg per diploid human cell.

Precision was determined by triplicate testing of one HR-HPV–negative sample and one HR-HPV–positive sample, aliquoted, and diluted to provide a Ct value close to the limit of detection as determined in the LOD experiment described in the preceding paragraphs. Three runs on separate days were performed.

Sample stability was determined by retesting aliquots of lysate, stored at room temperature (15°C to 30°C) or frozen at −20°C, after a 1-month and 3-month duration.

Statistical Analysis

Statistical analysis was performed using SPSS version 24 (IBM Corp., Armonk, New York). A paired *t*-test and a *t*-test were used to compare means. All reported variables were two-sided. Odds ratios were calculated using Cochran-Mantel-Haenszel to control for confounding factors.

Results

Of 123 FFPE specimens (mean sample age, 3.6 years; range, 1 to 15 years), 115 specimens were tested successfully on initial HR-HPV PCR. The 8 specimens with initial invalid results, probably resulting from inhibitors or poor DNA quality, were retested after a 1:4 dilution, which helped generate valid results in 2 additional cases, allowing for 117 cases (117/123, 95% technical success rate) to be analyzed. Four of the six failed cases (after dilution and second PCR test) were samples dated more than 13 years ago. The remaining two failed cases were mandibular resections, failed probably owing to PCR inhibitory substances in the decalcifying agents used during grossing. Of the 117 cases with valid PCR results, approximately half (46.2%, 54/117) were positive for HR-HPV. A majority of the HR-HPV–positive patients were males ($P = 0.02$) and had OPSCC as the primary site of involvement ($P \leq 0.001$). Although more positive cases were found in younger age groups (<62 years), this was not statistically significant ($P = 0.222$) (Table 1). HPV-16 was the most prevalent genotype among positive cases, accounting for 40.2% (47/117); whereas 3.4% (4/117) were positive for other high-risk HPV; 2.6% (3/117) had dual infections with HPV-16 and other HR-HPV. Consistent with the literature,[19] no cases of HPV-18 were identified. Within the oropharynx and the base of the tongue, 76.5% (39/51) of the cases were positive for HR-HPV (Figure 2A). The p16 IHC was positive in 43.5% (53/117) of the cases, and equivocal in 12% (14/117). FNA specimens had the most equivocal p16 results (43.8%; 7/16) (Figure 2B). Overall, the PCR test had a positive agreement of 91.5% and a negative agreement of 100% for the detection of HPV-related squamous cell carcinoma when compared with p16 IHC (Table 2).

Among the four discordant cases (p16 IHC positive but PCR negative), two cases from the oropharynx further tested negative by HR-HPV DNA-ISH, one FNA of a neck node was confirmed to be a p16-positive neuroendocrine tumor, and one case from the floor of the mouth tested positive by DNA-ISH, albeit with a very faint speckled nuclear staining pattern. Within the oropharynx, there was good correlation

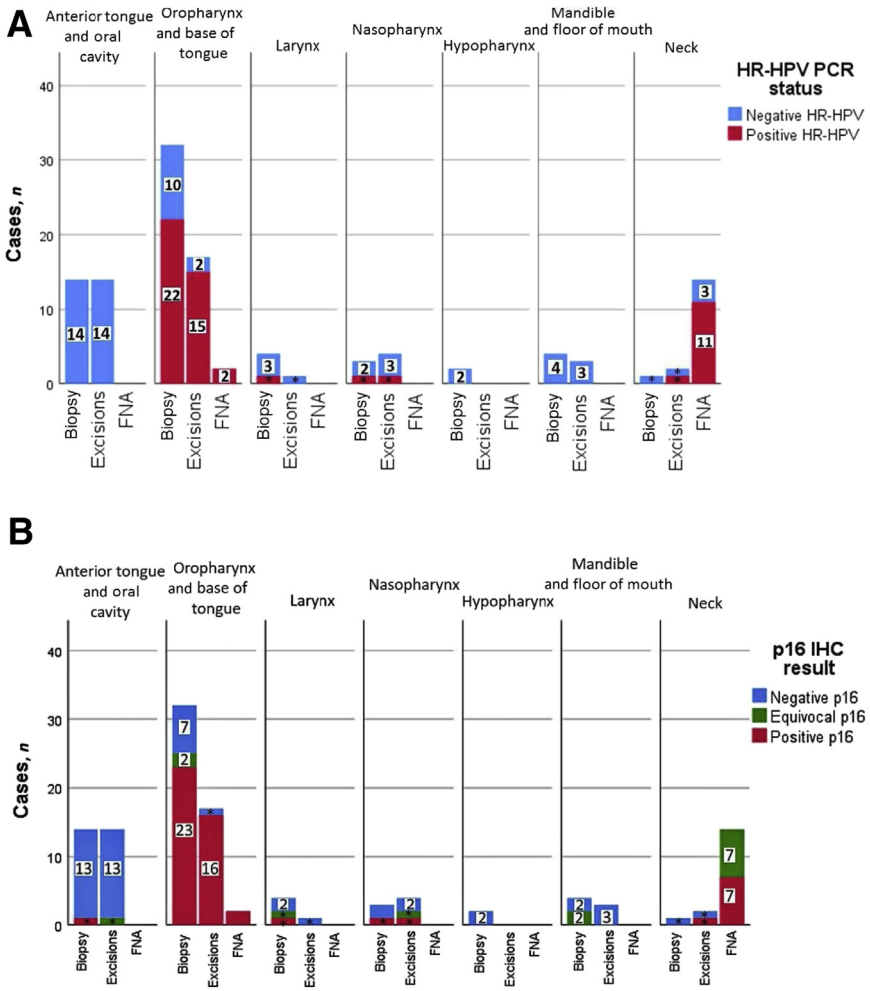

Figure 2 **A:** Results of high-risk human papillomavirus (HR-HPV) PCR testing by anatomic location and specimen type. **B:** Results of p16 immunohistochemical staining by anatomic location and specimen type. *One case each. FNA, fine-needle aspirate; IHC, immunohistochemistry.

between the PCR results and the p16 IHC results, with a positive agreement of 95.1% and a negative agreement of 100% (Table 2). The Ct values for HR-HPV (combining HPV-16 and other HR types) in the positive cases were significantly lower in the excisions (mean, 26.2; range, 22.4 to 32.7) and biopsies (mean, 26.9; range, 21.8 to 36.9) than the Ct values in FNA (mean, 32.4; range, 25.5–36.9) (Figure 3). The mean values of β-globulin were also significantly higher in the FNA specimens than the biopsies ($P < 0.0001$) and the excisions ($P < 0.0001$), reflecting lower human diploid cellular quantities in the FNAs (Figure 3).

To determine the analytical sensitivity of the test, a serial dilution of lysate from a highly positive sample (>95% p16-positive tumor and HPV-16 PCR positive) was tested. The last dilution in which HPV-16 was detectable by PCR corresponded to approximately 8 cells/mL (Figure 4). A repeat experiment verified this lower limit of detection (data not shown). However, because of the possibility of a wide range of HPV viral genome copies that may be present in one infected tumor cell,[20] we were not able to specify the limit of detection in HPV copies/mL for this test. The interassay and intra-assay precision was 100%, with consistent results on all runs for both positive and negative samples (data not shown). In addition, the CV for HPV-16 Ct values among the replicates was only 0.13%. All FFPE samples collected within 12 years were tested successfully except for 2 samples from mandibular excisions that may have had PCR inhibitory substances from the decalcification process. The processed tumor lysates, diluted in 50% ethanol, were stable for up to 3 months at room temperature (18°C to 25°C), with minimal change in Ct values for β-globulin and HPV-16 (0.2 to 0.3 Ct value increase over 3 months for both targets). Lysates frozen at −20°C also remained stable for up to 3 months (with a 0 to 0.1 increase in both HPV-16 and β-globulin Ct values).

Equivocal p16 IHC results were seen most frequently in FNA specimens (43.8%; 7/16) followed by biopsies (8.2%; 5/61), and least in the larger excisions (0.82%; 2/41). The trend probably reflected increased difficulty in interpreting p16 IHC results when tumor cells were scant. The seven equivocal FNA cases were all from neck lymph nodes and were tested successfully by the HR-HPV PCR, with five of them positive and the remaining two were negative. Six of these FNA cases were later further tested on the excision samples and the p16 IHC results were consistent with the PCR results. One of the PCR-negative FNA cases had a follow-up subsequent excision in which SCC was ruled

Table 2 Comparison of HR-HPV PCR Results and p16 IHC Results

HR-HPV PCR results by site	p16 IHC positive, *n*	p16 IHC negative, *n*	p16 IHC equivocal, *n*	Total, *n*
All sites				
HR-HPV PCR positive	49	0	5‡	54
HR-HPV PCR negative	4*	50	9†	63
Total	53	50	14	117
Oropharynx				
HR-HPV PCR positive	39	0	0	39
HR-HPV PCR negative	2	8	2	12
Total	41	8	2	51

*Two of the four cases subsequently tested negative by HPV-DNA *in situ* hybridization; the third case was a confirmed neuroendocrine tumor that also was p16 positive. The fourth case remained positive by HPV-ISH testing (albeit very faintly on ISH).

†Four of these cases were negative by HPV-DNA *in situ* hybridization.

‡All cases either had a positive HPV-ISH or a follow-up larger excision that was p16 positive.

HR-HPV, high-risk human papillomavirus; IHC, immunohistochemistry; ISH, *in situ* hybridization.

negative by histology (Table 3). The other seven cases of biopsies and excisions with equivocal p16 IHC results were all HPV-PCR negative. Three of these cases were confirmed to be negative by HPV DNA-ISH. One case was confirmed to be p16 IHC negative on the follow-up excision sample, and another case was diagnosed as an Epstein-Barr virus–related tumor. Confirmatory testing could not be performed on two cases because of unavailability of the leftover samples. After re-adjusting for the additional testing, the HR-HPV PCR results were consistent with the final diagnosis in the majority (85.7%; 12/14) of the samples with equivocal p16 IHC results (Table 3).

Discussion

Immunohistochemistry for p16 currently is considered to be an independent prognostic marker for the detection of HR-HPV infection in HNSCCs and recently was applied to a separate staging system in HNSCC.[8] Although the interpretation of p16 IHC usually is straightforward with good interobserver agreement, especially when defined criteria are applied, it does occasionally become difficult and subjective. Smaller biopsy specimens and FNA cases usually are harder to interpret,[13,21] and this was shown in our study with equivocal p16 IHC results reported most often in FNA cases. To our knowledge, this study is the first to prove the feasibility of performing HR-HPV PCR on the paraffin-embedded FNA materials (rather than fresh FNA needle washings), with clear and unequivocal results.

Our laboratory-modified HR-HPV PCR test showed a high concordance with p16 IHC, particularly in the oropharynx, in which the positive agreement was 95.1% and the negative agreement was 100%. Given the high concordance of this test, it may be implemented in an algorithmic approach for the detection of HR-HPV, in which it can complement the lower sensitivity of p16 in low tumor

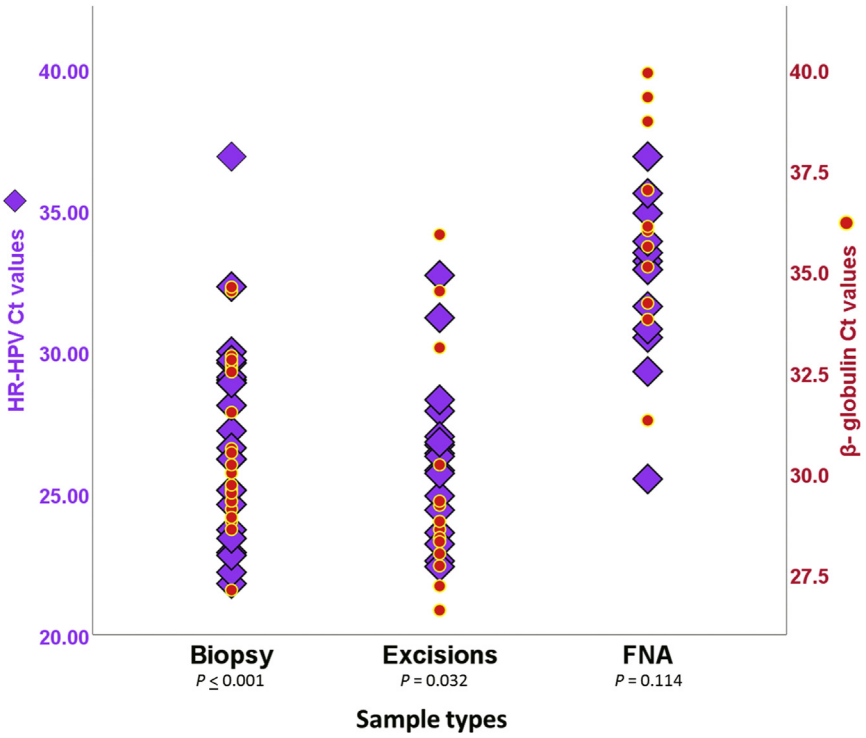

Figure 3 Dot plot of cycle threshold (Ct) values of β-globulin and high-risk human papillomavirus (HR-HPV) sorted by sample types for all positive cases. Paired *t*-test comparing β-globulin Ct and HR-HPV Ct values in each sample category.

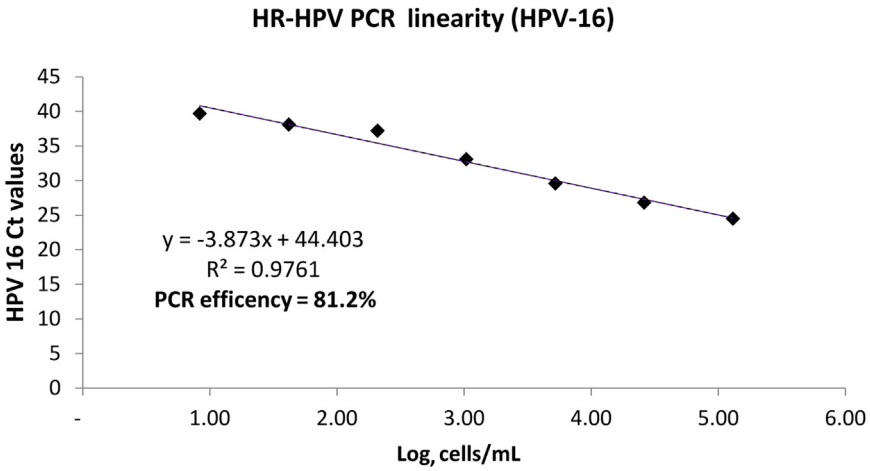

Figure 4 Fivefold serial dilution of tumor lysate from a strongly p16-positive tumor sample. PCR efficiency for human papillomavirus (HPV)-16 target was calculated as 81.2%. Ct, cycle threshold; HR-HPV, high-risk HPV.

quantity samples and help resolve cases with equivocal p16 IHC results. This test can be easily adopted by many laboratories owing to the wide availability of the Cobas 4800 system and relative lower cost compared with other molecular techniques (DNA-ISH or RNA-ISH). The throughput of this test can be up to 96 samples in a run, which is much higher than ISH tests. The test has very manageable hands-on time (an average of 45 minutes for up to two samples before loading). In addition, the turnaround time (within 8 hours) is significantly shorter than other confirmatory tests. Of note, the modified HR-HPV PCR test on the FFPE samples can be run concurrently with the regular cervical swab samples without any interference in the routine workflow of cervical HR-HPV screening. This study also showed excellent sample stability for this test, with up to 12 years for the FFPE blocks and up to 90 days at room temperature for the processed tissue lysate.

Some limitations of this test included the possibility for detection of transcriptionally silent, not biologically causative, virus (passenger virus) or viral DNA contaminant. This study showed the great analytical sensitivity of the discussed test. Consistent with the literature,[20] very high viral quantities were observed within the neoplastic cells, as shown by significantly higher Ct values of β-globulin (as a marker for the quantity of cells) than the HR-HPV Ct values (as a marker for the quantity of viruses) (Figure 3). Arguably, this may lead to cross-contamination of samples between microtome sectioning steps or during the lysate preparation process if care is not taken. Despite using very stringent techniques, including a fresh microtome blade replacement between samples, one PCR false-positive case (<1%; 1/123) that had an initial high Ct value of 39.6 cycles was encountered. This sample subsequently was retested as negative. Examination of actual outlier Ct values when close

Table 3 Evaluation of HR-HPV PCR Results in the p16 IHC Equivocal Cases

		HR-HPV PCR results				
Specimen site	Specimen type	HPV type	HPV Ct values	β-*globulin* Ct values	Additional test results	Final diagnosis compared with PCR results
Neck	FNA	HPV 16	32.9	33.8	P16-positive excision	Consistent
Neck	FNA	HPV 16	33.9	37.0	P16-positive excision	Consistent
Neck	FNA	HPV 16	25.5	31.3	P16-positive excision	Consistent
Neck	FNA	Other HR-HPV	36.9	39.3	P16-positive excision	Consistent
Neck	FNA	Other HR-HPV	35.6	37.0	P16-positive excision	Consistent
Neck	FNA	Negative	0.0	32.8	Excision negative for SCC	Consistent
Neck	FNA	Negative	0.0	31.5	P16-negative excision	Consistent
Mandible/mouth	Biopsy	Negative	0.0	40	EBV-positive tumor*	Consistent
Nasopharynx	Excision	Negative	0.0	34.8	HR-HPV ISH negative	Consistent
Oropharynx	Biopsy	Negative	0.0	31.8	P16-negative excision	Consistent
Anterior tongue	Excision	Negative	0.0	29.0	HR-HPV ISH negative	Consistent
Mandible/mouth	Biopsy	Negative	0.0	34.2	Not available	Indeterminate
Larynx	Biopsy	Negative	0.0	30.7	Not available	Indeterminate
Oropharynx	Biopsy	Negative	0.0	30.8	HR-HPV ISH negative	Consistent

*Confirmed as a case of nasopharyngeal carcinoma extending into the roof of the mouth.

Ct, cycle threshold; EBV, Epstein-Barr virus; FNA, fine-needle aspirate; HR-HPV, high-risk human papillomavirus; IHC, immunohistochemistry; ISH, *in situ* hybridization.

to the cut-off value (40.5 cycles) may be helpful in cases suspected for contamination (eg, discordant p16 result), especially when next to a highly positive sample (very low Ct value). The Cobas HPV test also is limited by not specifying other high-risk HPV types, an important aspect of disease epidemiology that may influence future vaccine subtypes.

In summary, we have developed and validated a modified Cobas HR-HPV PCR assay that is able to detect HR-HPV DNA directly in various FFPE HNSCC sample types with excellent sensitivity, specificity, and precision. The test can be easily implemented in many clinical laboratories, and can serve as a rapid and cost-effective method to help either confirm p16 IHC-positive HNSCC cases or resolve p16 IHC equivocal cases, especially in FNA cases with limited tissue materials. Additional larger prospective clinical studies are needed to fully elucidate the value of this method and to help develop newer testing algorithms for better diagnosis of HR-HPV−related HNSCCs.

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A.N.H. retrieved specimens from archives, designed the methodology, wrote the manuscript, and analyzed and curated data; N.Y. designed the methodology and curated data; T.J.B. advised on immunohistochemistry and anatomic pathology, reviewed slides, and investigated and curated data; and S.Y. supervised the project, validated the data, and wrote and edited the manuscript.

Supplemental Data

Supplementary data related to this article can be found at *https://doi.org/10.1016/j.jmoldx.2017.11.008*.

References

1. Gillison ML, Koch WM, Capone RB, Spafford M, Westra WH, Wu L, Zahurak ML, Daniel RW, Viglione M, Symer DE, Shah KV, Sidransky D: Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. J Natl Cancer Inst 2000, 92:709−720
2. Ang KKP, Harris J, Wheeler R, Weber R, Rosenthal DI, Nguyen-tân PF, Westra WH, Chung CH, Jordan RC, Lu C, Kim H, Silverman CC, Redmond KP, Gillison ML: Human papillomavirus and survival of patients with oropharyngeal cancer. N Engl J Med 2011, 363:24−35
3. Gildener-Leapman N, Lee J, Ferris RL: Tailored immunotherapy for HPV positive head and neck squamous cell cancer. Oral Oncol 2014, 50:780−784
4. Zaravinos A: An updated overview of HPV-associated head and neck carcinomas. Oncotarget 2014, 5:3956−3969
5. Lewis A, Kang R, Levine A, Maghami E: The new face of head and neck cancer: the HPV epidemic. Oncology (Williston Park) 2015, 29:616−626
6. Chaturvedi AK, Engels EA, Anderson WF, Gillison ML: Incidence trends for human papillomavirus-related and -unrelated oral squamous cell carcinomas in the United States. J Clin Oncol 2008, 26:612−619
7. Chaturvedi AK, Engels EA, Pfeiffer RM, Hernandez BY, Xiao W, Kim E, Jiang B, Goodman MT, Sibug-Saber M, Cozen W, Liu L, Lynch CF, Wantzensen N, Jordan RC, Altekruse S, Anderson WF, Rosenberg PS, Gillison ML: Human papillomavirus and rising oropharyngeal cancer incidence in the United States. J Clin Oncol 2011, 29:4294−4301
8. O'Sullivan B, Lydiatt WM, Haughey BH, Brandwein-Gensler M, Glastonbury CM, Shah JP: HPV-mediated (p16+) oropharyngeal cancer. AJCC Cancer Staging Manual, ed 8. Edited by Amin MB. Switzerland, Springer Nature, AG, 2017. pp. 113−121
9. Abreu ALP, Souza RP, Gimenes F, Consolaro MEL: A review of methods for detect human papillomavirus infection. Virol J 2012, 9:262
10. Bishop JA, Lewis JS, Rocco JW, Faquin WC: HPV-related squamous cell carcinoma of the head and neck: an update on testing in routine pathology practice. Semin Diagn Pathol 2015, 32:344−351
11. Westra WH: Detection of human papillomavirus (HPV) in clinical samples: evolving methods and strategies for the accurate determination of HPV status of head and neck carcinomas. Oral Oncol 2014, 50:771−779
12. Jordan RC, Lingen MW, Perez-Ordonez B, He X, Pickard R, Koluder M, Jiang B, Wakely P, Xiao W, Gillison ML: Validation of methods for oropharyngeal cancer HPV status determination in US cooperative group trials. Am J Surg Pathol 2012, 36:945−954
13. Xu B, Ghossein R, Lane J, Lin O, Katabi N: The utility of p16 immunostaining in fine needle aspiration in p16-positive head and neck squamous cell carcinoma. Hum Pathol 2017, 54:193−200
14. Lewis JS, Chernock RD, Ma X-J, Flanagan JJ, Luo Y, Gao G, Wang X, El-Mofty SK: Partial p16 staining in oropharyngeal squamous cell carcinoma: extent and pattern correlate with human papillomavirus RNA status. Mod Pathol 2012, 25:1212−1220
15. Warner WA, Sanchez R, Dawoodian A, Li E, Momand J: Identification of FDA-approved drugs that computationally bind to MDM2. Chem Biol Drug Des 2012, 80:631−637
16. Kerr DA, Pitman MB, Sweeney B, Arpin RN, Wilbur DC, Faquin WC: Performance of the Roche cobas 4800 high-risk human papillomavirus test in cytologic preparations of squamous cell carcinoma of the head and neck. Cancer Cytopathol 2014, 122:167−174
17. Kerr DA, Sweeney B, Arpin RN, Ring M, Pitman MB, Wilbur DC, Faquin WC: Automated extraction of formalin-fixed, paraffin-embedded tissue for high-risk human papillomavirus testing of head and neck squamous cell carcinomas using the Roche cobas 4800 System. Arch Pathol Lab Med 2016, 140:844−848
18. Robinson M, Schache A, Sloan P, Thavaraj S: HPV specific testing: a requirement for oropharyngeal squamous cell carcinoma patients. Head Neck Pathol 2012, 6 Suppl 1:83−90
19. Gillison ML, Chaturvedi AK, Anderson WF, Fakhry C: Epidemiology of human papillomavirus-positive head and neck squamous cell carcinoma. J Clin Oncol 2015, 33:3235−3242
20. Herberhold S, Hellmich M, Panning M, Bartok E, Silling S, Akgül B, Weiland U: Human polyomavirus and human papillomavirus prevalence and viral load in non-malignant tonsillar tissue and tonsillar carcinoma. Med Microbiol Immunol 2017, 206:93−103
21. Jalaly JB, Lewis JS, Collins BT, Wu X, Ma XJ, Luo Y, Bernadt CT: Correlation of p16 immunohistochemistry in FNA biopsies with corresponding tissue specimens in HPV-related squamous cell carcinomas of the oropharynx. Cancer Cytopathol 2015, 123:723−731