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Hybrid Capture 2 is as Effective as PCR Testing for High Risk Human Papillomavirus in Head and Neck Cancers

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Abstract

High risk human papillomavirus (HPV) infection is a common cause of oropharyngeal squamous cell carcinoma, especially in young male nonsmokers. Accurately diagnosing HPV-associated oral cancers is important, because they have a better prognosis and may be treated differently than smoking-related oral carcinomas. Various methods have been validated to test for high risk HPV in cervical tissue samples and they are in routine clinical use to detect dysplasia before it progresses to invasive disease. Similarly, future screening for HPV-mediated oropharyngeal dysplasia may identify patients before it progresses. Our objective was to compare four of these methods in a retrospective series of 87 oral and oropharyngeal squamous cell carcinomas that had archived fresh-frozen and paraffin-embedded tissue for evaluation. Patient age, gender, smoking history, and tumor location were also recorded. DNA prepared from fresh-frozen tissue was tested for HPV genotypes by multiplex PCR analysis (*Diatherix*), and high risk HPV screening was done with Hybrid Capture 2 (*Qiagen hc2*) and Cervista (*Hologic*). Histologic sections were immunostained for p16 (*mtm/Roche)*. HPV positive outcome was defined as agreement between at least two of the three genetic tests and used for X^2 analysis and calculations of diagnostic predictive value. As expected, high risk HPV-positive oral cancers were most common in the tonsil and base of tongue (oropharynx) of younger male (55 years vs 65 years) (p=0.0002) non-smokers (p=0.01). Most positive cases were HPV16 (33/36, 92%). Hybrid Capture 2 and Cervista were as sensitive as PCR and had fewer false positives than p16 immunohistochemistry.

Keywords

HPV; Oropharyngeal; PCR; Hybrid Capture 2; Cervista; p16

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INTRODUCTION

It is well accepted that the human papillomavirus (HPV) causes squamous cell carcinoma of the cervix [1,2], anus [3], and many cases in the oropharynx [4-10]. Recent trends suggest that the recognition of invasive HPV-positive oropharyngeal cancers in the United States is increasing, and the number of invasive cervical cancers is decreasing, due to improved patient screening [6, 11]. Therefore, identifying accurate and *practical* high risk HPV assays to screen for oropharyngeal cancers is important; especially since these viral-mediated carcinomas may be treated differently and have significantly better prognoses compared with smoking related oral squamous cell carcinoma [12-15].

The clinical success of accurate and practical cervical dysplasia screening is responsible for the more than 50% drop in invasive cervical carcinoma prevalence in the past 50 years [11]. Liquid-based cytology and high risk HPV testing have significantly improved screening sensitivity, which is now approaching 99% [1]. In fact, a negative HPV test coupled with a negative cytology result provides sufficient negative predictive value to allow for screening every 3-5 years [1]. The frequency of subclinical high risk HPV infections in the oropharynx is currently unknown, but it is likely that methods developed for cervical screening will also be employed when screening for pre-invasive oropharyngeal dysplasia [16-22].

Currently only a few small pilot studies have been published testing the efficacy of FDA approved and widely used cervical HPV screening assays like Hybrid Capture 2 (*Qiagen hc2)* [19], or Cervista (*Hologic)* [20], in head and neck cancers. Therefore, our objective was to compare the predictive value of these tests with PCR and p16 in a cohort of 87 oral and oropharyngeal carcinomas.

MATERIALS AND METHODS

Head and neck squamous cell carcinoma samples

Using an IRB approved protocol, we retrospectively identified and retrieved Oregon Health & Science University archived cases of oral squamous cell carcinoma that had both fresh-frozen tissue for DNA extraction and paraffin-embedded tissue blocks for histologic sections. These selection criteria yielded 87 confirmed cases for analysis. Chart review recorded gender, age, and any reported smoking history.

Hybrid Capture 2 high risk HPV testing

A portion of each frozen tissue specimen (10mg) was used for DNA extraction for the Hybrid Capture 2 assay per manufacturer's instructions (Qiagen Digene, Valencia, CA). Briefly, this assay is an *in vitro* nucleic acid hybridization microplate assay to detect 13 high risk HPV genotypes (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68). It uses RNA probes targeted to these high risk genotypes and then an antibody against RNA:DNA hybrids conjugated to an alkaline phosphatase reporter. Cleavage of the chemiluminescent substrate results in light emission, which is measured using a luminometer. For each specimen, relative light unit/cutoff (RLU/CO) values are calculated as the ratio of the specimen luminescence relative to the average luminescence of 1.0 pg/ml of high risk HPV standard. A RLU/CO value of greater than 1.0 was considered a high risk HPV-positive

result. Less than 1.0 was a negative result. Any RLU/CO values from 1.0 to 2.5 were retested. Four positive and four negative controls were run with each experiment per clinical guidelines employed for routine cervical sample testing in our CLIA approved laboratory.

Cervista high risk HPV DNA testing

A separate portion of each frozen tissue sample (10mg) was suspended in Thinprep media (Hologic, Marlborough, MA), yielding a concentration of 4mg tissue/mL. Unfortunately, 31/44 of the oropharyngeal biopsies and two tongue biopsies did not have sufficient tissue for Cervista testing; therefore, only 54/87 of the available samples were screened for the 14 high risk HPV genotypes (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) according to the manufacturers' instructions (Hologic) using the Cervista platform and HPV Invader HR Molecular Assay *(*Hologic). Briefly, high risk HPV specific oligonucleotides cycle rapidly on and off the target DNA, creating substrate for the proprietary Cleavase enzyme (Hologic). The action of the enzyme results in production of cleaved 5′ oligonucleotide flaps, which subsequently bind a universal hairpin fluorescence resonance energy transfer oligonucleotide that creates a second substrate for the Cleavase enzyme. Cleavage of this bond yields a fluorescent signal measured with the Tecan Infinite Microplate Reader (Hologic), representing a positive result. Human histone 2 was assayed in each mixture as an internal positive control.

Multiplex PCR HPV genotyping

DNA was extracted from a portion of the frozen tissue (10 mg) using Trizol (Life Technologies) for PCR analysis of 21 high risk HPV genotypes (types 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 67, 68, 69, 70, 73, 82) and four low risk genotypes (types 6, 11, 42, 44). Genotyping was performed by *DIATHERIX Laboratories* (Huntsville, AL) using a published protocol [23]. Briefly, the nested PCR master mix contained type-specific primers for the E6/E7 region to amplify the 25 genotypes. An endogenous positive-control gene iduronate 2-sulfatase was also amplified as a DNA quality control for each sample. HPV types and the control gene were amplified in a single multiplex PCR reaction that labeled PCR products with biotin for purification. The genotypes were determined using a suspension array for multiplex detection on a Luminex 100 instrument (Luminex, Austin, TX) and sequence-tagged beads specific for each HPV type and control gene [iduronate 2-sulfatase] Genaco Biomedical Products, Inc.). The hybridization reaction was read by a Qiagen LiquidChip. Any bead fluorescing above a pre-determined threshold was considered to be bound to PCR product, indicating that particular template was present in the PCR reaction. Known positive and negative controls confirmed specificity.

P16 immunohistochemistry

Formalin-fixed and paraffin-embedded histologic sections of each tumor biopsy were immunostained for $p16^{INK4a}$ [24, 25] per manufacturer instructions (CINtec, mtm/Roche laboratories AG, Heidelberg, Germany) using a Ventana BenchmarkXT autostainer (Ventana Medical Systems, Tucson, AZ). Sections were counterstained with hematoxylin and scored as either positive or negative for at least focal (10% of tumor) strong diffuse

staining per recommended guidelines widely employed for HPV-mediated cervical cancer [26].

Statistical analysis

Cases were considered true positive for high risk HPV if at least two of the three genetic tests were positive (PCR, Hybrid Capture 2, or Cervista). The sensitivity, specificity, overall accuracy, and predictive value of each test were calculated using 2 × 2 contingency tables with binomial 95% confidence intervals. Associations between positive HPV test outcome and patient metrics (i.e., gender, smoking status) were evaluated by Chi-square analysis with post hoc correction for multiple comparisons. Relationships between patient age relative to gender, smoking status, and HPV-mediated cancer was determined by two-way analysis of variance (ANOVA) with Bonferroni post hoc correction.

RESULTS

87 oral and oropharyngeal squamous cell carcinoma cases were identified in our tissue bank that had both fresh frozen samples for DNA extraction and paraffin blocks for histologic confirmation by two surgical pathologists (JH and TM) and p16 immunohistochemical staining. The oral biopsies came from various sites, including the oropharynx (tonsils [n=25] and base of tongue [n=19]), larynx (n=19), and the remainder of the oral cavity (ie, floor of mouth [n=10], roof of mouth [n=6], and remainder of tongue [n=8]). Less than half of these archived cases were positive for high risk HPV in at least two of the genetic assays (36/87, 41%) (Table 1). Most of the high risk HPV cases were type 16 (33/36, 92%) and came from the oropharynx (tonsil or base of tongue) (Table 2), while the larynx, floor of mouth, tongue, and roof of mouth were only rarely positive for high risk HPV (total of 3/43, 7%) (Table 2).

We observed two potential false negatives by multiplex PCR testing, if one accepts positive agreement between Hybrid Capture 2 and Cervista as sufficient evidence for a positive high risk HPV infection (Table 1). However, these two cases were also negative for p16 immunostaining; therefore, we cannot exclude the possibility that Hybrid Capture 2 and Cervista results may instead be false positives. We elected to use the former rather than the later as gold standard outcomes for test accuracy analysis (Table 3), because p16 immunohistochemistry is known to be less reliable in head and neck cancers [14] and our results also show that p16 staining leads to both false positives (n=5/51, 10%) and false negatives (1-3/36, 3-8%) (Tables 1 and 2). In our laboratory, the overall test accuracy was lowest for p16 immunostaining (91%) compared with the other three high risk HPV tests (97-100%) (Table 3). The negative predictive value of a negative p16 test was more reliable (94 [95% CI: 83-99] than the positive predictive value (87 [72-96]), despite the high prevalence of high risk HPV (41%).

Similar to other recent studies [27], we observed an inverse relationship between patient age and positive high risk HPV status (Table 4). Positive cases were more likely to be younger males (n=30 cases; mean age 55 +/− 1.6 years) compared with HPV negative males (n=34; mean age 65 +/−1.9 years) (p=0.0002). HPV positive male non-smokers were also younger (53+/−2.6 years) compared with HPV negative male non-smokers (70+/−6.2) (p=0.01). In our sample of 23 females, there was no statistically significant relationship between positive

high risk HPV, patient age (65+/−3.6 versus 60+/−2.5 years) (p=0.31), or smoking status (p=0.59), but females tended to be less likely to be HPV positive (26% of cases versus 47% of males) (p=0.08), despite having the same frequency of oropharyngeal cancer (11/23 females versus 33/64 males).

DISCUSSION

Head and neck squamous cell carcinomas are the sixth most common type of cancer world-wide with approximately 600,000 cases reported each year. About 10% of these cases arise in the tonsil or base of tongue [6, 11]. Although smoking and alcohol use remain leading causes of oral cancer, high risk HPV infection is now recognized as the leading cause of oropharyngeal carcinoma, especially in younger non-smoking males [4, 12, 27].

It is interesting that carcinomas of the tonsil and base of tongue in particular, show the greatest prevalence of HPV compared with the low prevalence in other oral cavity carcinomas [28]. Indeed, recent studies suggest that one reason HPV may be more likely to cause cancer in the cervix, anus, and tonsillar ring is similarities in immature cells at the transformation zone between squamous and glandular mucosa at these locations [29].

Oropharyngeal cancers are usually diagnosed when there is a grossly visible lesion or metastatic disease [15], similar to methods used for cervical cancer before the pap smear cytology revolution of the last century. Not surprisingly then, outcomes in oropharyngeal cancers are generally less favorable when compared with cervical cancers, which are much more likely to be identified and treated while they are still *in situ.* To detect oropharyngeal cancer before it becomes invasive, it seems inevitable that clinicians will eventually utilize the same preemptive screening approach to the tonsillar ring that has proven to be so effective in preventing cervical malignancy.

Despite the growing number of requests for HPV testing of head and neck biopsies, many laboratories are uncertain about the best diagnostic methods for testing; especially when balancing accuracy with practical considerations such as test complexity and availability. Most CLIA approved clinical pathology laboratories use FDA approved and/or internally validated cervical HPV screening tests such as Hybrid Capture 2, or Cervista, to evaluate cervical pap smears. They then use indirect assays like p16 immunohistochemistry in cervical biopsies as a specific marker of HPV-mediated neoplastic transformation [24-26]. Nonetheless, most head and neck surgeons, managing patients with oral squamous cell carcinomas, request HPV confirmation by PCR analysis, which is a more complex test that is usually available in only specialized reference laboratories. Of course, sending biopsies to off-site reference laboratories affects cost, turnaround time, and complicates the logistics of sample-linked diagnostic reporting.

In our study, we compared the test accuracy of PCR to more widely available clinical HPV tests like Hybrid Capture 2, Cervista, and p16. In the 1990s and early 2000s, PCR, or *in situ* hybridization, were the detection methods of choice for HPV testing in cervical biopsies. However, after Hybrid Capture 2 was introduced as a less complicated assay [22], it soon replaced PCR and *in situ* tissue assays for routine clinical testing by nearly all clinical

pathology laboratories. Although a few laboratories may use *in situ* hybridization for HPV, or new multiplex PCR platforms, like the one employed in our study, most employ Hybrid Capture 2 (Qiagen, Digene) or Cervista (Hologic) for cervical HPV testing. Notably, an advantage of HPV *in situ* assays may be improved specificity for potential neoplastic transformation compared with the more sensitive PCR, or Hybrid Capture 2 assays [30, 31], but it is not as specific as p16 staining [32]. *In situ* hybridization is also unlikely to be used to screen for high risk HPV in future oropharyngeal cytologic studies [33]. Nonetheless, the ability of *in situ* hybridization to improve the positive predictive value for clinically significant HPV infections may be an advantage. Indeed, the problem with PCR, Hybrid Capture 2, and Cervista when screening cervical specimens is judging the clinical significance of a positive test.

Positive HPV tests are common while HPV-mediated carcinoma is uncommon [1, 2]. That is to say, many sexually active women and men are positive for high risk HPV, but the infections are most likely transient and eventually cleared by the immune system. Indeed, the prevalence of high risk HPV is so high in young sexually active women that mandatory HPV co-testing is not recommended until after age 29 [1]. As many as 66% of atypical cervical pap smears are positive for high risk HPV, while the frequency of squamous cell carcinoma is less than 0.5% [2]. The prevalence of high risk HPV infection in oropharyngeal pap smears from the general population is currently unknown, but we suspect there will be similar positive predictive value problems due to transient non-neoplastic infections. Moreover, this problem of poor positive predictive value will be further exacerbated as vaccines decrease the prevalence of high risk viral infection in both males and females [34]. Negative predictive values will remain excellent, but positive HPV test results will be even less likely to be accurate and/or clinically relevant.

This could be especially true when using very sensitive assays like PCR. In fact, others have shown that about 15% of oropharyngeal carcinomas that are positive for high risk HPV by PCR have low viral loads and are negative for neoplastic E6/E7 expression [14]. Our data did not reveal any cases positive for PCR that were not also considered positive by either Hybrid Capture 2 or Cervista. However, we did have two unexpected cases that were negative by multiplex PCR and were positive by these other two clinical tests. Thus, either our PCR results were false negatives, or the other two tests were false positives. Internal controls were intact for both PCR and Cervista tests in both cases, which suggest the discrepancy was not a technical issue. One possible explanation could be disruption of the HPV L1 gene during integration into the host cell's genome [13] affecting the PCR primer site(s) in these two cases. This would not be entirely unusual, but would require essentially no intact episomal HPV DNA in these two samples. We did not sequence these two cases; therefore, we consider the results in these two cases not entirely conclusive despite including them as two false negatives in our overall accuracy calculations. Regardless of these two cases, however, our data suggest that commonly employed clinical tests used to screen for high risk HPV in cervical pap smears like Hybrid Capture 2 and Cervista have very similar overall accuracy as PCR. No cases positive by PCR were negative by Hybrid Capture 2.

In cervical biopsies, p16 immunohistochemistry is often used to improve the positive predictive value for neoplastic transformation and diagnose carcinoma at least *in situ*

[24-26]. This appears to be generally true for cervical biopsies because the cause of neoplastic transformation in the cervix is almost always related to persistent high risk HPV infections leading to genomic integration of the virus and E6/E7 oncogenic upregulation of indirect markers like p16 [24]. P16 immunostained pap smears are currently being developed to improve their positive predictive value and reduce the number of unnecessary surgical biopsies [35, 36], but this assay has not yet been approved for routine clinical testing. In addition, it is important to recognize that oropharyngeal cancers [14] and other types of carcinoma [37] may show diffuse strong p16 staining *independent* of HPV infection. Our study and others [14] have shown false positive p16 rates ranging from 10-15%. This is most likely because p16 is a tumor suppressor gene that may be upregulated in any cancer, but is nearly always upregulated in HPV-mediated carcinomas [24]. Another possibility may be variability in "scoring" p16 as either positive or negative. One recent study suggested that an optimal cut-off point of 35% staining of the tumor for p16 may provide the best sensitivity and a specificity [14]. However, criteria already established for scoring p16 stained cervical biopsies, such as at least focal diffuse full thickness staining [26], appeared to provide excellent negative predictive value in our study. When employing p16 immunohistochemistry in oropharyngeal cancers, in the absence of concurrent HPV testing, it seems best to rely on its negative predictive value, which in our hands using a 10% staining cutoff was 94% [95% CI: 83-99]. The positive predictive value for high risk HPV may be as low as 72% in our sample; therefore, in our opinion, p16 staining should not be the sole test to determine HPV status, or to guide clinical management.

Our data support the hypothesis that high risk HPV may reliably be detected by Hybrid Capture 2, or Cervista, compared with PCR testing in fresh tissue samples of head and neck squamous cell carcinomas. Our analysis of 87 cases is the largest to date testing the efficacy of Hybrid Capture 2 [19], or Cervista [20] in oral carcinomas. It is also the first to our knowledge to directly compare Hybrid Capture 2 with Cervista in oral carcinomas. Unfortunately many of our tonsillar biopsies had insufficient tissue for all three genetic tests and Cervista testing was not performed in those cases (33/87). Because the remaining cases had a lower prevalence of high risk HPV (fewer tonsil cases), the negative predictive value reported for Cervista may be biased. Nonetheless, there was complete agreement between Cervista and Hybrid Capture 2 in all 54 co-testing cases. In addition, we did not have liquid-based cytologic specimens to compare with our fresh-frozen tissue samples. This comparison should be done in future prospective studies, since clinical screening will likely use cytologic brushings to test the tonsillar ring for high risk HPV.

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REFERENCES

1. Saslow D, Solomon D, Lawson HW, et al. American cancer society, american society for colposcopy and cervical pathology, and american society for clinical pathology screening guidelines for the prevention and early detection of cervical cancer. Am J Clin Pathol. Apr; 2012 137(4):516–42. [PubMed: 22431528]
2. Sherman M, Lorincz A, Scott D, Wacholder S, Castle P, Glass A, Mielzynska-Lohnas I, Rush B, Schiffman M. Baseline cytology, human Papillomavirus testing, and risk for cervical neoplasia: a 10-year cohort analysis. J Natl Cancer Inst. 2003; 95(1):46–52. [PubMed: 12509400]
3. Pirog EC, Quint KD, Yantiss RK, Pirog EC, Quint KD, Yantiss RK. P16/CDKN2A and Ki-67 enhance the detection of anal intraepithelial neoplasia and condyloma and correlate with human papillomavirus detection by polymerase chain reaction. Am J Surg Pathol. 2010; 34(10):1449–55. [PubMed: 20871219]
4. Gillison ML, Broutian T, Pickard RK, et al. Prevalence of oral HPV infection in the United States, 2009-2010. JAMA. 2012; 307:693–703. [PubMed: 22282321]
5. Sudhoff HH, Schwarze HP, Winder D, et al. Evidence for a causal association for HPV in head and neck cancers. Eur Arch Otorhinolaryngol. 2011; 268:1541–1547. [PubMed: 21792686]
6. Ramqvist T, Dalianis T. Oropharyngeal cancer epidemic and human papillomavirus. Emerg Infect Dis. 2010; 16:1671–1676. [PubMed: 21029523]
7. Fakhry C, Rosenthal BT, Clark DP, Gillison ML. Associations between oral HPV16 infection and cytopathology: evaluation of an oropharyngeal “Pap-test equivalent” in high-risk populations. Cancer Prev Res (Phila). Sep; 2011 4(9):1378–84. [PubMed: 21836021]
8. Charfi L, Jouffroy T, de Cremoux P, et al. Two types of squamous cell carcinoma of the palatine tonsil characterized by distinct etiology, molecular features, and outcome. Cancer Lett. 2008; 260:72–78. [PubMed: 18060686]
9. Sedaghat AR, Zhang Z, Begum S, et al. Prognostic significance of human papillomavirus in oropharyngeal squamous cell carcinomas. Laryngoscope. 2009; 119:1542–1549. [PubMed: 19522004]
10. Klussman JP, Mooren JJ, Lehnen M, et al. Genetic signatures of HPV-related and unrelated oropharyngeal carcinoma and their prognostic implications. Clin Cancer Res. 2009; 15:1779–1786. [PubMed: 19223504]
11. U.S. Cancer Statistics Working Group. United States Cancer Statistics: 1999-2008 Incidenceand Mortality Web-based Report. Department of Health and Human Services,Centers for Disease Control and Prevention, and National Cancer Institute; Atlanta (GA): 2012. Available at: http://www.cdc.gov/uscs
12. Andrews E, Seaman W, Webster-Cyriaque J. Oropharyngeal carcinoma in non-smokers and non-drinkers: a role for HPV. Oral Oncology. 2009; 45:486–491. [PubMed: 19027350]
13. Jo S, Juhasz A, Zhang K, et al. Human papillomavirus infection as a prognostic factor in oropharyngeal squamous cell carcinomas treated in a prospective phase II clinical trial. Anticancer Res. 2009; 29:1467–1474. [PubMed: 19443352]
14. Sedaghat AR, Zhang Z, Begum S, et al. Prognostic significance of human papillomavirus in oropharyngeal squamous cell carcinomas. Laryngoscope. 2009; 119:1542–1549. [PubMed: 19522004]
15. Joo Y-H, Cho K-J, Park J-O, et al. Factors influencing the outcomes of primary surgery with postoperative radiotherapy for pN2 oropharyngeal squamous cell carcinoma. Oral Oncol. 2012; 48:90–94. [PubMed: 21924946]
16. Kingma DW, Allen RA, Caughron SK, et al. Comparison of molecular methods for detection of HPV in oral and oropharyngeal squamous cell carcinoma. Diagn Mol Pathol. 2010; 19:218–223. [PubMed: 21051999]
17. Agoston ES, Robinson SJ, Mehra KK, et al. Polymerase chain reaction detection of HPV in squamous carcinoma of the oropharynx. Anatomic Pathology. 2010; 134:36–41.

18. Jordan RC, Lingen MW, Perez-Ordonez B, et al. Validation of methods for oropharyngeal cancer HPV status determination in US Cooperative Group trials. Am J Surg Path. 2012; 36(7):945–954. [PubMed: 22743284]
19. Bishop JA, Maleki Z, Valsamakis A, et al. Application of the Hybrid Capture 2 assay to squamous cell carcinomas of the head and neck. Cancer Cytopath. 2012; 120:18–25.
20. Charalambos CS, Bibbo M, Wang Z. Assessment of fine needle aspiration specimen adequacy for high risk HPV detection and genotyping in oropharyngeal squamous cell carcinoma. Acta Cytologica. 2012; 56:196–198. [PubMed: 22378084]
21. Hoffman M, Ihloff AS, Gorogh T, et al. $p16_{INK4A}$ overexpression predicts translational active human papillomavirus infection in tonsillar cancer. Int J Cancer. 2010; 127:1595–1602. [PubMed: 20091864]
22. Clavel C, Masure M, Putaud I, et al. Hybrid capture II, a new sensitive test for human papillomavirus detection. Comparison with hybrid capture II and PCR results in cervical lesions. J Clin Pathol. 1998; 51:737–740. [PubMed: 10023335]
23. Han J, Swan D, Smith S, et al. Simultaneous amplification and identification of 25 human papillomavirus types with Templex technology. J. Clin Microbiol. 2006; 44(11):4157–62. [PubMed: 17005760]
24. Sano T, Oyama T, Kashiwabara K, Fukuda T, Nakajima T. Expression status of p16 protein is associated with human Papillomavirus oncogenic potential in cervical and genital lesions. Am J Pathol. Dec; 1998 153(6):1741–8. [PubMed: 9846965]
25. Keating J, Cviko A, Riethdorf S, Riethdorf L, Quade B, Sun D, Duensing S, Sheets E, Munger K, Crum C. Ki-67, cyclin E, and p16INK4 are complimentary surrogate biomarkers for human papilloma virus-related cervical neoplasia. Am J Surg Pathol. 2001; 25(7):884–91. [PubMed: 11420459]
26. Shroyer, K.; Chivukula, M.; Ronnett, B.; Morgan, T. CINtec p16 Cervical Histology Compendium & Staining Atlas. Mtm labs; 2011.
27. van Monsjou HS, van Velthuysen MLF, van den Brekel MWM, et al. Human papillomavirus status in young patients with head and neck squamous cell carcinoma. Int J Cancer. 2012; 130:1806–1812. [PubMed: 21607949]
28. Machado J, Reis PP, Zhang T, et al. Low prevalence of human papillomavirus in oral cavity carcinomas. Head Neck Oncol. 2010; 2:1–6. [PubMed: 20157425]
29. Herfs M, Yamamoto Y, Laury A, et al. A discrete population of squamocolumnar junction cells implicated in the pathogenesis of cervical cancer. Proc Natl Acad Sci U S A. Jun 26; 2012 109(26):10516–21. [PubMed: 22689991]
30. Guo M, Gong Y, Deavers M, et al. Evaluation of a commercialized in situ hybridization assay for detecting human papillomavirus DNA in tissue specimens from patients with cervical intraepithelial neoplasia and cervical carcinoma. J Clin Microbiol. 2008; 46(1):274–80. [PubMed: 17977987]
31. Schlecht N, Brandwein-Gensler M, Nuovo G, et al. A comparison of clinically utilized human papillomavirus detection methods in head and neck cancer. Mod Pathol. 2011; 24:1295–1305. [PubMed: 21572401]
32. Kong C, Balzer B, Troxell M, et al. p16 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]
33. Alameda F, Marinoso M, Bellosillo B, et al. Detection of HPV by in situ hybridization in thin-layer (ThinPrep) cervicovaginal samples. Tumour Biol. 2011; 32(3):603–9. [PubMed: 21302019]
34. Kim J. Weighing the benefits and costs of HPV vaccination of young men. NEJM. 2011; 364(5): 393–5. [PubMed: 21288093]
35. Schmidt D, Bergeeron C, Denton K, Ridder R. p16/Ki-67 Dual-stain cytology in the triage of ASCUS and LSIL Papanicolaou cytology. Cancer Cytopathology. 2011; 119:158–166. [PubMed: 21442767]
36. Waldstrom M, Christensen R, Ornskov D. Evaluation of p16/Ki-67 dual stain in comparison with an mRNA HPV test on liquid-based cytology samples with LSIL. Cancer Cytopathology. 2013; 121:136–45. [PubMed: 22987560]

37. Steinestel J, Cronauer MV, Müller J, et al. Overexpression of $p16^{INK4a}$ in urothelial carcinoma in situ is a marker for MAPK-mediated epithelial-mesenchymal transition but is not related to human papillomavirus infection. PLoS ONE. 2013; 8(5):e65189. [PubMed: 23724131]

Table 1

High risk HPV positive specimens by testing method.

Specimen	Location	PCR	Hybrid Capture	Cervista	P16	HPV Agreement	HPV type
1	Tonsil	Positive	Positive	ND	Positive	Positive	16
2	Tonsil	Positive	Positive	ND	Positive	Positive	16
3	Tonsil	Positive	Positive	ND	Positive	Positive	16
4	Tonsil	Positive	Positive	ND	Positive	Positive	16
5	Tonsil	Positive	Positive	ND	Positive	Positive	16
6	Tonsil	Positive	Positive	ND	Positive	Positive	16
7	Tonsil	Positive	Positive	ND	Positive	Positive	16
8	Tonsil	Positive	Positive	ND	Positive	Positive	16
9	Tonsil	Positive	Positive	ND	Positive	Positive	16
10	Tonsil	Positive	Positive	ND	Positive	Positive	16
11	Tonsil	Positive	Positive	ND	Positive	Positive	16
12	Tonsil	Positive	Positive	ND	Positive	Positive	16
13	Tonsil	Positive	Positive	ND	Positive	Positive	16
14	Tonsil	Positive	Positive	ND	Positive	Positive	16
15	Tonsil	Positive	Positive	ND	Positive	Positive	16
16	Tonsil	Positive	Positive	ND	Positive	Positive	16
17	Tonsil	Positive	Positive	ND	Positive	Positive	16
18	Tonsil	Positive	Positive	ND	Positive	Positive	16
19	Tonsil	Positive	Positive	ND	Positive	Positive	33
20	Tonsil	Positive	Positive	Positive	Positive	Positive	35
21	Tonsil	Negative^	Positive	Positive	Negative	Positive	16/18
22	BOT	Positive	Positive	ND	Positive	Positive	16
23	BOT	Positive	Positive	ND	Positive	Positive	16
24	BOT	Positive	Positive	ND	Positive	Positive	16
25	BOT	Positive	Positive	ND	Positive	Positive	16
26	BOT	Positive	Positive	ND	Positive	Positive	16
27	BOT	Positive	Positive	ND	Positive	Positive	16
28	BOT	Positive	Positive	ND	Positive	Positive	16
29	BOT	Positive	Positive	ND	Positive	Positive	16
30	BOT	Positive	Positive	ND	Positive	Positive	16
31	BOT	Positive	Positive	ND	Positive	Positive	16
32	BOT	Positive	Positive	ND	Positive	Positive	16
33	BOT	Positive	Positive	ND	Positive	Positive	16
34	Tongue	Positive	Positive	Positive	Positive	Positive	16
35	ROM	Positive	Positive	Positive	Negative	Positive	16
36	Larynx	Negative^	Positive	Positive	Negative	Positive	51/56

BOT: base of tongue; ROM: roof of mouth; ND: no data due to insufficient DNA for diagnosis. 36/87 (41%) met criteria for HPV agreement between at least two tests; almost all were from the oropharynx.

^Two cases were negative by PCR, but positive by Hybrid Capture and Cervista (types 16/18 and 51/56 genotypes from Cervista (mix 1 and mix 3 positive results, respectively). 51/87 (59%) cases were negative by all four assays, but most of these were not oropharyngeal.

Table 2

High risk HPV results by tumor location and testing method.

	High risk HPV testing method			
Site	**PCR Positive**	**Hybrid Capture 2**	**Cervista**	**p16 Immunohistochemistry**
Oropharynx (n=44)	32/44 (73%)	33/44 (75%)	5/13^	35/44 (80%)
Larynx (n=19)	0/19	1/19 (5%)	1/19 (5%)	1/19 (5%)
Floor of mouth (n=10)	0/10	0/10	0/10	1/10 (10%)
Tongue (n=8)	1/8 (13%)	1/8 (13%)	0/6^	1/8 (13%)
Roof of mouth (n=6)	1/6 (17%)	1/6 (17%)	1/6 (17%)	0/6 (0%)

PCR, Hybrid Capture 2, and p16 immunohistochemistry were available for all 87 cases.

^However there was insufficient sample for Cervista testing in 31 oropharyngeal (19 tonsils and 12 base of tongue) and two distal tongue cancer cases. All positive Cervista cases were also positive by Hybrid Capture 2.

Table 3

Test characteristics with 95% confidence intervals relative to gold standard high risk HPV outcome.

High Risk HPV Test	Sensitivity [95% CI]	Specificity [95% CI]	Overall Test Accuracy	Negative Predictive Value [95% CI]	Po sitive Predictive Value [95% CI]
Multiplex PCR	94 [81-96]	100 [93-100]	97%	96 [87-99]	100 [90-100]
Hybrid Capture 2	100 [90-100]	100 [93-100]	100%	100 [93-100]	100 [90-100]
Cervista^	100 [59-100]	100 [92-100]	100%	100 [92-100]	100 [59-100]
P16 Immunostaining	92 [78-98]	90 [79-97]	91%	94 [83-99]	87 [72-96]

HPV testing was considered “gold standard” positive if at least two out of three high risk HPV genetic testing methods agreed. Cases were considered negative if PCR, Hybrid Capture 2, and Cervista testing were negative. This yielded 36 positive and 51 negative outcomes for comparison with each individual test. The prevalence of high risk HPV in this cohort was 41% (36/87), because of selection bias based on increased numbers or oropharyngeal squamous cell carcinomas (44/87) in our sample.

^33/87 samples were not tested by Cervista due to insufficient tissue for analysis. Overall accuracy is the weighted average of the test’s sensitivity and specificity relative to the prevalence of high risk HPV in the sample.

Table 4

Subject demographics related to high risk HPV status in head and neck squamous cell carcinoma.

	Mean age (range)	Gender		Smoking	
HPV		**Male**	**Female**	**Smoker**	**Non-smoker**
Positive (n=36)	57 (31-74)**	30/64 (47%)	6/23 (26%)	19/36 (53%)	17/36 (47%)*
Negative (n=51)	63 (44-89)	34/64 (53%)	17/23 (74%)^	38/51 (75%)	13/51 (25%)

HPV testing was considered positive if at least two out of four high risk HPV testing methods agreed. Cases were considered negative if PCR, Hybrid Capture 2, and Cervista testing were negative.

*X^2 p-value=0.25;

**two-way ANOVA p-value=0.003.

^X^2 p-value=0.08.