Imaging, Diagnosis, Prognosis

Clinical Cancer Research

Evaluation of Human Papilloma Virus Diagnostic Testing in Oropharyngeal Squamous Cell Carcinoma: Sensitivity, Specificity, and Prognostic Discrimination

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Abstract

Purpose: Human papillomavirus-16 (HPV16) is the causative agent in a biologically distinct subset of oropharyngeal squamous cell carcinoma (OPSCC) with highly favorable prognosis. In clinical trials, HPV16 status is an essential inclusion or stratification parameter, highlighting the importance of accurate testing.

Experimental Design: Fixed and fresh-frozen tissue from 108 OPSCC cases were subject to eight possible assay/assay combinations: p16 immunohistochemistry (p16 IHC); *in situ* hybridization for high-risk HPV (HR HPV ISH); quantitative PCR (qPCR) for both viral E6 RNA (RNA qPCR) and DNA (DNA qPCR); and combinations of the above.

Results: HPV16-positive OPSCC presented in younger patients (mean 7.5 years younger, $P = 0.003$) who smoked less than HPV-negative patients ($P = 0.007$). The proportion of HPV16-positive cases increased from 15% to 57% ($P = 0.001$) between 1988 and 2009. A combination of p16 IHC/DNA qPCR showed acceptable sensitivity (97%) and specificity (94%) compared with the RNA qPCR "gold standard", as well as being the best discriminator of favorable outcome (overall survival $P = 0.002$). p16 IHC/HR HPV ISH also had acceptable specificity (90%) but the substantial reduction in its sensitivity (88%) impacted upon its prognostic value ($P = 0.02$). p16 IHC, HR HPV ISH, or DNA qPCR was not sufficiently specific to recommend in clinical trials when used in isolation.

Conclusions: Caution must be exercised in applying HPV16 diagnostic tests because of significant disparities in accuracy and prognostic value in previously published techniques. *Clin Cancer Res; 17(19); 6262–71. ©2011 AACR.*

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth commonest cancer worldwide, accounting for approximately 4% of all tumors (1). Despite changes in behavioral exposure to traditional risk factors for HNSCC (2, 3), the incidence of oropharyngeal squamous cell carcinoma (OPSCC) has shown a steady rise over recent years (4, 5). Figures from the Scottish Cancer Registry suggest that OPSCC shows the most rapidly rising incidence of any anatomic tumor site, exceeding that of both cutaneous melanoma and adenocarcinoma of esophagus (6). In 2009, the International Agency for Research on Cancer recognized human papillomavirus type 16 (HPV16) to be the causal agent in OPSCC (7). However, epidemiologic evidence from several countries points to marked variations in the extent to which HPV16 is involved in OPSCC (8–11).

HPV status has been shown to be an important prognostic biomarker in OPSCC (12, 13) with a HR for overall survival around 0.4 from systematic reviews of clinical trials (14). Understandably, calls have been made to standardize the definitions and clarify the best test or combination of tests for accurate diagnosis (15). Currently, a variety of detection methods are available (16), each with specific benefits and detractions. In addition, considerable

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Translational Relevance

Oropharyngeal squamous cell carcinoma (OPSCC) caused by human papillomavirus-16 (HPV16) has distinct behavior and prognosis; hence, clinical trials specifically for these cases are now being developed. The accuracy of allocation of tumors to either HPV-positive or -negative categories is of immediate clinical impact. A cohort of OPSCC patients was subject to a variety of diagnostic tests, using both fresh-frozen and fixed tissue samples to determine sensitivity, specificity, and prognostic discrimination. A combination of p16 immunohistochemistry (p16 IHC)/DNA quantitative PCR (qPCR) showed acceptable sensitivity (97%) and specificity (94%) as well as being the best discriminator of favorable outcome (overall survival = 0.002). p16 IHC/*in situ* hybridization for high-risk HPV (HR HPV ISH) also had acceptable specificity (90%), but the substantial reduction in its sensitivity (88%) impacted upon its prognostic value ($P = 0.02$). p16 IHC, HR HPV ISH, or DNA qPCR was not sufficiently specific to be used in isolation as diagnostic tests. These are the first U.K. HPV16 OPSCC data available, showing a dramatic increase in the role for HPV from 14% to 57% over 2 decades.

variation in sensitivity and specificity exists between the tests defining HPV status (17), such that the utility of some has been questioned.

In the head and neck oncology clinic, the introduction of HPV testing is justified to provide a more representative prognosis to the patient, and in this context we will investigate the relative efficiency of various tests in predicting survival. Another important application of widespread HPV testing is to facilitate recruitment into clinical trials. New trials are now being developed to exploit the evident contrast in biology of HPV-positive cases compared with conventional smoking- and alcohol-derived HNSCCs. Where de-escalation of therapy is the motivation behind a trial, the hazards to patient safety of inaccurately assigning HPV-negative tumors to an HPV-positive category are clear. We hypothesize that a "gold standard" test, that is, most reliable way to detect a biologically relevant HPV infection (18), is the detection of viral mRNA expression carried out by quantitative PCR (qPCR) techniques on fresh-frozen–derived samples. Although this gold standard may prove valuable in a research setting, it would be logistically difficult to introduce into a routine pathology service where diagnostic algorithms are based on the assessment of formalin-fixed, paraffin-embedded (FFPE) tissue. Against this standard, we will therefore compare the prognostic ability and sensitivity/specificity of 7 other detection methods or combinations: p16 immunohistochemistry (p16 IHC); high-risk HPV *in situ* hybridization (HR HPV ISH); p16 IHC/HR HPV ISH combination; DNA qPCR; p16 IHC/DNA qPCR combination; DNA/RNA qPCR, and p16 IHC/RNA qPCR combination.

The analysis of OPSCC cases with individual diagnostic tests is not new; the application of a comprehensive diagnostic test panel to OPSCC samples is novel and offers to define the standard for HPV-positive OPSCC diagnostic testing. In addition, this research will provide data clarifying the role of HPV16 from a cohort of OPSCC patients from the United Kingdom, a region for which no published prognostic data currently exist.

Materials and Methods

Patients and clinical specimens

A retrospective analysis of tissue bank records was made for all cases of OPSCC between 1988 and 2009 with available tissue samples.

All samples were collected after informed consent under previously granted ethical approval (South Sefton Research Ethics Committee, EC.47.01-6; North West 5 Research Ethics Committee, EC.09.H1010.5) from individuals treated in the Liverpool H&N Oncology Service, a multidisciplinary unit serving a geographically stable population of approximately 2 million individuals within Merseyside and Cheshire, United Kingdom. One hundred and eight cases of OPSCC were identified, all of which adhered to strict tumor site classification (19) made at the time of diagnosis and entry to the tissue bank. Cases that could not be reliably designated as squamous cell carcinoma from oropharyngeal sites were excluded. Cases with banked tissue originated from 3 distinct time periods: 1988–1997, 2004–2007, and 2008–2009.

Tissue samples included fresh-frozen tumor specimens (stored at −80°C; $n = 100$) and FFPE tissue blocks ($n = 97$). Case notes, electronic patient records, pathology reports, and the results of a U.K. Office of National Statistics (ONS) database search were reviewed to collate demographic details (age at diagnosis, gender, subsite of tumor, history of tobacco smoking, alcohol consumption, and nodal stage) and clinical outcomes (disease-specific survival and overall survival). All patients from whom samples were derived received surgery and, where necessary, adjuvant radiotherapy/chemoradiotherapy.

Tissue microarray preparation

Tissue microarrays (TMA) were constructed from FFPE tissue blocks of OPSCC, using a manual tissue arrayer (MTA-I; Beecher Instruments), as previously described (20). Briefly, hematoxylin and eosin–stained sections were used to identify areas of tumor and normal mucosa in the donor block. Three tumor cores and 1 matched normal mucosal core (height = 4 mm; diameter = 0.6 mm) were transferred from the donor block to the recipient block by a predetermined asymmetrical distribution. Hematoxylin and eosin–stained sections of the TMAs were examined to confirm accurate sampling.

DNA/RNA extraction and cDNA synthesis

The AllPrep DNA/RNA Mini Kit (Qiagen) was used to purify genomic DNA and total RNA simultaneously from

fresh-frozen tissue samples (2 mm^3). All tissue preparation was conducted in a class II biological safety cabinet with new sterile disposable consumables for each specimen to avoid cross-contamination. An in-column RNase-free DNase (Qiagen) treatment was incorporated in the RNA extraction protocol to eliminate DNA carryover in the RNA preparations. The purified DNA and RNA samples were quantified by spectrophotometry with NanoDropTM 1000 (Thermo Fischer Scientific). Total RNA (500–600 ng) was used for cDNA synthesis, using QuantiTect Reverse Transcription Kit (Qiagen). This process included a further genomic DNA elimination step prior to reverse transcription. As a final quality control step, cDNA samples were amplified with primers for 2 microsatellites at 9p21 and 17p13 loci (21) to ensure an absence of genomic DNA contamination, which was confirmed in all cases.

Quantification of tumor cell proportion within fresh-frozen samples was undertaken to ensure that extracted DNA/RNA was representative of tumor rather than surrounding stroma or inflammatory cells. The analysis of FFPE slides corresponding to frozen samples of 20 randomly selected cases was conducted.

High-risk HPV detection (HPV16, -18, and -33)

HPV16 E6 DNA qPCR. Primers and an FAM-MGB–labeled TaqMan probe were designed (synthesized by Applied Biosystems) and optimized to specifically amplify the HPV16 E6 region (sequence and PCR conditions listed in Table 1). Commercially available primers and a VIC-TAMRA–labeled probe for the single-copy gene *RNase P* (TaqMan RNase P Control Reagents, Applied Biosystems) were used as an endogenous reference in each multiplex reaction. A total reaction volume of 25 μL in each reaction contained 1× TaqMan Gene Expression Master Mix (Applied Biosystems), 500 nmol/L of each primer, 250 nmol/L of probe, 1× RNase P primer/probe mix, and 100 ng genomic DNA. Real-time PCR reactions were done in duplicate for all samples on an Applied Biosystems 7500 FAST system. The HPV16-positive cervical cancer cell lines CaSki (U.K. Health Protection Agency Culture Collections—87020501) and SiHa (ATCC-LGC-HTB-35) were used as positive controls and as calibrators for the assay. Normal bronchial epithelial lung cell line DNA was used as a negative control in each 96-well plate.

The detection threshold for HPV-positive status was set in accordance with the previously reported frequency of *E6* gene copies per diploid genome for CaSki (869 copies; ref. 22). Assuming an HPV16-driven tumor is composed of a dominant clonal population of cells, we scored as positive those samples with 1 or more *E6* gene copy/diploid genome. A sample was only deemed positive if the threshold was met in both of the duplicate runs.

HPV16 E6 RNA qPCR. Duplicate real-time RNA (cDNA) PCR reactions were carried out with the same primer and probe combinations for the HPV16 *E6* gene under identical conditions, as detailed above, on the Applied Biosystems 7500 FAST System.

Primers and an FAM-MGB–labeled TaqMan probe were designed to specifically amplify the HPV18 E6 region (synthesized by Applied Biosystems) and optimized with the cervical cell line HeLa (UK Health Protection Agency Culture Collections—93021013). Primer and probe sequences and PCR conditions are listed in Table 1. A commercially available HVP33 detection system (Primer Design HPV33 Kit) was used to analyze HPV33 *E6* gene expression. Both HPV18 and HPV33 expression analyses were conducted on the Applied Biosystems 7500 FAST System.

Human VIC-MGB–labeled ACTB primers and probe (Applied Biosystems) were incorporated as an endogenous control to facilitate internal normalization and relative gene expression quantification for all expression assays. The mean of duplicate reactions was used to calculate relative gene expression by the $2^{-\Delta\Delta CT}$ method (23).

p16 immunohistochemistry. p16 IHC was carried out with a proprietary kit (CINtec Histology; mtm laboratories AG) on a Ventana Benchmark autostainer (Ventana Medical Systems Inc.). A tonsil squamous cell carcinoma with high p16 expression was used as a positive control. The primary antibody was omitted from negative controls. p16 IHC was scored as positive if there was strong and diffuse nuclear and cytoplasmic staining present in greater than

Table 1. Primer and probe sequences and PCR conditions

Target	Primer and probe sequences		
	Forward sequence	Reverse sequence	Probe sequence
HPV16 E6	CTGCGACGTGAGGTATATGACTTT	ACATACAGCATATGGATTCCCATCT	CTTTTCGGGATTTATGC
HPV18 E6	AAACCGTTGAATCCAGCAGAA	GTCGTTCCTGTCGTGCTCG	TTGCAGCACGAATGG

	PCR conditions	
	Melting time	Anneal/Extension time
HPV16 E6	95°C 15 s	61°C 60 s
HPV18 E6	95°C 15 s	60°C 60 s

70% of the malignant cells (23). All other staining patterns were scored as negative.

***High-risk HPV* in situ *hybridization*.** HR HPV ISH was carried out using proprietary reagents [Inform HPV III Family 16 Probe (B); Ventana Medical Systems Inc.] on a Benchmark Autostainer (Ventana Medical Systems Inc.). The Inform HPV III Family 16 Probe (B) detects high-risk genotypes HPV16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, and -66. Three control samples were used: FFPE CaSki cells (HPV16-positive; 400–600 copies per cell), HeLa cells (HPV18 positive; 10–50 copies per cell), and C-33A (HPV negative; Ventana Medical Systems Inc.). The HR HPV ISH test was scored as positive if there was any blue reaction product that colocalized with the nuclei of malignant cells (24).

Interpretation of HPV tests. The p16 IHC and HR HPV ISH tests were assessed independently by 2 head and neck pathologists, using a binary classification (positive vs. negative). The results were collated, and discordant scores were resolved at a meeting between the pathologists to establish a consensus interpretation.

Statistical analysis

Kaplan–Meier estimates were used to show both disease-specific and overall survival by HPV status as defined by our gold standard, where only those positive in duplicate runs of RNA qPCR were deemed as reliably diagnosed HPV16-driven OPSCC. The χ^2 and Kruskal–Wallis tests were used for comparison of demographic and tumor-specific features between periods of sample collection and HPV-positive and -negative subgroups. Kaplan–Meier estimates and sensitivity and specificity of 7 alternative tests (p16 IHC; HR HPV ISH; DNA qPCR; and combined analysis tests: p16 IHC/HR HPV ISH; p16 IHC/DNA qPCR; DNA/RNA qPCR; and p16 IHC/RNA qPCR) were carried out. The log-rank (Mantel–Cox) test was used for comparison between survival curves according to each of the detection methods. Disease-specific survival was defined as death from or due to OPSCC, and overall survival was defined as death from any cause. Both disease-specific survival and overall survival were calculated at 36 months follow-up beyond the date of initial diagnosis.

Table 2. Cohort characteristics by time period of collection

	Time period			Overall	Statistical significance
	1988–1997	2004–2007	2008–2009	1998–2009	
Patient/tumor data					
No. of patients	40 (37%)	37 (34%)	31 (29%)	108 (100%)	
Age at diagnosis, y					
Mean	60.2	57.2	57.8	58.5	NS
Median	61.7	56.8	58.7	58.6	
Sex					
Female	8 (20%)	10 (27%)	7 (23%)	25 (23%)	NS
Male	32 (80%)	27 (73%)	24 (77%)	83 (77%)	
Tumor site					
Tonsil	22 (55%)	17 (46%)	20 (64%)	59 (55%)	NS
Soft palate	8 (20%)	7 (19%)	3 (10%)	18 (17%)	
Base of tongue	7 (18%)	9 (24%)	4 (13%)	20 (18%)	
Oropharynx (not further specified)	3 (7%)	4 (11%)	4 (13%)	11 (10%)	
Smoking					
Nonsmoker	2 (7%)	12 (37%)	9 (31%)	23 (25%)	$P = 0.018$
<20 pack-year history	16 (51%)	7 (22%)	7 (24%)	30 (33%)	
≥20 pack-year history	13 (42%)	13 (41%)	13 (45%)	39 (42%)	
Alcohol consumption					
Nondrinker	4 (13%)	6 (19%)	4 (13%)	14 (15%)	NS
<28 units/wk	12 (40%)	13 (42%)	15 (48%)	40 (44%)	
≥28 units/wk	14 (47%)	12 (39%)	12 (39%)	38 (41%)	
Nodal stage					
N0	9 (35%)	10 (28%)	8 (27%)	27 (29%)	NS
N1 (without ECS)	3 (11%)	5 (14%)	4 (13%)	12 (13%)	
N1 (with ECS) and N2/3	14 (54%)	21 (58%)	18 (60%)	53 (58%)	
Tissue available					
Fresh frozen	36 (90%)	33 (89%)	31 (100%)	100 (93%)	NS
FFPE	31 (78%)	36 (97%)	30 (97%)	97 (90%)	

Abbreviation: ECS, extracapsular.

The quality of both DNA and RNA was assessed by a Kruskal–Wallis test of the ΔC_t of the relevant reference gene (*RNase P* for DNA qPCR and *β-actin* for RNA qPCR) to ensure that era of collection did not impact upon detection.

Results

Cohort characteristics

The characteristics of the overall group and comparisons between the 3 periods of collection are seen in Table 2. The numbers of cases collected and the clinical and demographic characteristics showed no significant differences with respect to era of collection.

Availability for testing, sample quality, and consistency between repeats

DNA and RNA qPCR. Ninety-eight of 108 (91%) and 95 of 108 (88%) of samples were evaluable for HPV status by DNA and RNA qPCR respectively, with all samples providing analyzable results. The cycles to reach threshold (C_t) for relevant reference genes (in both RNA and DNA analyses) showed no trend for deterioration by year of sample collection. Therefore, we established that there was no conclusive evidence of DNA or RNA degradation over time. Tumor cell proportion within fresh-frozen samples was estimated at greater than 50% for all samples and greater than 80% for two-thirds of samples analyzed.

TMA: p16 IHC and HR HPV ISH. FFPE blocks were available for tissue microarray inclusion for 97 of 108 cases and, subsequently, p16 IHC and HR HPV ISH results were produced from at least 1 or more representative tumor cores for each case (97 of 97, 100%). Complete consistency of p16 IHC and HR HPV ISH results between all tumor cores originating from the same FFPE block was seen in 36 of 41 (88%) and 20 of 29 (69%) cases, respectively. A combined threshold of $\geq$2/3 core concordance for combined p16 IHC and HR HPV ISH was achieved by 97 of 97 (100%) cases. Additional p16 IHC of whole sections was carried out in 5 cases in which a complete absence of staining in the TMA cores occurred in the face of positive HPV tests. This internal control confirmed true-negative scores by the presence of positive p16 IHC within tissue components such as follicular dendritic cells, tonsillar crypt epithelium, and fibroblasts.

HPV status

The HPV16 status within each time period, and the overall total, is expressed as a trend for 1988–2009 in Table 3. The percentage of cases attributable to HPV16 by RNA qPCR increased from 14% to 57% in the period ($P = 0.001$). Compared with this standard, the sensitivity of the 7 tests and combinations ranged from 88% to 97% and the specificity from 82% to 100% (Table 3). The increase in incidence remained statistically significant irrespective of the test used, although the 2008–2009 measures of HPV rates varied markedly between 52% for combined DNA/RNA qPCR and 77% for p16 IHC. With the exception of a single case, all samples that were positive by RNA qPCR were also positive by DNA qPCR; however, 8 DNA qPCR-positive cases were negative by RNA qPCR. Three of 95 (3%) cases were positive for either HPV18 (1 of 95) or HPV33 (2 of 95) E6 expression, whereas one of these cases in the latter group showed gene expression for both HPV16 and HPV33 *E6*. FFPE tissue was not available for this case; however, the second HPV33-positive case did show a positive result for HR HPV ISH in the absence of p16 staining. The single case shown to be positive for HPV18 was p16 IHC/HR HPV ISH positive whereas negative by both HPV16 DNA and RNA qPCR.

HPV16 status versus clinical characteristics

The HPV16-positive group was younger than the HPV16-negative group (mean 53.3 vs. 60.8 years, $P = 0.003$; Table 4). Patient ages fitted in a normal distribution (one-sample Kolmogorov–Smirnov test, $P = 0.997$) and,

Table 3. Sensitivity/specificity of tests and trends of incidence over time

HPV16 status by test RNA qPCR "gold standard"	Sensitivity (Compared with RNA qPCR)	Specificity (Compared with RNA qPCR)	Number of HPV positive by presentation era: 1988–1997	2004–2007	2008–2009	Total 1988–2009	Statistical significance of difference by presentation era
			5/36 (14%)	12/29 (41%)	17/30 (57%)	34/95 (36%)	0.001
p16 IHC	94%	82%	6/31 (19%)	13/36 (36%)	23/30 (77%)	42/97 (43%)	<0.001
HR HPV ISH	88%	88%	4/31 (13%)	14/36 (39%)	18/30 (60%)	36/97 (37%)	0.001
Combined p16/HR HPV ISH	88%	90%	4/31 (13%)	12/36 (33%)	18/30 (60%)	34/97 (35%)	0.001
DNA qPCR	97%	87%	8/35 (23%)	15/33 (46%)	17/30 (57%)	40/98 (41%)	0.02
Combined p16/DNA qPCR	97%	94%	3/26 (12%)	13/32 (41%)	17/30 (57%)	33/88 (38%)	0.002
Combined p16/RNA qPCR	94%	100%	3/27 (11%)	12/28 (43%)	16/29 (55%)	31/84 (37%)	0.008
Combined DNA qPCR/RNA qPCR	94%	100%	3/35 (9%)	12/29 (41%)	15/29 (52%)	30/93 (32%)	0.001

NOTE: $P = 0.001$ for increasing HPV positive over time by RNA qPCR.

significantly, the modest 7.5 years difference seen between mean ages exceeded the 6.8-year difference seen between the median ages. The other notable clinical characteristic correlating with HPV status was smoking history. Of the 82 patients for whom reliable smoking history could be determined, the nonsmokers and those smoking less than 20 pack-years were more common in the HPV16-positive group (Pearson's χ^2, $P = 0.007$). There were no significant differences between the groups in gender, tumor site, cervical lymph node stage, or alcohol consumption.

HPV16 testing methods as prognostic biomarkers: survival analysis

Kaplan–Meier survival curves using the assigned gold standard RNA qPCR test showed a significant prognostic benefit for overall survival ($P = 0.003$) and disease-specific survival ($P = 0.005$; Figs. 1 and 2). Kaplan–Meier estimates of mean survival for the other tests are shown in Table 5. Although very similar to the gold standard RNA qPCR outcome measures, the test combination conferring greatest prognostic significance for both disease-specific survival and overall survival was p16 IHC/DNA qPCR (overall survival, $P = 0.002$; disease-specific survival, $P = 0.005$). The least satisfactory tests in this regard were the sole use of p16 IHC or HR HPV ISH and also the combination of both. Although remaining statistically significant, the differences in overall survival ($P = 0.021$, 0.011, and 0.016, respectively) vary by an order of magnitude by comparison with the gold standard. All tests using target amplification of DNA and RNA performed relatively well in differentiating survival outcomes for both overall survival and disease-specific survival, although it is important to note that DNA qPCR lacked specificity (87%).

Discussion

This study reports the first incidence data for HPV16 in OPSCC with outcome data in a U.K. cohort. The proportion of HPV-mediated OPSCC cases has increased from 14% to 57% between 1998 and 2009, reflecting known trends seen in other developed countries (11, 14). As well as comprehensive clinical, demographic, and outcome

Table 4. Characteristics of HPV-positive versus HPV-negative cases

	HPV status by RNA qPCR analysis		Total	Statistical significance
	Negative	Positive		
Patient/tumor data				
No. of patients	61 (64%)	34 (36%)	95 (100%)	
Age at diagnosis, y				
Mean	60.8	53.3		0.003
SEM	1.4	1.7		
Sex				
Female	16 (26%)	6 (18%)	22 (23%)	NS
Male	45 (74%)	28 (82%)	73 (77%)	
Tumor site				
Tonsil	30 (49%)	22 (65%)	52 (55%)	
Soft palate	13 (21%)	4 (12%)	17 (18%)	NS
Base of tongue	11 (18%)	6 (18%)	17 (18%)	
Oropharynx (not further specified)	7 (11%)	2 (6%)	9 (9%)	
Nodal stage				
N0	15 (31%)	9 (28%)	24 (30%)	
N1 without ECS	8 (16%)	3 (9%)	11 (13%)	NS
N2/3 or N1 with ECS	26 (53%)	20 (63%)	46 (57%)	
Total	49 (100%)	32 (100%)	81 (100%)	
Smoking				
Nonsmoker	8 (16%)	13 (42%)	21 (26%)	
<20 pack-year history	16 (31%)	11 (36%)	27 (33%)	0.007
≥20 pack-year history	27 (53%)	7 (23%)	34 (42%)	
Total	51 (100%)	31 (100%)	82 (100%)	
Alcohol consumption				
Nondrinker	6 (14%)	5 (16%)	11 (14%)	
<28 units/wk	14 (33%)	18 (56%)	32 (43%)	NS
≥28 units/wk	23 (53%)	9 (28%)	32 (43%)	
Total	43 (100%)	32 (100%)	75 (100%)	

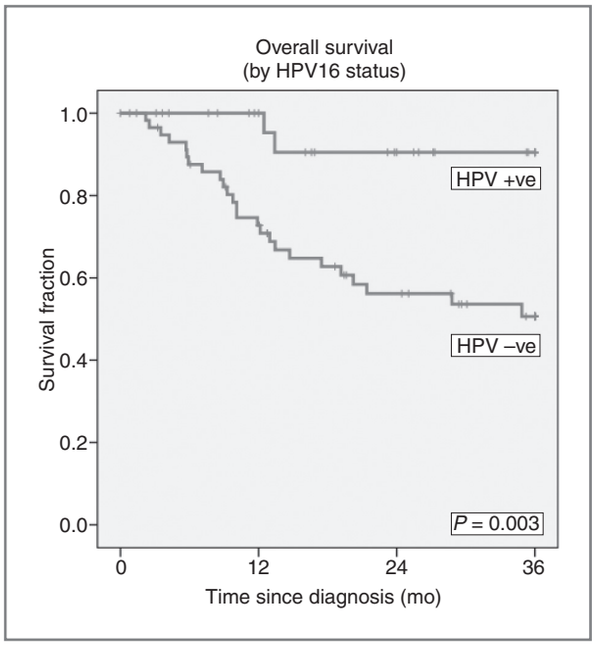

Figure 1. Three-year (36-month) Kaplan–Meier estimates showing overall survival versus HPV status (measured by RNA qPCR) are displayed including log-rank (Mantel–Cox) results. Red, HPV positive; green, HPV negative. $P = 0.003$.

data, this cohort offers analysis of tests based on both high-quality fresh frozen and fixed tumor tissues, offering a particularly detailed comparison between the different HPV detection methods previously advocated. The marked variability in HPV test results, despite exacting

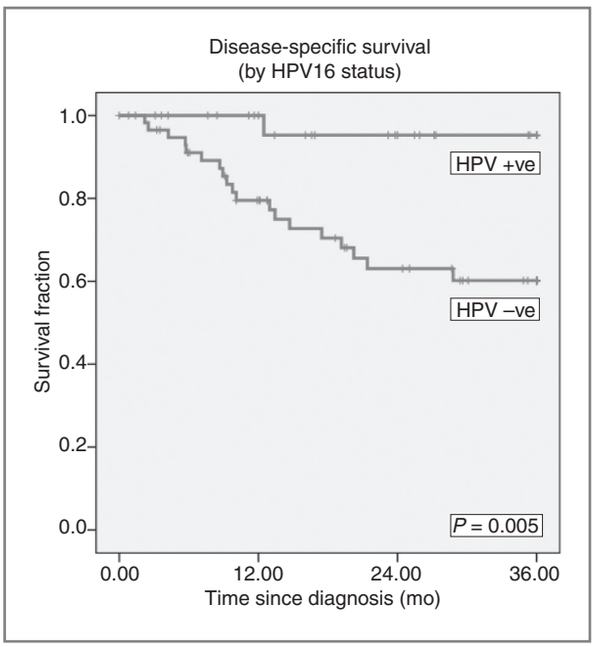

Figure 2. Three-year (36-month) Kaplan–Meier estimates showing disease-specific survival versus HPV status displayed including log-rank (Mantel–Cox) results. Red, HPV positive; green, HPV negative. $P = 0.005$.

(i.e., quantitative/duplicate) techniques used, has clinical significance for both the prediction of prognosis and the selection of patients for clinical trials.

The contribution and clinical importance of high-risk HPV subtypes other than HPV16 seem to be minimal by comparison with gynecologic and anogenital malignancy (7). Consequently, we feel that the use of "consensus" HPV PCR primers in HNSCC cases is difficult to justify, not least as this would merely confirm presence of viral DNA rather than the stronger burden of proof that viral oncogene expression bears when considering virally mediated malignancy. Kreimer and colleagues (25) in their systematic review of prevalence and HPV-type distribution in the head and neck found 86.7% of OPSCC patients were HPV16 positive whereas HPV18- and HPV33-positive cases, the subsequent largest percentage of types, accounted for only 1% each. Using viral oncogene expression, our findings are comparable, with HPV16 accounting for 94% of all HPV-positive cases and HPV18 and 33 representing a small subset (3% and 6%, respectively).

Van Houten and colleagues (26) alluded to the inherent variability in the sensitivity of tests that may lead to potential overestimation of the role of HPV16 OSPCC. We show that p16 IHC alone significantly "overcalls" HPV status, providing suboptimal prognostic information by comparison with other measures. p16 IHC was initially described as a surrogate for HPV status by Klussmann and colleagues (2) and was later applied in OPSCC survival analysis by Lassen and colleagues (13) in their description of HPV status within the Danish Head and Neck Cancer Group (DAHANCA) 5 Trial. When used in isolation, p16 IHC will identify tumors with excess p16 protein due to both the effects of viral E7 protein and through, as yet unexplained, non-HPV–mediated mechanisms (27). To improve specificity as a test, p16 IHC can be combined with a test for HPV DNA (by PCR or ISH), allowing classification into 1 of 4 groups (28) depending on a score for the 2 components. The review by Robinson and colleagues (16) on HPV testing included a pooling of results from 6 studies examining 496 tumors using such a classification and found p16-positive/HPV-negative results in 5% of cases and p16-negative/HPV-positive results in 8%. On the basis of our current series, the p16 IHC/HR HPV ISH classification shows a p16-positive/HPV-negative rate of 8% and a p16-negative/HPV-positive rate in 2% of cases. Both of the p16-negative/HPV-positive cases were negative by both DNA qPCR and RNA qPCR; however, 1 sample was positive by HPV33 qRNA analysis. Of particular interest, however, is the finding that RNA qPCR results highlight 2 of 97 cases (2%) that were p16 positive/HR HPV ISH negative. Such a finding of false-negative results reflects reduced sensitivity for the combined p16 IHC/HR HPV ISH test in determining tumor HPV16 status. In comparison, combined p16 IHC/DNA qPCR showed 6 of 88 (7%) cases that were p16 positive/HPV negative, none of which showed HPV16 E6 expression (RNA qPCR). The presence of HPV16 DNA was detected in 8 cases (20% of DNA qPCR-positive samples) where expression was not evident. Given the stringent efforts used

Table 5. Kaplan–Meier estimates of survival by HPV status as defined by each test

	Mean, mo	SE	95% CI		*P*
			Lower	Upper	
Disease-specific survival					
RNA qPCR					
HPV negative	26.7	1.7	23.3	30.1	0.005
HPV positive	34.9	1.1	32.7	37.0	
p16 IHC					
HPV negative	27.2	1.9	23.6	30.9	0.018
HPV positive	33.5	1.4	30.7	36.2	
HR HPV ISH					
HPV negative	27.6	1.8	24.1	31.0	0.02
HPV positive	34.0	1.3	31.4	36.7	
p16 IHC/HR HPV ISH					
HPV negative	27.7	1.7	24.3	31.1	0.027
HPV positive	33.9	1.4	31.2	36.7	
DNA qPCR					
HPV negative	26.1	1.9	22.5	29.9	0.008
HPV positive	33.8	1.3	31.3	26.2	
DNA qPCR/p16 IHC					
HPV negative	26.7	1.9	23.0	30.4	0.005
HPV positive	34.9	1.0	32.9	37.0	
RNA qPCR/p16 IHC					
HPV negative	26.8	1.9	23.0	30.6	0.007
HPV positive	34.9	1.1	32.7	37.0	
Combined DNA/RNA qPCR					
HPV negative	26.7	1.7	23.3	30.1	0.006
HPV positive	34.8	1.1	32.6	37.1	
Overall survival					
RNA qPCR					
HPV negative	24.7	1.8	21.2	28.2	0.003
HPV positive	33.8	1.5	30.9	36.7	
P16 IHC					
HPV negative	25.7	1.9	21.9	29.5	0.021
HPV positive	31.8	1.7	28.5	35.2	
HR HPV ISH					
HPV negative	25.7	1.8	22.2	29.3	0.011
HPV positive	33.0	1.6	29.8	36.2	
p16 IHC/HR HPV ISH					
HPV negative	25.9	1.8	22.4	29.5	0.016
HPV positive	32.9	1.7	29.5	36.2	
DNA qPCR					
HPV negative	24.4	1.9	20.7	28.0	0.007
HPV positive	32.1	1.6	28.9	35.3	
DNA qPCR/p16 IHC					
HPV negative	24.8	1.9	21.0	28.6	0.002
HPV positive	33.9	1.4	21.0	36.7	
RNA qPCR/p16 IHC					
HPV negative	24.8	2.0	21.0	28.7	0.003
HPV positive	33.8	1.5	30.9	36.7	
Combined DNA/RNA qPCR					
HPV negative	26.7	1.8	21.2	28.2	0.004
HPV positive	33.7	1.5	20.7	30.1	

to avoid contamination at each step in this analysis, we feel that this most likely reflects detection of innocent bystander infection in the absence of true virally mediated malignancy. Clearly, the reduced specificity of DNA qPCR limits its utility in most settings.

The Ventana Inform III HR HPV ISH probe detects 12-high risk HPV types including HPV16, -18, and -33. The performance of this probe defines as positive a small subset of cases, although this result is reduced by inclusion of p16 IHC in a combined test. It is possible that the HR HPV cocktail is detecting HPV types other than those tested by RNA qPCR; however, contribution to OPSCC of other HR HPV types (beyond HPV16 and -18) in isolation is unlikely (9).

If the calls for inclusion of OPSCC patients into appropriately designed and stratified clinical trials are to be met (14, 15, 29), then it is vital that accurate classification of HPV status be made prior to enrolment and with a validated, clinically appropriate test. This is now more than a theoretical problem, as several trials focused around the de-escalation of treatment to HPV-positive groups and in early-phase trials of HPV-directed agents and immunotherapy have recently opened. Our data suggest that HPV16 status determination with the p16 IHC/DNA qPCR combination test offers a valuable alternative to RNA analysis, with excellent prognostic value and 97% sensitivity/94% specificity. As a potential alternative with less logistic constraints in routine pathology practice, the combination of p16 IHC/HR HPV ISH is worthy of consideration, consistent with the diagnostic algorithms suggested by Westra and colleagues (30). In our data, specificity for p16 IHC/HR HPV ISH, albeit with an HPV high-risk cocktail probe rather than a type-specific probe, was maintained (90%) but at a cost to sensitivity (88%) that may be deemed undesirable. In routine clinical practice, this compromise may be acceptable, acknowledging that underrepresentation of HPV-positive cases will generally have less serious consequences. Clinical trials in HNSCC frequently struggle to adequately recruit, and in those focusing within one anatomic subsite, this difficulty may be exaggerated. To maximize sensitivity, that is, potential recruitment while maintaining specificity, that is, patient safety, the choice of satisfactory test is more limited. Faced with the potential "loss" of approximately 10% of eligible patients, using p16 IHC/HR HPV ISH, the benefits to sensitivity of using DNA or RNA PCR assays seem to easily balance the additional logistic costs.

With respect to survival, this research reinforces previously reported favorable outcomes for individuals with HPV-positive tumors (12, 14, 31, 32), as shown by improved disease-specific and overall survival. It is apparent that, with the exception of p16 IHC or HR HPV ISH in isolation, most of the other assays available provide a reasonable prognostic guide.

The additional prognostic value of combining HPV16 and smoking history has not been undertaken, as the number of nonsmokers in this study is small. Hence, further division of the cohorts would preclude meaningful statistical analysis. We speculate that adding smoking data adds accuracy to some other published HPV detection methods that have proven inaccuracies (e.g., P16IHC), as nonsmoking will doubtless be strongly correlated with HPV16-positive category.

The data presented highlight that the significant recent changes in etiology among OPSCC cases seen in other nations are also present in the United Kingdom. It is thought that the improved survival seen in younger patients will result in an increasing and potentially unnecessary burden of late toxicities arising from combined modality therapy. In addition, data such as these may be helpful in gaining a U.K. perspective for decision making surrounding prophylactic vaccination of young males against HR HPV (including HPV16) in addition to females. We show mean age at diagnosis for HPV-positive cases to be midway through the sixth decade, in keeping with other authors (33). Although the natural history of HPV infection in the oral cavity is, as yet, unclear (34), any benefits of an introduction of vaccination of boys prior to the age of first sexual contact (12–13 years) may be realized only after 30 to 35 years.

In conclusion, we present a rigorous analysis of diagnostic tests, judging their value against the most clinically relevant demands of diagnostic accuracy and prognostic relevance. It is hoped that the design of forthcoming clinical trials, aimed at both de-escalating therapy in HPV-mediated OPSCC and perhaps intensifying therapy for HPV-negative cases, will be informed by these results.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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