Evaluation of human papilloma virus diagnostic testing in oropharyngeal squamous cell carcinoma: sensitivity, specificity and prognostic discrimination.

Running Title: Evaluation of HPV16 testing in oropharyngeal cancer

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Statement of Translational Relevance:

Oropharyngeal squamous cell carcinoma (OPSCC) caused by Human papillomavirus-16 (HPV-16) has distinct behaviour and prognosis hence clinical trials specifically for these cases are now being developed. The accuracy of allocation of tumours to either HPV positive or negative categories is of immediate clinical impact. A cohort of OPSCC 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 IHC / DNA qPCR demonstrated acceptable sensitivity (97%) & specificity (94%) as well as being the best discriminator of favourable outcome (OS = 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). Neither p16 IHC, HR HPV ISH nor DNA qPCR were sufficiently specific to use in isolation as diagnostic tests. This is the first UK HPV-16 OPSCC data available, showing a dramatic increase in role for HPV from 14% - 57% over two decades.

Abstract

Purpose: Human Papillomavirus-16 (HPV16) is the causative agent in a biologically distinct subset of oropharyngeal squamous cell carcinoma (OPSCC) with highly favourable 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 hybridisation for high risk HPV (HR HPV ISH); quantitative PCR for both viral E6 RNA (RNA qPCR) & 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 demonstrated acceptable sensitivity (97%) & specificity (94%) compared with the RNA qPCR "gold standard", as well as being the best discriminator of favourable outcome (OS 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). Neither p16 IHC, HR HPV ISH nor DNA qPCR were sufficiently specific to recommend in clinical trials when used in isolation.

Conclusions: Caution must be exercised in applying HPV16 diagnostic tests due to significant disparities in accuracy and prognostic value in previously published techniques.

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Introduction;

Head & Neck (H&N) Squamous Cell Carcinoma (SCC) is the sixth commonest cancer worldwide accounting for approximately 4% of all tumours (1). Despite changes in behavioural exposure to traditional risk factors for H&N SCC(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 OPSCC demonstrates the most rapidly rising incidence of any anatomical tumour site, exceeding that of both cutaneous melanoma and adenocarcinoma of oesophagus (6). In 2009, the International Agency for Research on Cancer (IARC) recognised Human Papillomavirus type 16 (HPV16) to be causal in OPSCC(7). However, epidemiological evidence from several countries points to marked variations in the extent to which HPV16 is involved in OPSCC(8-11).

HPV status has been demonstrated to be an important prognostic biomarker in OPSCC(12, 13) with a hazard ratio for overall survival around 0.4 for systematic reviews of clinical trials(14). Understandably calls have been made to standardise 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. Additionally, considerable variation in sensitivity and specificity exists between the tests defining HPV status(17), such that the utility of some has been questioned.

In the H&N oncology clinic, the introduction of HPV testing is justified in order 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 in order to exploit the evident contrast in biology of HPV positive cases compared with conventional smoking and alcohol derived HNSCC. Where de-escalation of therapy is the motivation behind a trial, the hazards to patient safety of inaccurately assigning HPV negative tumours to an HPV positive category are clear. We hypothesise that a “gold standard” test, i.e. most reliable way to detect a biologically relevant HPV infection(18), is detection of viral mRNA expression carried out using quantitative PCR (qPCR) techniques on fresh-frozen derived samples. Whilst 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 FFPE tissue. Against this standard we will therefore compare the prognostic ability, and sensitivity/specificity of seven other detection methods or combinations:p16 immunohistochemistry (p16 IHC); high risk HPV in situ hybridisation (HR HPV ISH); p16 IHC / HR HPV ISH combination; DNA qPCR; p16 IHC / DNA qPCR combination; RNAqPCR, and p16 IHC / RNA qPCR combination.

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

Materials and methods

Patients & 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 two million individuals within Merseyside and Cheshire, United Kingdom. 108 cases of OPSCC were identified, all of which adhered to strict tumour site classification(19) made at the time of diagnosis and entry to the tissue bank. Cases that could not be reliably designated as SCC from oropharyngeal sites were excluded. Cases with banked tissue originated from three distinct time periods; 1988-1997, 2004-2007 & 2008-2009.

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

Tissue microarray preparation

Tissue microarrays (TMAs) were constructed from formalin-fixed paraffin-embedded (FFPE) tissue blocks of OPSCC using a manual tissue arrayer (MTA-I, Beecher Instruments, USA), as previously described(20). Briefly, haematoxylin and eosin (H&E) stained sections were used to identify areas of tumour and normal mucosa in the donor block. Three tumour cores and one matched normal mucosal core (height 4mm, diameter 0.6mm) were transferred from the donor block to the recipient block employing a predetermined asymmetrical distribution. H&E stained sections of the TMAs were examined to confirm accurate sampling.

DNA/RNA extraction & 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 carry over in the RNA preparations. The purified DNA and RNA samples were quantified by spectrophotometry using NanoDropTM 1000 (Thermo Fischer Scientific). 500-600ng of the total RNA was used for complimentary DNA (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 using primers for two microsatellites at 9p21 and 17p13 loci(21) to ensure an absence of genomic DNA contamination, which was confirmed in all cases.

Quantification of tumour cell proportion within fresh frozen samples was undertaken to ensure that extracted DNA/RNA was representative of tumour rather than surrounding stroma or inflammatory cells. Analysis was made of FFPE slides corresponding to frozen samples of twenty randomly selected cases.

HR HPV Detection (HPV16, -18 & -33)

HPV16 E6 DNA Quantitative Polymerase Chain Reaction (DNA qPCR)

Primers and a FAM-MGB labelled Taqman probe were designed (synthesised by Applied Biosystems) and optimised to specifically amplify the HPV16 E6 region (sequence and PCR conditions listed in Table 1). Commercially available primers and a VIC-TAMRA labelled 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 1X Taqman Gene Expression Master Mix (Applied Biosystems), 500 nM of each primer, 250 nM of probe, 1X RNAse P primer/probe mix and 100 ng genomic DNA. Real-time PCR reactions were performed in duplicate for all samples on an Applied Biosystems 7500 FAST system. The HPV16 positive cervical cancer cell lines, CaSki (UK 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 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)(22). Assuming an HPV16 driven tumour is composed of a dominant clonal population of cells, we scored as positive those samples with ≥1 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 Quantitative Polymerase Chain Reaction (RNA qPCR)

Duplicate real-time RNA (cDNA) PCR reactions were carried out using 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 a FAM-MGB labelled Taqman probe were designed to specifically amplify the HPV18 E6 region (synthesised by Applied Biosystems) and optimised using 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 (PrimerDesign™ genesig HVP33 Kt, Southampton, UK) was used to analyse HPV33 E6 gene expression. Both HPV18 and HPV33 expression analysis was conducted on the Applied Biosystems 7500 FAST system.

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

p16 immunohistochemistry

p16 immunohistochemistry (IHC) was carried out using a proprietary kit (CINtec Histology, mtm laboratories AG, Germany) on a Ventana Benchmark Autostainer (Ventana Medical Systems Inc, USA). A tonsil SCC 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 70% of the malignant cells (23). All other staining patterns were scored as negative.

High risk HPV in situ hybridisation

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

Interpretation of HPV tests

The p16 IHC and high risk HPV ISH tests were assessed independently by two H&N 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 demonstrate both DSS and OS 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 Chi-square and Kruskal-Wallis tests were used for comparison of demographic and tumour specific features between periods of sample collection and HPV positive and negative subgroups. Kaplan-Meier estimates and sensitivity and specificity of seven alternative tests (p16 IHC; HR HPV ISH; DNA qPCR; RNA qPCR and combined analysis tests: p16 IHC/HR HPV ISH; p16 IHC/DNA 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. DSS was defined as death from or due to OPSCC, and OS was defined as death from any cause. Both DSS and OS were calculated at thirty-six (36) months follow up beyond the

date of initial diagnosis.

DNA and RNA quality was assessed by a Kruskal-Wallis test of the ΔCT 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 (Table2)

The characteristics of the overall group and comparisons between the three 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

98/108 (91%) and 95/108 (88%) of samples were evaluable for HPV status by DNA and RNA qPCR respectively, with all samples providing analysable results. The cycles to reach threshold (CT) for relevant reference genes (in both RNA and DNA analysis) 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. Tumour cell proportion within fresh frozen samples was estimated at greater than 50% for all samples and greater than 80% for two thirds of samples analysed.

TMA: P16 IHC & HR HPV ISH

FFPE blocks were available for tissue microarray inclusion for 97/108 cases and subsequently, p16 IHC and HR HPV ISH results were produced from at least one or more representative tumour core for each case (97/97, 100%). Complete consistency of p16 IHC and HR HPV ISH results between all tumour cores originating from the same FFPE block was seen in 36/41 (88%) and 20/29 (69%) cases, respectively. A combined threshold of ≥2/3 core concordance for combined p16 IHC and HR HPV ISH was achieved by 97/97 (100%) cases. *Additional p16 IHC of whole sections was carried out in 5 cases where 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 (Table 3)

The HPV16 status within each time period, and the overall total, is expressed as a trend 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 seven 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 2008/9 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. 3/95 (3%) of cases were positive for either HPV18 (1/95) or HPV33 (2/95) E6 expression, whilst one of these cases in the latter group demonstrated both HPV16 and HPV33 E6 gene expression. FFPE tissue was not available for this case, however the second HPV33 positive case did demonstrate 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 whilst negative by both HPV16 DNA and RNA qPCR.

HPV16 status vs. clinical characteristics (Table 4)

The HPV16 positive group were younger than the HPV16 negative group (mean 53.3 vs. 60.8 yrs, p=0.003). Patient ages fitted in a normal distribution (1-sample Kolmogorov-Smirnov test, p=0.997) and, significantly, the modest 7.5 yrs difference seen between mean ages exceeded the 6.8 yrs 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 non-smokers and those smoking <20 pack years were more common in the HPV16 positive group (Pearson's chi square, p=0.007). There were no significant differences between the groups in gender, tumour site, cervical lymph node stage or alcohol consumption.

HPV16 testing methods as prognostic biomarkers: survival analysis (Table 5)

Kaplan-Meier survival curves using the assigned gold standard RNA qPCR test showed a significant prognostic benefit for OS (P= 0.003) and DSS (P=0.005) (Figures 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 and overall survival was p16 IHC/ DNA qPCR (OS, p=0.002

and DSS, 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 OS (p=0.021, 0.011 & 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 OS and DSS, 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 United Kingdom 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 data, this cohort offers analysis of tests based on both high quality fresh frozen and fixed tumour tissue, offering a particularly detailed comparison between the different HPV detection methods previously advocated. The marked variability in HPV test results, despite exacting (i.e. quantitative / duplicate) techniques employed, 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, appears to be minimal by comparison with gynaecological 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 bares when considering virally mediated malignancy. Kreimer et al(25) in their systematic review of prevalence and HPV type distribution in the head and neck found 86.7% of OPSCC were HPV16 positive whilst 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 & 33 representing a small subset (3% and 6% respectively).

Van Houten et al(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 sub optimal prognostic information by comparison to other measures. p16 IHC was initially described as a surrogate for HPV status by Klussmann et. al.(2) and was later applied in OPSCC survival analysis by Lassen et al. (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 tumours with excess p16 protein due to both the effects of viral E7 protein but also

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 one of four groups(28) depending on a score for the two components. Robinson et. al.'s review(16) of HPV testing included a pooling of results from six studies examining 496 tumours using such a classification and found p16 positive/HPV negative in 5% of cases and p16 negative/HPV positive results in 8%. Based on our current series, the p16 IHC/HR HPV ISH classification demonstrates 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 one sample was positive by HPV33 qRNA analysis. Of particular interest however is the finding that RNA qPCR results highlight 2/97 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 tumour HPV16 status. By comparison, combined p16 IHC/DNA qPCR showed 6/88 (7%) cases that were p16 positive/HPV negative, none of which demonstrated 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 employed to avoid contamination at each step in this analysis, we feel that this most likely reflects detection of innocent bystander infection rather 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 twelve high risk HPV types including HPV16, 18 & 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 using RNA qPCR, however contribution to OPSCC of other HR HPV types (beyond HPV16 & -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 focussed 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 suggests 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 et. al. (30). In our data, specificity for p16 IHC/ HR HPV ISH, albeit with a 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 under-representation of HPV positive cases will generally have less serious consequences. Clinical trials in HNSCC frequently struggle to adequately recruit, and in those focussing within one anatomical sub-site, this difficulty may be exaggerated. In order to maximise sensitivity i.e. potential recruitment whilst maintaining specificity i.e. 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 employing DNA or RNA PCR assays appear to easily balance the additional logistic costs.

With respect to survival, this research reinforces previously reported favourable outcomes for individuals with HPV positive tumours(12, 14, 31, 32), as demonstrated by both improved DSS and OS. 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 non-smokers 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 typing methods that have proven inaccuracies (e.g. P16IHC) as non-smoking will doubtless be strongly correlated with HPV-16 positive category.

The data presented highlights that the significant recent changes in aetiology amongst 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 United Kingdom 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 6th 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 realised only after 30-35 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.

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Figure Legends:

Figure 1

3-Year (36 month) Kaplan Meier estimates demonstrating overall survival (OS) versus HPV status (measured by RNA qPCR) are displayed including log rank (Mantel–Cox) results.

Key: Red = HPV positive; Green = HPV negative

P=0.003

Figure 2

3-Year (36 month) Kaplan Meier estimates demonstrating disease specific survival (DSS) versus HPV status displayed including log rank (Mantel–Cox) results.

Key: Red = HPV positive; Green = HPV negative

P=0.005

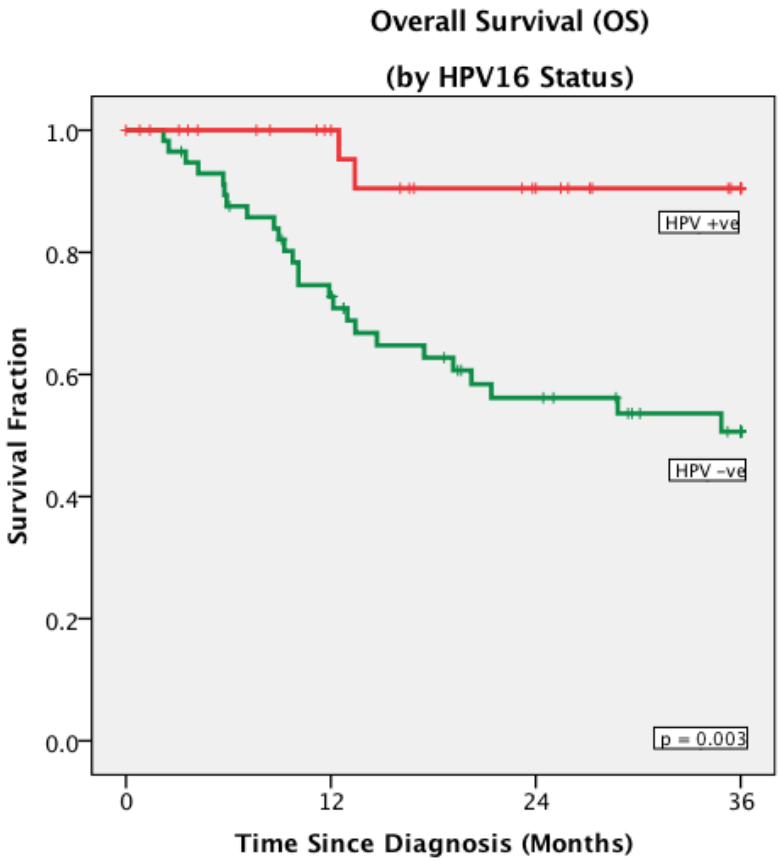

Overall Survival (OS)
(by HPV16 Status)
Survival Fraction
1.0
0.8
0.6
0.4
0.2
0.0
HPV +ve
HPV -ve
p = 0.003
0
12
24
36
Time Since Diagnosis (Months)

Figure 1

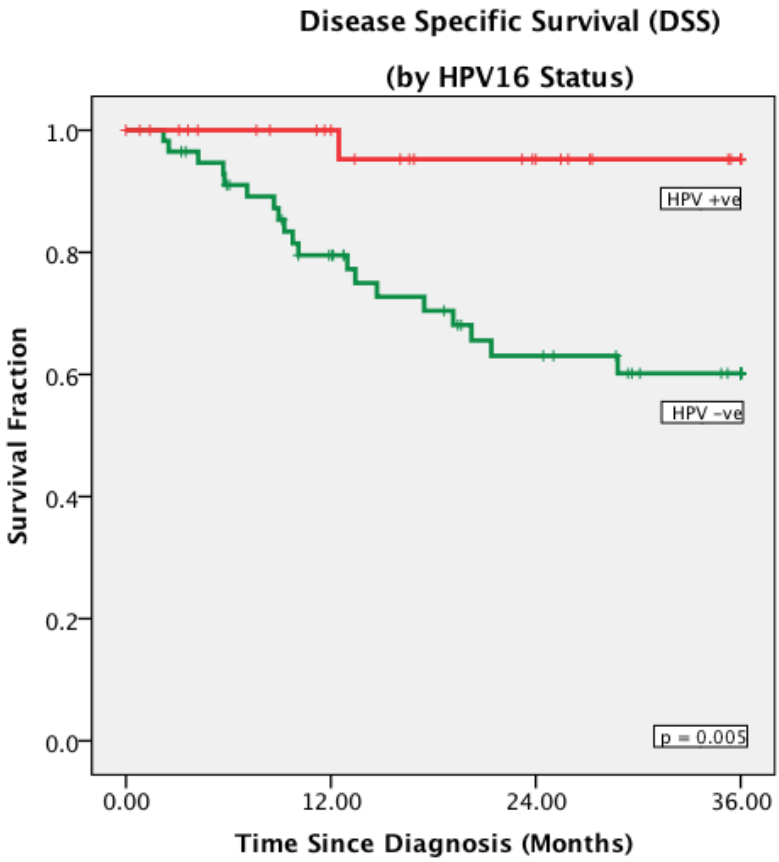
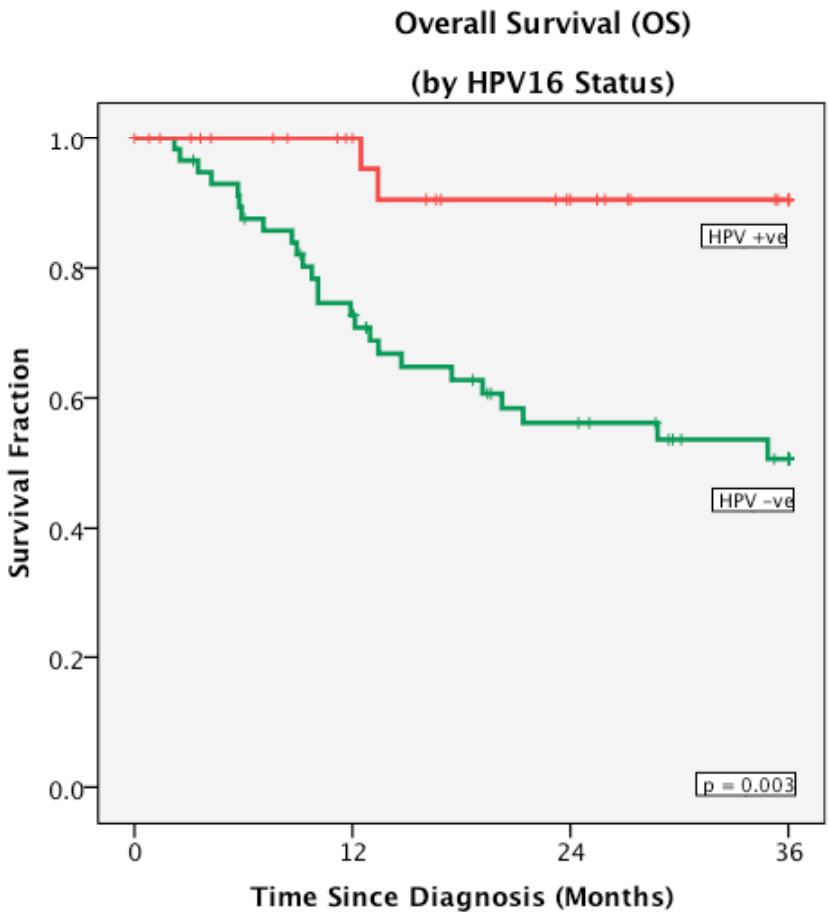

Disease Specific Survival (DSS)
(by HPV16 Status)
Survival Fraction
1.0
0.8
0.6
0.4
0.2
0.0
HPV +ve
HPV -ve
p = 0.005
0.00
12.00
24.00
36.00
Time Since Diagnosis (Months)

Figure 2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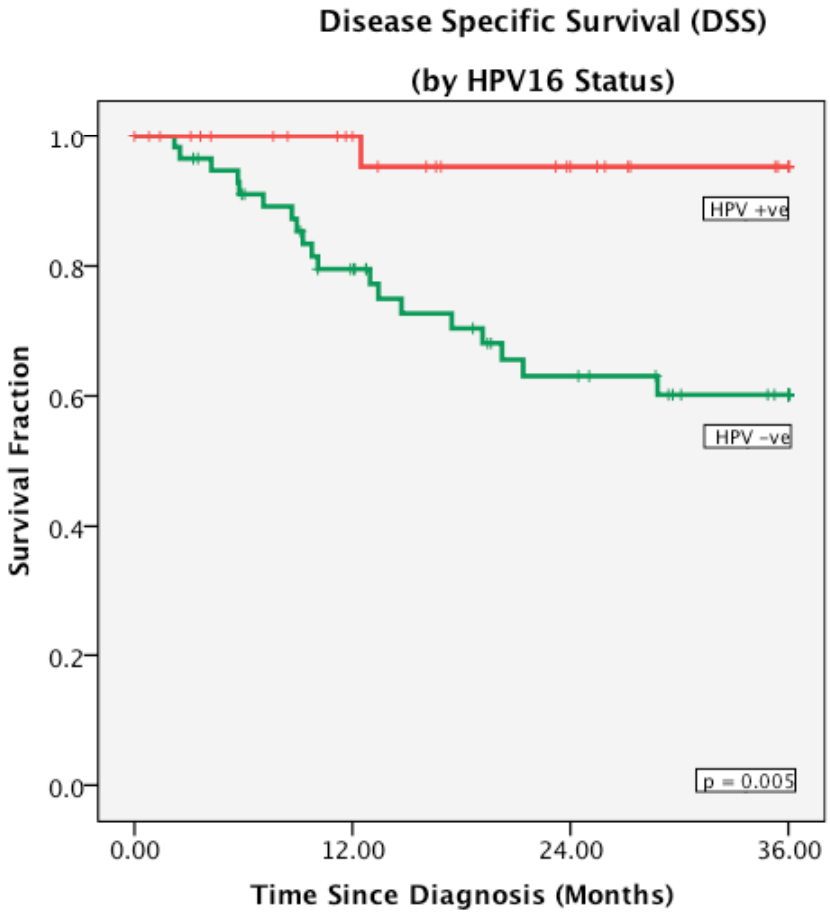
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p = 0.005
0.00
12.00
24.00
36.00
Time Since Diagnosis (Months)

Table 1 Primer & Probe Sequences
PCR Conditions

Target	F Sequence	R Sequence	Probe Sequence
HPV16 E6	CTGCGACGTGAGGTATATGACTTT	ACATACAGCATATGGATTCCCATCT	CTTTTCGGGATTTATGC
HPV18 E6	AAACCGTTGAATCCAGCAGAA	GTCGTTCCTGTCGTGCTCG	TTGCAGCACGAATGG

Target	Melting	Anneal/Extension
HPV16 E6	95°C 15s	61°C 60s
HPV18 E6	95°C 15s	60°C 60s

Table 2 Cohort characteristics by time period of collection

	Time Period			Overall	Statistical Significance
	1988-1997	2004-2007	2008-2009	**1998-2009**	
Patient/Tumour Data					
No. of Patients	40 (37%)	37 (34%)	31(29%)	**108 (100%)**	
Age at Diagnosis (years)					
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%)**	
Tumour 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 spec.)	3 (7%)	4 (11%)	4 (13%)	**11 (10%)**	
Smoking					
Non-smoker	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					
Non-drinker	4 (13%)	6 (19%)	4 (13%)	**14 (15%)**	NS
<28 Units/Week	12 (40%)	13 (42%)	15 (48%)	**40 (44%)**	
≥28 Units/Week	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) & 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%)**	

Abbrevations: ECS, extracapsular; FFPE, formalin fixed parafin embedded

Table 3 Sensitivity/specificity of tests & trends of incidence over time

	Sensitivity	Specificity	Number of HPV+ve by Presentation Era			Total	Statistical Significance
HPV16 Status by Test	(compared to RNA qPCR)		1988-1997	2004-2007	2008-2009	1988-2009	p =
RNA qPCR "Gold Standard"			**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 +ve over time by RNA qPCR

Table 4 Characteristics of HPV positive vs. HPV negative cases

		HPV Status by combined RNA qPCR Analysis		Total	Statistical Significance
		Negative	Positive		p =
Patient/Tumour Data					
No. of Patients		61 (64%)	34 (36%)	**95 (100%)**	
Age at Diagnosis (years)					
Mean		60.8	53.3		0.003
S.E. of Mean		1.4	1.7		
Sex					
Female		16 (26%)	6 (18%)	**22 (23%)**	NS
Male		45 (74%)	28 (82%)	**73 (77%)**	
Tumour 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 spec.)		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					
Non-smoker		8 (16%)	13 (42%)	**21 (26%)**	
<20 pack-year Hx		16 (31%)	11 (36%)	**27 (33%)**	0.007
≥20 pack-year Hx		27 (53%)	7 (23%)	**34 (42%)**	
	Total	*51 (100%)*	*31 (100%)*	***82 (100%)***	
Alcohol Consumption					
Non-drinker		6 (14%)	5 (16%)	**11 (14%)**	
<28 Units/Week		14 (33%)	18 (56%)	**32 (43%)**	NS
≥28 Units/Week		23 (53%)	9 (28%)	**32 (43%)**	
	Total	*43 (100%)*	*32 (100%)*	***75 (100%)***	

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

Disease Specific Survival		Mean (months)	SE	95% Confidence Interval		p value
				Lower	Upper	
RNA qPCR	HPV -ve	26.7	1.7	23.3	30.1	0.005
	HPV +ve	34.9	1.1	32.7	37.0	
p16 IHC	HPV -ve	27.2	1.9	23.6	30.9	0.018
	HPV +ve	33.5	1.4	30.7	36.2	
HR HPV ISH	HPV -ve	27.6	1.8	24.1	31.0	0.02
	HPV +ve	34.0	1.3	31.4	36.7	
p16 IHC/HR HPV ISH	HPV -ve	27.7	1.7	24.3	31.1	0.027
	HPV +ve	33.9	1.4	31.2	36.7	
DNA qPCR	HPV -ve	26.1	1.9	22.5	29.9	0.008
	HPV +ve	33.8	1.3	31.3	26.2	
DNA qPCR/p16 IHC	HPV -ve	26.7	1.9	23.0	30.4	0.005
	HPV +ve	34.9	1.0	32.9	37.0	
RNA qPCR/p16 IHC	HPV -ve	26.8	1.9	23.0	30.6	0.007
	HPV +ve	34.9	1.1	32.7	37.0	
Combined DNA/RNA qPCR	HPV -ve	26.7	1.7	23.3	30.1	0.006
	HPV +ve	34.8	1.1	32.6	37.1	

Overall Survival		Mean (months)	SE	95% Confidence Interval		p value
				Lower	Upper	
RNA qPCR	HPV -ve	24.7	1.8	21.2	28.2	0.003
	HPV +ve	33.8	1.5	30.9	36.7	
p16 IHC	HPV -ve	25.7	1.9	21.9	29.5	0.021
	HPV +ve	31.8	1.7	28.5	35.2	
HR HPV ISH	HPV -ve	25.7	1.8	22.2	29.3	0.011
	HPV +ve	33.0	1.6	29.8	36.2	
p16 IHC/HR HPV ISH	HPV -ve	25.9	1.8	22.4	29.5	0.016
	HPV +ve	32.9	1.7	29.5	36.2	
DNA qPCR	HPV -ve	24.4	1.9	20.7	28.0	0.007
	HPV +ve	32.1	1.6	28.9	35.3	
DNA qPCR/p16 IHC	HPV -ve	24.8	1.9	21.0	28.6	0.002
	HPV +ve	33.9	1.4	21.0	36.7	
RNA qPCR/p16 IHC	HPV -ve	24.8	2.0	21.0	28.7	0.003
	HPV +ve	33.8	1.5	30.9	36.7	
Combined DNA/RNA qPCR	HPV -ve	26.7	1.8	21.2	28.2	0.004
	HPV +ve	33.7	1.5	20.7	30.1	