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ABSTRACT

Squamous cell carcinomas of the hypopharynx (HPSCC) and oropharynx (OPSCC) have markedly different patient outcomes. Differences in HPV prevalence between these two patient groups may account for some of this difference, but other molecular markers of prognosis or pathological phenotype have not been established. Copy number gain of oncogenes is a well-established molecular change contributing to HNSCC development. Quantitative PCR was used to explore copy number gains of specific genes (3q - *PIK3CA, TP63;* 11q13.3 - *CCND1, ANO1*) in tumour DNA recovered from HPSCC (n=48) and OPSCC (n=52) patients. Associations between copy number gain, patient demographics, HPV/p16INK4a status and pathological stage were examined. HPV/p16 prevalence in HPSCC and OPSCC groups was 2.1% and 46.0% respectively. HPSCCs had frequent gains of *CCND1* (56.3%) and *ANO1* (56.3%) but few gains of *PIK3CA* (6.3%). By contrast, OPSCCs had significantly fewer *CCND1* (23.1%) and *ANO1* (17.3%) gains, and significantly more *PIK3CA* (26.9%) gains. A mutually exclusive relationship between HPV/p16 and 11q13.3 gains was observed in OPSCCs, while *PIK3CA* and *TP63* gains were similar across HPV-associated and smoking/alcohol-associated patients. *ANO1* gain was significantly linked to tumour pathology in HPSCC, associating with nodal metastasis and smaller and less invasive tumours at presentation (p=0.010). Our results provide a convincing link between a specific molecular change and disease phenotype that appears unique to our HPSCC population, supporting a model of 11q13.3 in promoting metastatic disease progression in HNSCC, and suggest a role for *ANO1* as a molecular marker of metastatic disease.

244 words

KEY WORDS Hypopharyngeal Carcinoma, Oropharyngeal Carcinoma, 11q13.3, HPV/p16INK4a, copy number gain, lymph node metastasis

INTRODUCTION

Head and neck cancers remain a major cause of cancer-associated morbidity and mortality worldwide. Squamous cell carcinomas (HNSCC, accounting for more than 90% of head and neck cancers) are now recognised as having at least two distinct aetiological origins. Tobacco and alcohol exposure account for 70-80% of cases, but a growing subset in younger patients without this typical risk profile and associated with high risk Human Papillomavirus (HPV) infection are increasingly common in some populations (Gillison et al., 2008; Junor et al., 2012). Crucially, these different patient subsets have distinct clinical outcomes - with HPV-associated disease fairing favourably in treatment response, recurrence and survival (Ragin et al., 2007; Klussmann et al., 2007; Fakhry et al., 2008; Junor et al., 2012; Salazar et al., 2014), providing the basis for a risk stratification process based on the molecular detection of HPV in tumours. Guidelines for HPV annotation of head and neck tumours have been developed for the UK by the Royal College of Pathologists (Helliwell et al., 2013), although currently only for OPSCC patients where the link between HPV and prognosis is most apparent. To date, HPV and its co-marker p16INK4A are the only biomarkers in widespread clinical use in the management of HNSCC.

Large-scale next generation sequencing (NGS) projects have profiled HNSCC across multiple molecular and cellular levels, and many themes from early cytogenetic work (including broad, binary distinctions between smoking/alcohol and HPV-associated disease) have now been expanded upon. The consistent observation in early work of a mutually exclusive relationship between amplification of the 11q13.3 chromosomal band (found in high frequency across HNSCC, and other cancers) and the presence of HPV in tumours remains one of the most striking differences observed within HNSCCs (Smeets et al., 2006; Ragin et al., 2006; Klussmann et al., 2009; Lechner et al., 2013; Seiwert et al., 2015). This separation may be attributable to the divergent mechanisms by which chemical carcinogens (via genetic amplification and consequent over-expression of Cyclin D1 gene *CCND1* within the 11q13.3 amplicon - often in combination with genetic or functional loss of restriction point Cyclin Kinase Inhibitor p16INK4A) and HPV (via HPV oncoproteins E6 and E7) achieve dysregulation of the cell cycle. In contrast, certain genetic changes have emerged as common to both HPV and smoking/alcohol-associated HNSCC. For

example, mutation, amplification and over-expression of elements of the PI3 kinase pathway (in particular the p110 catalytic subunit gene *PIK3CA* at 3q26.3) have been consistently observed across both aetiological groups (Smeets et al., 2006; Wilting et al., 2009; Lechner et al., 2013; Cancer Genome Atlas Network, 2015; Keck et al., 2015; Seiwert et al., 2015).

Integrated NGS analysis has also expanded upon binary aetiological classification, with new sub-classes now defined by broad common patterns of copy number change, mutation, transcription and cellular features (now including atypical, classical, mesenchymal/inflammatory and basal classes, as described in Cancer Genome Atlas Network, 2015; Keck et al., 2015; Seiwert et al., 2015). Some key areas remain under-explored, however, including whether common specific changes underpin a 'switch' from locally invasive to nodal or distant metastatic disease, or underpin the markedly differing outcomes of tumours from different anatomical sites. Any such markers have the potential to be exploited as risk stratification tools, or new therapeutic targets.

In this study, we aimed to explore how copy number gain of HNSCC-associated genes might differ between oropharyngeal (OPSCC) and hypopharyngeal (HPSCC) tumours, and how the observed genetic changes might link to clinico-pathological features across the two patient groups. Although less common than other head and neck tumours, HPSCCs have some of the worst outcomes of head and neck tumours (Pulte et al., 2010), and remain relatively under-researched from a molecular perspective. We developed a qPCR assay to determine copy number gain in specific genes from the commonly amplified 3q arm (*PIK3CA, TP63*) and 11q13.3 amplicon (*CCND1, ANO1*). We used this assay to explore the frequency of copy number gains in HPSCC and OPSCC tumours, and using linked patient data we examined the relationships between the observed copy number gains and clinico-pathologic characteristics of the two patient groups.

MATERIALS AND METHODS

Patients

Patients referred to the Edinburgh Cancer Centre with histologically confirmed HPSCC (2003-2012) or OPSCC (2011 to 2012) were identified and relevant demographic and clinico-pathologic data

extracted from electronic and paper records. Current smokers were defined as patients smoking at presentation, ex-smokers as patients who reported stopping smoking prior to presentation. Social drinkers were defined as patients drinking within recommended weekly alcohol limits (males <18 units, females <14 units), current or past heavy drinkers as patients drinking in excess of these limits at, or prior to presentation, respectively.

HPV & p16 testing, genomic DNA extraction

Sections of formalin fixed paraffin embedded (FFPE) tumour blocks (approved for use by the South East of Scotland BioResource - project reference SR-090) were reviewed and selected by a pathologist to confirm histological diagnosis. Immunohistochemistry (IHC) for p16 was carried out on 3μm sections using a monoclonal antibody to p16 (CINtec Histology, mtm Laboratories) on a Leica Bond III automated immunostainer. IHC for p16 was scored independently by two pathologists, with double review of any cases where there was not consensus, and considered positive if there was strong diffuse nuclear and cytoplasmic staining present in >70% of malignant cells (Singhi et al., 2010).

Tumour genomic DNA was extracted from corresponding sections of FFPE tissue blocks, and from FFPE blocks of normal tonsil tissue (as reference human genomic DNA for qPCR and copy number calculation) as described previously (Junor et al., 2012). Tumour sections comprised approximately 66% tumour tissue in the majority of cases. HPV testing was performed on extracted genomic DNA using the Optiplex HPV Genotyping Assay (Diamex, Heidelberg, Germany), as described previously (Cuschieri et al., 2013). DNA extracts were further purified and concentrated by ethanol precipitation and re-suspension in nuclease-free water, and samples selected for qPCR based on amount of DNA available (>30ng total DNA) and spectrophotometric quality (260nm/280nm absorption >1.8; 260nm/230nm absorption >2.0). Of 87 HPSCC FFPE samples tested for HPV, 48 were found to have sufficient amounts & quality of DNA for qPCR, and were included in the final genetic amplification analysis (HPSCC n=48). Of 60 OPSCC FFPE samples

tested for HPV, 52 were found to have sufficient amounts & quality of DNA for qPCR (OPSCC n=52).

Reference & target site selection for qPCR

Target sites for examining copy number gain were selected in genes commonly reported as amplified in HNSCC – two genes on the 3q chromosomal arm (*PIK3CA, TP63*) and two genes in well-characterised 11q13.3 amplicon (*CCND1, ANO1*). As a reference gene we selected a chromosomal region infrequently reported as amplified or deleted across the literature - 2p23 - and within this, the *EIF2B4* gene, given its putative constitutive role (initiation of protein translation), no reported involvement in malignant disease, and no current reports of copy number variation. Primer pairs were selected using the Primer3 software tool, with specificity confirmed using the BLAST search tool. Reference and target amplicons were generated using PCR with pooled human genomic DNA (Promega) as template (reaction conditions and primers shown in Supplemental Materials S1). PCR products were cloned into the Promega pGem-T-easy vector (as per manufacturer's instructions). Sanger sequencing was used to confirm plasmid amplicon specificity.

qPCR & copy number determination

qPCR was performed using the Maxima SYBR green master mix (Thermo Scientific) and the Stratagene Mx3000p thermos-cycler (Agilent Technologies). Reaction conditions were optimised (Supplemental Materials S1) and product specificity assessed using melt curve analysis, using commercial human genomic DNA as substrate. As a valid reference sample for copy number determination in tumour DNA, qPCR was performed and reference copy number values derived from a pool of human genomic DNA extracted from 31 FFPE tissue blocks of normal tonsils with qPCR-confirmed normal copy number (using the ddCt method with above mentioned commercial human genomic DNA as reference). These samples were deemed analogous to the FFPE tumour collection with regard to storage conditions, sample age (2002-2012) and extraction procedure, providing a more valid calibrator for tumour copy number calculation. qPCR for each target and reference gene was performed in replicate on tumour (sample) and tonsil (reference/calibrator)

genomic DNA. Estimated copy number of target genes in tumour samples was evaluated by relative quantitation using the ddCt method (as described previously in Ginzinger et al., 2000, Huang et al., 2002) and final copy number calls assigned based on an assumed normal copy number of two in the pooled normal tonsil DNA (as confirmed by comparison to commercially available normal human genomic DNA).

Analysis

To explore relationships between copy number gain, risk factors and tumour stage, patients were grouped according to the presence or absence of target site copy numbers greater than or equal to four (to account for possible stromal/normal tissue contamination in FFPE tumour samples, as described previously in Huang et al., 2006). Patient groups were compared by tumour site (HPSCC vs OPSCC) and HPV/p16 status (HPV negative/p16 negative vs HPV positive/p16 negative vs HPV negative/p16 positive vs HPV positive/p16 positive). Presence of any high risk HPV type in a sample was used to classify patients as "HPV positive", and for purposes of univariate analysis, patients were grouped according to their combined HPV and p16 status (HPV positive/p16 positive versus not HPV positive/p16 positive, including HPV positive/p16 negative, HPV negative/p16 positive and HPV negative/p16 negative – referred to collectively as "HPV/p16 negative" henceforth). Patients were then grouped by presence or absence of copy number gain at target sites within *PIK3CA, TP63, CCND1* or *ANO1*, and association between gains and clinico-pathologic features (sex, smoking, drinking, TNM stage, HPV/p16 status) were examined using univariate analysis. Pearson's Chi-square (unless otherwise stated) or Fisher's Exact Test ('f') were used for univariate analysis between patient groups; age differences were examined using Student's t-test; analysis was performed using IBM SPSS v22.

RESULTS

Demographics, risk factors and pathological stage across anatomical and HPV/p16 groups

Comparing patients by anatomical site (Table 1), HPSCC and OPSCC patients differed significantly with regard to sex ($p<0.001$; HPSCCs predominantly male, compared to relative gender equivalence amongst OPSCC), smoking ($p=0.002$; HPSCC predominantly current

smokers, OPSCC predominantly never or ex-smokers) and heavy drinking (p=0.012; almost half HPSCCs having ex- or current history of heavy drinking, and twice as likely to have heavy drinking history than OPSCCs).

The two anatomical groups differed significantly with regard to HPV/p16 status (p<0.001) - only one HPSCC tumour was confirmed as HPV positive/p16 positive (all other tumours were HPV negative/p16 negative), this single sample was HPV-18 positive. In contrast 23 of 52 (44.2%) OPSCC tumours were confirmed as HPV positive/p16 positive – with HPV-16 being the commonest high-risk type. HPV and p16 status were significantly associated (p<0.001) – only four tumours were HPV positive/p16 negative and two HPV negative/p16 positive. Comparing the HPV/p16 negative patient groups across the two anatomical sites (i.e. HPV/p16 negative HPSCC vs HPV/p16 negative OPSCC, Table 2) found these patient groups to be more similar with regard to smoking and drinking history - the two groups remained distinct only with regard to sex (p=0.001).

Comparing patients (across both anatomical sites) by HPV/p16 status highlighted significant differences in risk factor history between these two aetiological groups (Table 3). Only one of 24 (4.2%) HPV/p16 positive patients was a current smoker, compared to 46 of 74 (63.9%) HPV/p16 negative patients (p<0.001). Similarly only two (9.1%) HPV/p16 positive patients were past or current heavy drinkers, compared to 32 (45.7%) HPV/p16 negative patients (p=0.002). Pathological stage at presentation also differed between HPV/p16 groups – HPV/p16 positive primary tumours were more likely to be lower T stage (18 of 24 (75.0%) HPV/p16 positive tumours being T1-3 at presentation, compared to 38 of 74 (51.4%) HPV/p16 negative tumours, p=0.034), and more likely to be node metastasis positive at presentation (23 of 24 (95.8%) HPV/p16 positive tumours being node positive at presentation, compared to 49 of 74 (66.2%) HPV/p16 negative tumours, p=0.004). Combining T and N stage found HPV/p16 positive patients significantly more likely to present with smaller or less locally invasive primary but with node positive disease (17 of 24 (70.8%) HPV/p16 positive patients T1-3/N positive at presentation, compared to 25 of 74 (33.8%) HPV/p16 negative patients; p=0.001).

Frequency and pattern of target copy number gains across anatomical and HPV/p16 groups

The frequency and pattern of copy number gains at target sites within *PIK3CA, TP63, CCND1* and *ANO1* genes differed significantly across anatomical and HPV/p16 groups (Tables 1-3; Figure 1). Comparing anatomical groups (Table 1; Figure 1i), copy number gain of *PIK3CA* was significantly more common in OPSCC patients (14 of 52 (26.9%) OPSCCs having *PIK3CA* gain, compared to only three of 48 (6.3%) HPSCCs; p=0.006). By contrast, copy number gains of *CCND1* and *ANO1* were significantly more common in HPSCC patients (27 of 48 (56.3%) of HPSCCs having *CCND1* gain, 27 of 48 (56.3%) having *ANO1* gain, compared to 12 (23.1%) and 9 (17.3%) of 52 OPSCCs respectively; p<0.001 in both instances). Frequency of *TP63* copy number gain was similar across both HPSCC (9 of 48 (18.8%)) and OPSCC (10 of 52 (19.2%)) groups (p=0.951).

Grouping patients by HPV/p16 status revealed significant differences in *CCND1* and *ANO1* copy number gain frequency (Table 2; Figure 1ii). No HPV/p16 positive tumours were found to have *ANO1* gain (compared to 37 of 74 (50.0%) HPV/p16 negative tumours), while only one of 24 (4.2%) HPV/p16 positive tumour had *CCND1* copy number gain (compared to 35 of 74 (47.3%) HPV/p16 negative tumours)(p<0.001 in both instances). Frequency of copy number gains at *PIK3CA* and *TP63* target sites were not significantly different between HPV/p16 positive and negative groups (11 of 74 (14.9%) HPV/p16 negative tumours had *PIK3CA* gains compared to 6 of 24 (25.0%) HPV/p16 positive tumours (p=0.255), and 15 of 74 (20.3%) HPV/p16 negative tumours had *TP63* gains compared to 4 of 24 (16.7%) HPV/p16 positive tumours (p=0.698).

Comparing the HPV/p16 negative subsets of HPSCC and OPSCC (Table 3; Figure 1iii), *PIK3CA* copy number gain remained significantly more common in OPSCCs (8 of 27 (29.6%)) than HPSCCs (3 of 47 (6.4%))(p=0.01f). A higher frequency of *CCND1* and *ANO1* copy number gains in HPSCC tumours was again apparent - 10 of 27 (37.0%) HPV/p16 negative OPSCCs had *CCND1* gains, and only eight of 27 (29.6%) had *ANO1* gains, compared to 27 of 47 (57.4%) HPV/p16 negative HPSCCs with *CCND1* or *ANO1* gains (p=0.091 and p=0.021 respectively).

Synchronous copy number gains were observed with *ANO1* and *CCND1* in both anatomical groups (24 HPSCC tumours and 9 OPSCC tumours harbouring both *CCND1* and *ANO1* gains together; p<0.001 in both instances). *TP63* gains were more frequent in HPSCCs and OPSCCs with *ANO1/CCND1* gains, but these results were not statistically significant. In HPSCC, *PIK3CA* gain was mutually exclusive to *ANO1* gain (p=0.077f) – this pattern was not observed in OPSCC.

Association of target copy number gains with pathological stage

Grouping patients by the presence or absence of copy number gains at target sites revealed a distinct pattern of association between *ANO1/CCND1* gain and pathological stage in HPSCC patients, but not in OPSCC patients (Tables 4, 5 & 6; Figure 2). In HPSCC patients, copy number gains of *ANO1* were significantly associated with smaller or less invasive (T1-3 vs T4) primary tumours (18 of 25 (72.0%) T1-3 HPSCCs had *ANO1* gain, compared to 9 of 23 (39.1%) T4 tumours; p=0.022), and with node positive disease at presentation (22 of 33 (66.7%) node positive HPSCCs had *ANO1* gain, compared to 5 of 15 (33.3%) node negative tumours; p=0.031)(Figure 2a). This association was also significant in smaller (T1-3) / node positive HPSCC tumours (15 of 19 (78.9%) T1-3/node positive HPSCCs had *ANO1* gain, compared to 12 of 29 (41.4%) of T4 or node negative T1-3 tumours; p=0.010). Similar (but non-significant) associations between gains of *CCND1* and *TP63* and smaller, node positive HPSCC tumours were also observed (Table 1), though the majority of tumours with copy number gains at *CCND1* (24 of 27 (88.9%)) and *TP63* (7 of 9 (77.8%)) target sites also had *ANO1* gains. No significant pattern of association between *ANO1* copy number gain (or any other target site) and pathological stage was found in OPSCC tumours, regardless of HPV/p16 status (Tables 2 & 3; Figure 2b). No significant patterns of association between any copy number gain and risk factor history or patient demographic were found in any patient group (by anatomical site or HPV status).

DISCUSSION

The advent of scalable next generation sequencing technologies brings with it the potential to stratify HNSCCs into new subclasses, framed by tumour aetiology (HPV vs smoking/alcohol) but defined by distinct common patterns spanning multiple levels of molecular and cellular change

(Lechner et al., 2013; Walter et al., 2013; Cancer Genome Atlas Network, 2015; Keck et al., 2015; Seiwert et al., 2015). In the present study, we targeted one aspect of genetic change that can underlie malignant progression - copy number gain - at specific sites in four genes strongly associated with head and neck cancer – *PIK3CA*, *TP63*, *CCND1* and *ANO1*. We find distinct frequencies and patterns of copy number gain across different anatomical and HPV/p16 patient subsets, with *PIK3CA* gains significantly more common in OPSCC, and *CCND1* and *ANO1* gains significantly more common in HPSCC. Crucially, we find distinct associations between these molecular changes and pathological features amongst different tumour subgroups, suggesting a possible molecular-pathogenic link between copy number gains in the 11q13.3 band (*ANO1, CCND1*) and early nodal metastasis in HPSCC.

In our study, the finding of a subset of HPSCCs with *ANO1/CCND1* copy number gains that present with node positive and smaller, less locally invasive primary tumours provides a convincing link between a specific molecular change and disease phenotype amongst a specific subset of HNSCCs. Of 27 HPSCCs found to have *ANO1* copy number gains, 22 (81.5%) were node-positive at presentation. This finding could suggest a direct contributing role for *ANO1* and/or *CCND1* copy number gain (likely in the form of the well-documented high level amplification of the 11q13.3 band leading to over-expression of these and/or other amplicon-resident genes) toward an early 'switch' from local invasion to nodal metastasis in HPSCC, resulting in the 'smaller primary / node positive' association observed. Our findings in HPSCC are consistent with other models examining links between 11q13.3 amplification and metastatic potential in HNSCC (Muller et al., 1994; Welkoborsky et al., 2000; Miyamoto et al, 2002; Hermsen et al., 2005; Myo et al., 2005; Rothschild et al., 2006; Ayoub et al., 2010; Sugahara et al., 2011; Pattje et al., 2013; van Kempen et al., 2015).

Several genes within the 11q13.3 amplicon have been proposed as drivers and markers of recurrence and metastasis, including *ANO1* and *CCND1*. Ano1 (a transmembrane calcium-activated chloride channel) has been suggested to contribute to malignant progression by stabilising EGFR at membrane surfaces (thereby promoting mitogenic stimulus to the cell)

(Britschgi et al., 2013; Bill et al., 2015), but may also directly engage with MAP kinase pathways to promote growth and survival (Duvvuri et al., 2012; Sui et al., 2014). Although it is often reported in association with metastasis and recurrence in translational studies, other studies have reported effects on proliferation and local invasion (Ruiz et al., 2012; Shiwarski et al., 2014; Jia et al., 2015), suggesting the tumourigenic functions of Ano1 may be more complex than a 'grow to go' switch. Cortactin (*CTTN* gene) has also been suggested as a potential metastatic driver from the 11q13.3 band, mediating an epithelial-to-mesenchymal switch through interactions with cytoskeletal components (Patel et al., 1998; van Rossum et al., 2006; Rothschild et al., 2006; Luo et al., 2006; Gibcus et al., 2008; Yamada et al., 2010). Regardless of mechanism, our results suggest that *ANO1* copy number gain could act as a strong predictive marker for HPSCC tumours with a high risk of nodal metastasis. Crucial to this supposition would be further studies examining local versus nodal (or distant metastatic) recurrence across the *ANO1* gain positive patients, or re-staging in those *ANO1* gain positive patients with node negative disease at presentation.

Interestingly, our results do not support a similar role for *ANO1/CCND1* copy number gain in nodal metastasis in the HPV/p16 negative OPSCC subgroup. It is plausible that the relevant pathways may be affected downstream of the 11q13.3 genes themselves, or by different means (epigenetic change, mutation) in OPSCC. However this finding could also indicate that the requirements for lymphatic invasion and cell spread differ between the mucosa of the oropharynx and hypopharynx, thus altering the selection pressures between the two tumour types and resulting in different molecular signatures observed in our study.

A mutually exclusive association between 11q13.3 amplification, smoking history and HPV/p16 has been widely reported across many populations (Smeets et al., 2006; Ragin et al., 2006; Klussmann et al., 2009; Lechner et al., 2013; Seiwert et al., 2015). Our findings are consistent with this model, observing again an inverse relationship between HPV/p16 status and the presence of *CCND1* and *ANO1* copy number gains. The pathogenic basis for this genetic distinction likely stems from the selection pressure to overcome the restriction point of G1-to-S cell cycle control in cancer cells. Smoking/alcohol-associated HNSCCs are frequently characterised by amplification and over-

expression of Cyclin D1, often complimented by loss of the G1 restriction point Cyclin Kinase Inhibitor p16INK4A, which itself binds and inactivates the Cyclin D1/CDK4 complex. HPV-associated tumours predominantly preserve p16INK4A, and (as observed here) typically preserve normal *CCND1* copy number. This reflects the actions of viral oncogene E7 in HPV-associated tumours, which binds and inactivates pRB (releasing the pro-proliferative E2F transcription activating factor) effectively committing cells to S phase down-stream of Cyclin D1 / CDK4 / p16INK4A changes, and bypassing the selection pressure to alter Cyclin D1 or p16INK4A found in smoking/alcohol-associated tumours.

Of course, this does not preclude the co-existence of HPV and 11q13.3 amplification in HNSCC, as observed in one patient sample in our study (nor the presence of HPV and the absence of p16, also observed). In some cases, the presence of HPV (whether as 'by-stander' infection or co-factor) may not be the primary driver of tumour progression as it is in those HPV/p16 'dual positive' patients, and pre-existing genetic changes (TP53 mutation, p16INK4A loss, or even 11q13.3 changes) may predominate over the actions of HPV E6 and E7. Such instances are most likely in those HPV-positive patients with a smoking history, as suggested in other studies (Keck et al., 2015) and supported by our own findings. Although these (and other) relationships are somewhat dependent on definitions and detection of HPV/p16 status (in our study grouping all patients without a combined HPV-positive result and p16-positive result) and are often only possible in the diminishing groups of patients with both HPV and smoking histories, the use of both p16 IHC and direct HPV detection adds validity to these observations, and of the distinct aetiological groups existing within our population.

The well-established inverse relationship between HPV and 11q13.3 amplification raises another intriguing question relating to molecular-pathological relationships in HNSCC. In our study population, HPV disease almost universally presents with nodal metastasis (some times in the absence of identifiable primary) but these patients are almost universally devoid of 11q13.3 amplification. Given that, by default, 11q13.3 amplification cannot be contributing to nodal metastasis in HPV-associated disease, what factors (host or viral) are driving a 'metastatic switch'

in HPV/p16 positive patients? In the context of our findings that *ANO1/CCND1* gains do not appear to influence a 'small primary / node positive' pathological pattern in HPV/p16 negative OPSCCs, it would be interesting to explore whether the specific selection pressures or molecular changes underpinning nodal metastasis are convergent across the HPV/p16 positive and negative OPSCC groups (thereby presenting common biomarkers or future therapeutic targets for metastatic disease across both patient groups), or whether yet more distinct molecular changes underpin this critical disease change in the two patient groups.

From a certain perspective, HPV can be considered a 'protective' factor over host genome integrity – by virtue of the 'supra-genomic' mechanisms of action of E6 and E7 in enabling unfettered cell proliferation, HPV can essentially supersede two critical selection pressures in tumour cells (knock out of p53; over-ride of G1-to-S restriction point). In so doing, HPV-associated tumours are more likely to maintain functional p53 (and p16INK4a), providing at least the potential for a degree of genomic integrity, which may well underlie the better patient outcomes observed in HPV-associated disease. However, as demonstrated in our study, the presence of HPV alone is not sufficient for malignant progression, with *TP63* and *PIK3CA* copy number gains detectable at equivalent rates in HPV and smoking/alcohol-associated OPSCCs. In addition to exploring the factors associated with nodal metastasis in HPV-associated tumours (as discussed above), it would be of great interest to assess whether E6 and/or E7 expression at a protein level (and other factors linked to this like viral genome integration) correlate to patterns of copy number gain or other mutations, or indeed to patient outcomes.

PIK3CA (encoding the catalytic p110 subunit of PI3 kinase complex) is a well-established oncogene - mutated, amplified and over-expressed across many common cancers, p110alpha can drive proliferation and survival in tumour cells via activation of central mediator AKT and other down-stream elements of the pathway. NGS projects have consistently found *PIK3CA* gene changes spanning both HPV-associated and smoking/alcohol-associated HNSCC groups (with many reporting increased prevalence of *PIK3CA* change in HPV-associated disease) (Nichols et al., 2013; Cancer Genome Atlas Network, 2015; Keck et al., 2015; Seiwert et al., 2015), suggesting

a possible common evolutionary requirement for maintaining proliferation or de-differentiation across a majority of HNSCCs. Interestingly, while we did observe *PIK3CA* gains across both the HPV/p16-positive and HPV/p16-negative OPSCCs at rates consistent with the literature, *PIK3CA* copy number gain was a rare event in our HPSCC population. This could suggest HPSCCs evolve with different pro-proliferative signalling mutations, or that the involvement of the PI3 kinase pathway in HPSCCs occurs via mechanisms other than copy number gain or amplification. The lack of association between *PIK3CA* (or *TP63*) and pathological stage in our study, combined with the relatively equal distribution across HPV-associated and smoking/alcohol-associated disease add weight to the supposition that these genes have a fundamental role in maintaining proliferation (rather than, for example, driving metastasis) in the subset of tumours harbouring these changes.

Technical differences could potentially contribute to the differences observed in *PIK3CA* copy number gain frequency between our study and previous work. However this seems unlikely owing to a number of observations and measures. First, in an effort to counter any effect of variability of tumour-to-stroma in our samples, and to reduce any effect of different sample age on results, we selected samples for final analysis based on spectrophotometric quality and quantity of nucleic acid, and performed our analysis based on presence or absence of 'large' detectable copy number gains (i.e. more than 4 copies of a target marker, calculated using the ddCt method). Second, our observation of the well-recognised inverse relationship between HPV/p16 and 11q13.3 copy number gains, together with the strong statistical correlation of *CCND1* and *ANO1* gains together in the majority of gain-positive samples provides further re-assurance that our PCR assay results reflect plausible copy number changes in the extracted tumour DNA.

The basis for the reported poor outcomes of HPSCC patients is likely to be multi-factorial. Outcomes may relate to i.) the anatomical position of this tumour subset, ii.) the often cited extensive co-morbidity, and iii.) the lower burden of HPV associated disease (Ernoux-Neufcoeur et al., 2011; Joo et al., 2013; Wendt et al., 2014; Castellsague et al., 2016) in this patient group. In addition to demonstrating the low prevalence of HPV in this patient subset, our results show that the molecular signatures of our HPSCC patients are significantly different to those of our OPSCC

patients at specific genes. Whether these differences (or other molecular differences not explored in our study) underlie prognosis in our patients remains unclear. Evidence for a prognostic effect of 11q13.3 amplification in HNSCC is inconsistent (Ruiz et al., 2012; Muller et al., 1997; Klussman et al., 2009; Hermsen et al., 2005; Rodrigo et al., 2000; Namazie et al., 2002; Ashman et al., 2003; Wreesmann et al., 2004; Xavier et al., 2012), but recent meta-analysis identified *ANO1* (along with FADD) as the strongest potential host prognostic biomarkers across HNSCC (Reddy et al., 2016).

Our study focussed exclusively on the detection of copy number gains of specific genes in the 11q13.3 and 3q sites, however several other genetic changes have been proposed to occur synchronously with these changes in HNSCC (as reviewed in Gollin 2014). Co-amplification between 11q13.3 and 11q22 has recently been confirmed in NGS data (Cancer Genome Atlas Network, 2015), proposed to stem from a selective pressure for amplification and over-expression of both FADD (11q13.3) and BIRC2 (11q22) in HNSCC, with likely anti-apoptotic effect. In other studies, 11q13.3 amplification has been associated with deletions of regions distal to 11q14 (Bockmuhl et al., 2002; Parikh et al., 2007), and deletions of 3p regions, each likely to involve loss of tumour suppressor genes which may contribute to disease progression and clinical outcome. Such associations remain under-reported in HPSCC tumours, and future studies in our sample population would benefit from more comprehensive analysis of the effects of combined gain/loss patterns. Given the high frequency of *ANO1*/*CCND1* copy number gain in HPSCC, it would also be interesting to explore whether tumours from this anatomical site fall predominantly within the basal molecular subtype (where 11q13.3 amplification is observed most frequently in other HNSCCs, as described in Cancer Genome Atlas Network, 2015), and whether this could influence treatment choice in future.

Larger scale studies with sample sizes great enough to control for pathological stage, co-morbidity and treatment modality would be of great value in any future exploration of the apparent molecular-pathogenic differences between our HPV/p16 negative HPSCC and OPSCC patient groups. Given the strong link with nodal metastasis, future studies of tumour recurrence and outcomes in HPSCC in relation to Ano1 and Cyclin D1 amplification and over-expression (and other genes in the

11q13.3 region) are also merited. This could form the basis for a (non-HPV) risk stratification process – with a combination of markers (potentially including *ANO1*) used to identify those patients with high risk of metastatic disease, thereby altering their management to include systemic therapy, surgical nodal clearance, or more intensive follow-up or re-staging.

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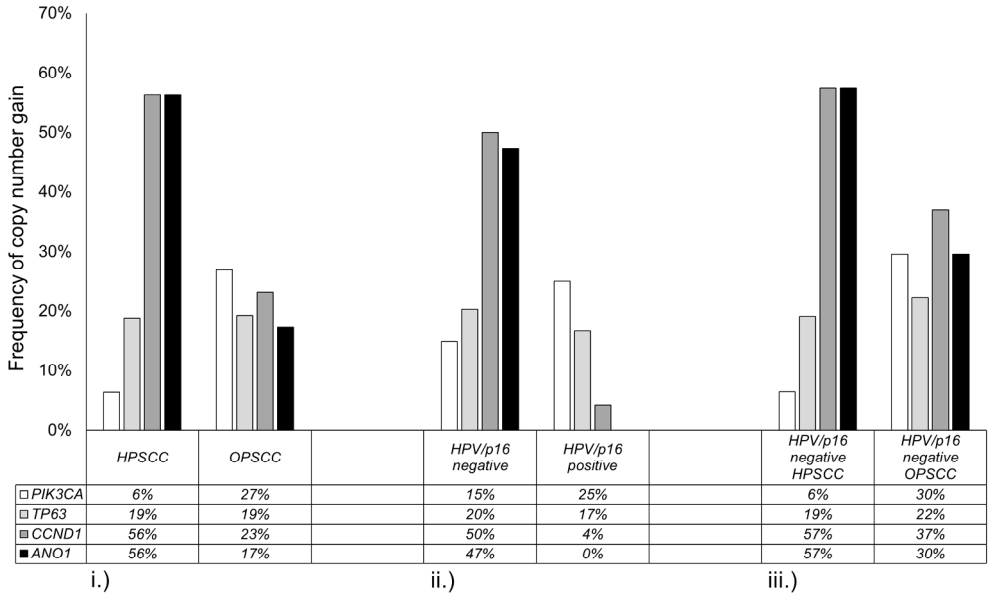

Figure 1. Frequency of copy number gains at target genes across HNSCC patients grouped by i.) anatomical tumour site (HPSCC n=48; OPSCC n=52), ii.) HPV/p16 status (HPV/p16 negative n=47; HPV/p16 positive n=27) and iii.) anatomical tumour site in HPV/p16 negative tumours (HPV/p16 negative HPSCC n=47; HPV/p16 negative OPSCC n=27).

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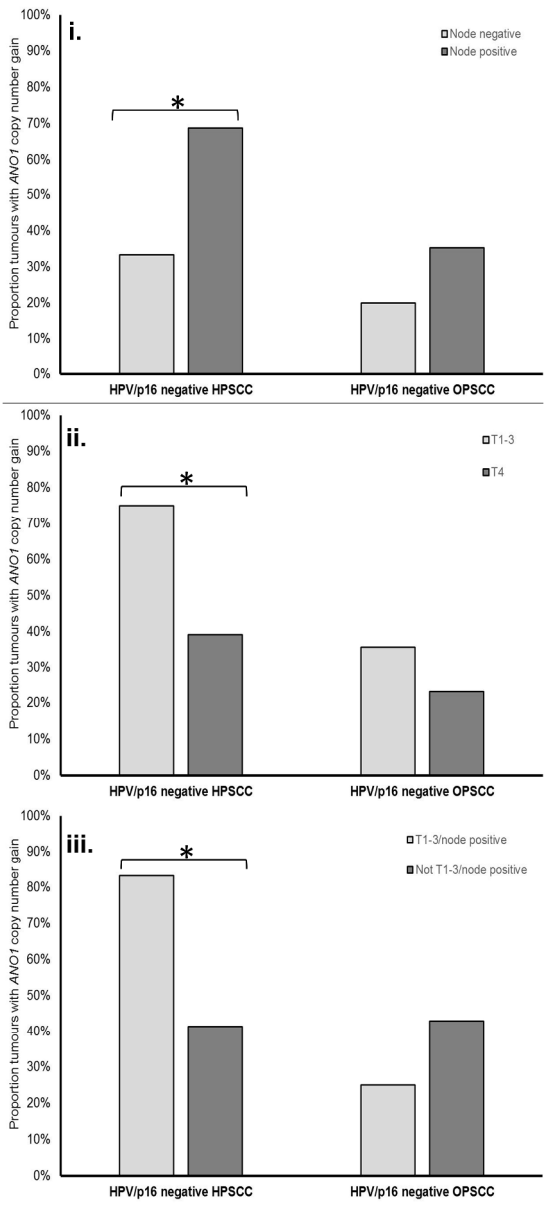

Figure 2. Nodal metastasis and T stage are significantly associated with ANO1 copy number gains in HPV/p16 negative HPSCC, but not in HPV/p16 negative OPSCC tumours. ANO1 copy number gains were significantly more frequent in HPV/p16 negative HPSCC tumours with lymph node metastasis (i, p=0.031), smaller or less invasive tumours as determined by T stage (ii, p=0.022), and in those tumours presenting as both node-positive and T1-3 at presentation (iii, p=0.010). ANO1 copy number gain was not significantly different across pathological stage in HPV/p16 OPSCC tumours. * indicates statistically significant difference (Pearson's Chi Square test).

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Clinico-pathologic characteristic		HPSCC (n=48)	OPSCC (n=52)	p value
Age (mean)		65.92	62.83	0.137
Sex	Male	**39 (81.3)**	**24 (46.2)**	**<0.001**
	Female	**9 (18.8)**	**28 (53.8)**	
Smoking	Never smoker	**7 (14.6)**	**11 (21.2)**	**0.002***
	Past smoker	**9 (18.8)**	**24 (46.2)**	
	Current smoker	**31 (64.6)**	**16 (30.8)**	
	nk	1 (2.1)	1 (1.9)	
Drinking	Never drinker	**4 (8.3)**	**9 (17.3)**	**0.012***
	Social drinker	**19 (39.6)**	**28 (53.8)**	
	Past heavy drinker	**5 (10.4)**	**0 (0)**	
	Current heavy drinker	**17 (35.4)**	**12 (23.1)**	
	nk	3 (6.3)	3 (5.8)	
Stage	T1-3	25 (52.1)	32 (61.5)	0.34
	T4	23 (47.9)	20 (38.5)	
	T1-3/Node positive	19 (39.6)	23 (44.2)	0.638
	Node positive	33 (68.8)	40 (76.9)	
HPV/p16 status	HPV negative/p16 negative	**47 (97.9)**	**21 (42.0)**	**<0.001***
	HPV negative/p16 positive	**0 (0.0)**	**2 (4.0)**	
	HPV positive/p16 negative	**0 (0.0)**	**4 (8.0)**	
	HPV positive/p16 positive	**1 (2.1)**	**23 (46.0)**	
	nk	0 (0)	1 (1.9)	
Copy number gain	*PIK3CA*	**3 (6.3)**	**14 (26.9)**	**0.006**
	TP63	9 (18.8)	10 (19.2)	0.951
	CCND1	**27 (56.3)**	**12 (23.1)**	**<0.001**
	ANO1	**27 (56.3)**	**9 (17.3)**	**<0.001**

Table 1. Clinico-pathologic characteristics of hypopharyngeal squamous cell carcinoma (HPSCC) and oropharyngeal squamous cell carcinoma (OPSCC) patients in the study. Values shown are number (% column total); nk – not known. TNM – as defined by UICC TNM Classification (6th edition); T – tumour size, N – lymph node status. P value indicates significance using Pearson's Chi-square test; ^{f} indicates Fisher's Exact test; ^{t} indicates Student's t-test. * indicates a p-value calculated from grouping patients together (never & social vs ex & current heavy; never & ex smoker vs current smoker; HPV/p16 positive vs HPV/p16 negative as defined in methods).

Clinico-pathologic characteristic		HPV negative/ p16 negative (n=68)	HPV negative/ p16 positive (n=2)	HPV positive/ p16 negative (n=4)	HPV positive/ p16 positive (n=24)		HPV/p16 negative (n=74)	HPV/p16 positive (n=24)	p value
Age (mean)		-	-	-	-		65.2	61.5	0.173
Sex	Male	49 (72.1)	1 (50.0)	0 (0.0)	13 (54.2)		50 (67.6)	13 (54.2)	0.234
	Female	19 (27.9)	1 (50.0)	4 (100.0)	11 (45.8)		24 (32.4)	11 (45.8)	
Smoking	Never smoker	9 (13.6)	0 (0.0)	0 (0.0)	8 (33.3)		**9 (12.5)**	**8 (33.3)**	**<0.001***
	Past smoker	16 (24.2)	0 (0.0)	1 (25.0)	15 (62.5)		**17 (23.6)**	**15 (62.5)**	
	Current smoker	41 (62.1)	2 (100.0)	3 (75.0)	1 (4.2)		**46 (63.9)**	**1 (4.2)**	
Drinking	Never drinker	6 (9.2)	0 (0.0)	0 (0.0)	6 (27.3)		**6 (8.6)**	**6 (27.3)**	**0.002***
	Social drinker	31 (47.7)	0 (0.0)	1 (33.3)	14 (63.6)		**32 (45.7)**	**14 (63.6)**	
	Past heavy drinker	5 (7.7)	0 (0.0)	0 (0.0)	0 (0.0)		**5 (7.1)**	**0 (0.0)**	
	Current heavy drinker	23 (35.4)	2 (100.0)	2 (66.7)	2 (9.1)		**27 (38.6)**	**2 (9.1)**	
Smoking & Drinking	Current smoker & drinker	40 (63.5)	2 (100.0)	2 (66.7)	1 (4.5)		**44 (64.7)**	**1 (4.5)**	**0.005**
Stage	T1-3	35 (51.5)	2 (100.0)	1 (25.0)	18 (75.0)		**38 (51.4)**	**18 (75.0)**	**0.034**
	T4	33(48.5)	0 (0.0)	3 (75.0)	6 (25.0)		**36 (48.6)**	**6 (25.0)**	
	Node positive	44 (64.7)	2 (100.0)	3 (75.0)	23 (95.8)		**49 (66.2)**	**23 (95.8)**	**0.004**
	T1-3/Node positive	22 (32.4)	2 (100.0)	1 (25.0)	17 (70.8)		**25 (33.8)**	**17 (70.8)**	**0.001**
Copy number gain	*PIK3CA*	7 (10.3)	1 (50.0)	3 (75.0)	6 (25.0)		11 (14.9)	6 (25.0)	0.255
	TP63	14 (20.6)	0 (0.0)	1 (25.0)	4 (16.7)		15 (20.3)	4 (16.7)	0.698
	CCND1	34 (50.0)	1 (50.0)	2 (50.0)	1 (4.2)		**37 (50.0)**	**1 (4.2)**	**<0.001**
	ANO1	32 (47.1)	1 (50.0)	2 (50.0)	0 (0.0)		**35 (47.3)**	**0 (0.0)**	**<0.001**

Table 2. Clinico-pathologic characteristics of patients grouped by HPV and p16 status in the study. Values shown are number (% column total); nk – not known. TNM – as defined by UICC TNM Classification (6th edition); T – tumour size, N – lymph node status. P value indicates significance using Pearson's Chi-square test; [f] indicates Fisher's Exact test; [t] indicates Student's t-test. * indicates a p-value calculated from grouping patients together (never & social vs ex & current heavy; never & ex smoker vs current smoker; HPV/p16 positive vs HPV/p16 negative as defined in methods).

Clinico-pathologic characteristic		HPV/p16 negative HPSCC (n=47)	HPV/p16 negative OPSCC (n=27)	p value
Age (mean)		66.1	63.7	0.327t
Sex	Male	**38 (80.9)**	**12 (44.4)**	**0.001**
	Female	**9 (19.1)**	**15 (55.6)**	
Smoking	Never smoker	7 (15.2)	2 (7.7)	0.755
	Past smoker	9 (19.6)	8 (30.8)	
	Current smoker	30 (65.2)	16 (61.5)	
Drinking	Never drinker	4 (9.1)	2 (7.7)	0.66
	Social drinker	19 (43.2)	13 (50.0)	
	Past heavy drinker	5 (11.4)	0 (0.0)	
	Current heavy drinker	16 (36.4)	11 (42.3)	
Smoking & Drinking	Current smoker & drinker	29 (67.4)	15 (60.0)	0.665
Stage	T1-3	24 (51.1)	14 (51.9)	0.948
	T4	23 (48.9)	13 (48.1)	
	Node positive	32 (68.1)	17 (63.0)	
	T1-3/Node positive	18 (38.3)	7 (25.9)	0.279
Copy number gain	*PIK3CA*	**3 (6.4)**	**8 (29.6)**	**0.010f**
	TP63	9 (19.1)	6 (22.2)	0.752
	CCND1	27 (57.4)	10 (37.0)	0.091
	ANO1	**27 (57.4)**	**8 (29.6)**	**0.021**

Table 3. Clinico-pathologic characteristics of HPV/p16 negative subsets of HPSCC and OPSCC patients in the study. Values shown are number (% column total); nk – not known. TNM – as defined by UICC TNM Classification (6th edition); T – tumour size, N – lymph node status. P value indicates significance using Pearson's Chi-square test; ^{f} indicates Fisher's Exact test; ^{t} indicates Student's t-test. * indicates a p-value calculated from grouping patients together (never & social vs ex & current heavy; never & ex smoker vs current smoker; HPV/p16 positive vs HPV/p16 negative as defined in methods).

Clinico-pathologic characteristic		*PIK3CA*	p	*TP63*	p	*CCND1*	p	*ANO1*	p
Sex	Male	3 (7.7)	0.528f	7 (17.9)	0.767	24 (61.5)	0.122f	23 (59.0)	0.335f
	Female	0 (0.0)		2 (22.2)		3 (33.3)		4 (44.4)	
Smoking	Ex or never smoker	2 (12.5)	0.218f	3 (18.8)	0.96	9 (56.3)	0.927	8 (50.0)	0.598
	Current smoker	1 (3.2)		6 (19.4)		17 (54.8)		18 (58.1)	
Drinking	Never or social drinker	1 (4.3)	0.524f	4 (17.4)	0.655	10 (43.5)	0.175	11 (47.8)	0.449
	Ex heavy or current heavy drinker	2 (9.1)		5 (22.7)		14 (63.6)		13 (59.1)	
Smoking & drinking	Current smoker & drinker	1 (3.3)	0.264f	6 (20.0)	0.641f	15 (51.7)	0.919	16 (55.2)	0.592
Stage	T1-3	0 (0.0)	0.102f	7 (28.0)	0.089f	17 (68.0)	0.087	**18 (72.0)**	**0.022**
	T4	3 (13.0)		2 (8.7)		10 (43.5)		**9 (39.1)**	
	Node positive	1 (3.0)	0.172f	7 (21.2)	0.415f	21 (63.6)	0.126	**22 (66.7)**	**0.031**
	T1-3/Node positive	0 (0.0)	0.211f	6 (31.6)	0.073	**14 (73.7)**	**0.049**	**15 (78.9)**	**0.011**
HPV/p16 status	HPV negative/p16 negative	3 (6.4)	0.938f	9 (19.1)	0.813f	27 (57.4)	0.438f	27 (57.4)	0.438f
	HPV negative/p16 positive	-		-		-		-	
	HPV positive/p16 negative	-		-		-		-	
	HPV positive/p16 positive	0 (0.0)		0 (0.0)		0 (0.0)		0 (0.0)	

Table 4. Frequency and association of gene copy number gains across demographic, risk factor and pathological features of HPSCC patients. Values shown are number (% row total). TNM – as defined by UICC TNM Classification (6th edition); T – tumour size, N – lymph node status. P value indicates significance using Pearson's Chi-square test; ^{f} indicates Fisher's Exact test.

Clinico-pathologic characteristic		*PIK3CA*	p value	*TP63*	p value	*CCND1*	p value	*ANO1*	p value
Sex	Male	6 (25.0)	0.772	6 (25.0)	0.266f	5 (20.8)	0.722	4 (16.7)	0.602f
	Female	8 (28.6)		4 (14.3)		7 (25.0)		5 (17.9)	
Smoking	Ex or never smoker	7 (20.0)	0.079f	7 (20.0)	0.619f	**5 (14.3)**	**0.028f**	**3 (8.6)**	**0.020f**
	Current smoker	7 (50.0)		3 (18.8)		**7 (43.8)**		**6 (37.5)**	
Drinking	Never or social drinker	8 (21.6)	0.323f	5 (13.5)	0.300f	7 (18.9)	0.254f	5 (13.5)	0.300f
	Ex heavy or current heavy drinker	4 (33.3)		3 (25.0)		4 (33.3)		3 (25.0)	
Smoking & drinking	Current smoker & drinker	6 (40.0)	0.106f	2 (13.3)	0.515f	6 (40.0)	0.066f	**5 (33.3)**	**0.051f**
Stage	T1-3	11 (34.4)	0.125	7 (21.9)	0.408f	9 (28.1)	0.228f	6 (18.8)	0.519f
	T4	3 (15.0)		3 (15.0)		3 (15.0)		3 (15.0)	
	Node positive	10 (25.0)	0.409f	8 (20.0)	0.582f	7 (17.5)	0.091f	6 (15.0)	0.340f
	T1-3/Node positive	8 (34.8)	0.255	5 (21.7)	0.475	4 (17.4)	0.386	3 (13.0)	0.366f
HPV/p16 status	HPV negative/p16 negative	4 (19.0)		5 (23.8)		7 (33.3)		5 (23.8)	
	HPV negative/p16 positive	1 (50.0)		0 (0.0)		1 (50.0)		1 (50.0)	
	HPV positive/p16 negative	3 (75.0)		1 (25.0)		2 (50.0)		2 (50.0)	
	HPV positive/p16 positive	6 (26.1)		4 (17.4)		1 (4.3)		0 (0.0)	
	HPV/p16 negative	8 (29.6)	0.781	6 (22.2)	0.474	**10 (37.0)**	**0.005**	**8 (29.6)**	**0.004f**
	HPV/p16 positive	6 (26.1)		4 (17.4)		**1 (4.3)**		**0 (0.0)**	

Table 5. Frequency and association of gene copy number gains across demographic, risk factor and pathological features of OPSCC patients. Values shown are number (% row total). TNM – as defined by UICC TNM Classification (6th edition); T – tumour size, N – lymph node status. P value indicates significance using Pearson's Chi-square test; [f] indicates Fisher's Exact test.

Clinico-pathologic characteristic		*PIK3CA*	p value	*TP63*	p value	*CCND1*	p value	*ANO1*	p value
Sex	Male	3 (25.0)	0.484f	3 (25.0)	0.557f	5 (41.7)	0.481f	4 (33.3)	0.516f
	Female	5 (33.3)		3 (20.0)		5 (33.3)		4 (26.7)	
Smoking	Ex or never smoker	1 (10.0)	0.081f	3 (30.0)	0.420f	3 (30.0)	0.391f	2 (20.0)	0.312f
	Current smoker	7 (43.8)		3 (18.8)		7 (43.8)		6 (37.5)	
Drinking	Never or social drinker	4 (26.7)	0.655f	3 (20.0)	0.654f	5 (33.3)	0.598f	4 (26.7)	0.655f
	Ex heavy or current heavy drinker	3 (27.3)		2 (18.2)		4 (36.4)		3 (27.3)	
Smoking & drinking	Current smoker & drinker	6 (40.0)	0.118f	2 (13.3)	0.301f	6 (40.0)	0.470f	5 (33.3)	0.399f
Stage	T1-3	5 (35.7)	0.385f	3 (21.4)	0.638f	7 (50.0)	0.147f	5 (35.7)	0.385f
	T4	3 (23.1)		3 (23.1)		3 (23.1)		3 (23.1)	
	Node positive	5 (29.4)	0.651f	4 (23.5)	0.613f	6 (35.3)	0.563f	6 (35.3)	0.349f
	T1-3/Node positive	3 (42.9)	0.332f	1 (14.3)	0.498f	3 (42.9)	0.525f	3 (42.9)	0.332f

Table 6. Frequency and association of gene copy number gains across demographic, risk factor and pathological features of HPV/p16 negative OPSCC patients. Values shown are number (% row total). TNM – as defined by UICC TNM Classification (6th edition); T – tumour size, N – lymph node status. P value indicates significance using Pearson's Chi-square test; ^{f} indicates Fisher's Exact test.

Supplemental Material S1

Gene Symbol (name)	Genome location (co-ordinates*)
PIK3CA (PI3 kinase alpha)	3q26 (179148114...179235137)
TP63 (p63)	3q28 (189631160...189897279)
CCND1 (Cyclin D1)	11q13 (69641105...69654474)
ANO1 (Anoctamin 1)	11q13.3 (69985875...70189545)
EIF2B4 (Eukaryotic Initiation Factor 2B delta)	2p23.3 (27364352...27370457)

Target & reference genes; *co-ordinates correspond to Homo sapiens reference genome Assembly GRCh38.p2

PRIMER	SEQUENCE	ANNEAL TEMP (°C)	LENGTH	PRODUCT SIZE	FINAL qPCR CONC
gPIK3CAleft	TGATGGCTGCTGACTTACCA	59	20	91	300nM
gPIK3CAright	GTGGCAAACTTCACTGTCGT	59	20		300nM
gTP63left	CAAGCATAGCACGACAGTCC	59	20	109	300nM
gTP63right	CTGTACTACTCTCGCCTGGG	59	20		300nM
gCCND1left	AGCCTGTAACGAACTCCCAA	59	20	135	300nM
gCCND1right	GCATTTCCCCAGACGTCATC	59	20		300nM
gANO1left	AGTAAGTGGGGAATGGGGTG	59	20	122	300nM
gANO1right	CCCCTGGCTTTTCTGGTAGA	59	20		300nM
gEIF2Bleft	AACCAGAGCGTCAACAGGTA	59	20	126	300nM
gEIF2Bright	ACTACTCTGACCCAAGCACC	59	20		300nM

Target & reference primers, amplicons & optimum qPCR conditions

Cycles	Temperature (oC) / duration (second)
1	95 / 120
40	95 / 20
	59 / 20
	72 / 20[F]
1	55-95 / 300*

qPCR reaction conditions – final reaction conditions were 1 X Maxima SYBR Green Master Mix (Thermo), 300-500nM forward primer, 300-500nM reverse primer (see above for primer-specific final concentrations), 2.5ng DNA template (tumour DNA, tonsil DNA or pooled human genomic DNA (Promega)). [F]Fluorescence read at end of each extension step; * varying temperature fluorescence read throughout for melt curve analysis.