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Identification of Prognostic Molecular Biomarkers in 157 HPV-positive and HPV-negative Squamous Cell Carcinomas of the Oropharynx

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Abstract

The incidence of oropharyngeal squamous cell carcinoma (OPSCC) has been increasing due to high-risk HPV infection. We explored the significance of genetic alterations in HPV-positive (HPV-P) and HPV-negative (HPV-N) OPSCC patients on long-term outcome. A total of 157 cases of primary resected OPSCC diagnosed from 1978 to 2005 were subjected to a targeted exome sequencing by MSK-IMPACT™ interrogating somatic mutations in 410 cancer-related genes. Mutational profiles were correlated to recurrence and survival outcomes. OPSCC included 47% HPV-positive (HPV-P) and 53% HPV-negative (HPV-N) tumors arising in the base of tongue (BOT, 43%), palatine tonsil (30%) and soft palate (SP, 27%). HPV negative status, SP location and smoking were associated with poorer outcome. Poorer overall survival was found in *NOTCH1*-mutated HPV-P (p=0.039), and in *SOX2*-amplified HPV-N cases (p=0.036). Chromosomal arm gains in 8p and 8q, and 16q loss were more common in HPV-P (p=0.005, 0.04 and 0.01, respectively), while 9p, 18q and 21q losses were more frequent in HPV-N OPSCC (p=0.006, 0.002 and 0.01, respectively). Novel, potentially functional *JAK3, MYC* and *EP300* intragenic deletions were found in HPV-P, and *FOXP1, CDKN2A, CCND1* and *RUNX1* intragenic deletions and one *FGFR3* inversion were detected in HPV-N tumors. HPV-N/*TP53*-wild type OPSCC harbored recurrent mutations in *NOTCH1/3/4* (39%), *PIK3CA, FAT1,* and *TERT*. In comparison to their oral and laryngeal counterparts, HPV-N OPSCC were genetically distinct. In OPSCC, HPV status, tumor subsite and smoking determine outcome. Risk-stratification can be further refined based on the mutational signature, namely *NOTCH1* and *SOX2* mutation status.

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Keywords

HPV; oropharynx; squamous cell carcinoma; *NOTCH1*; *SOX2*

INTRODUCTION

The incidence of oropharyngeal squamous cell carcinoma (OPSCC) has increased over the past several decades, largely due to the increasing number of human papillomavirus (HPV)-induced tumors [1, 2]. In comparison to HPV-negative (HPV-N) squamous cell carcinoma cases, which typically occur in older men with a significant history of tobacco exposure and increased alcohol consumption, HPV-related OPSCC affects patients who are younger, have less comorbidities, respond favorably to treatment and have remarkably better overall survival (OS) [3–6]. The differences in etiology, clinical presentation and response to treatment suggests that HPV-positive (HPV-P) and HPV-N SCC might be biologically distinct although the main putative mechanisms of early carcinogenesis may be similar in their final result. While the loss of functioning p53 and $p16^{INK4A}$-Cyclin D1-RB pathways in HPV-negative tumors occur through highly recurrent pathogenic *TP53* and *CDKN2A* mutations, these mutations are relatively uncommon in HPV-P SCC [7] and the inactivation of p53 and RB occurs as a result of binding of these tumor suppressors by HPV E6 and E7, respectively [8, 9]. Over the past few years, several genomic studies have emerged highlighting the mutational profiles of HPV-P and HPV-N SCC [7, 10–13]. However, the majority of these prior studies include a relatively small number of cases arising from the oropharynx [7], are focused on all HPV-P tumors irrespective of the site of origin [12], and often compare alterations to HPV-N tumors from the oral cavity, larynx, hypopharynx rather than HPV-N oropharynx tumors. In addition, these prior studies lack clinical outcomes data rendering correlations of genomic changes to outcome very difficult if not impossible [13]. In the present study, we have overcome these limitations by focusing only on oropharyngeal SCC with an approximately equal number of HPV-P and HPV-N tumors from a large retrospective cohort of surgically resected primary OPSCC. This has allowed a more accurate comparison of the genomic alterations between HPV-P and HPV-N oropharyngeal cancer. The long-term follow-up data on survival and recurrence has also enabled us to identify molecular signatures which impact on survival and recurrence for both HPV-P and HPV-N oropharyngeal cancer.

MATERIALS AND METHODS

I. Study cohort

The study was approved by the institutional review board of Memorial Sloan Kettering Cancer Center (MSKCC, New York, NY). A total of 157 patients who were diagnosed with primary OPSCC and underwent surgical resection with curative intent at out institution between 1978 and 2005 with available archival tumor specimens were included in the present study. Patients with recurrent disease, those who received any prior treatment or with tumors arising from other sites with extension to the oropharynx were excluded from the study. The clinical and pathologic features were reviewed. The high risk human papillomavirus (HR-HPV) status in each case was determined based on a positive p16 cytoplasmic and nuclear immunohistochemical stain in at least 70% tumor cells as

previously described [14] and by detection of HPV sequence reads using the bioinformatics algorithm for mapping of off-target reads to the HPV genome [15]. HPV RNA ISH was performed in 5 select cases [16].

II. DNA extraction and targeted capture massively parallel sequencing

Genomic DNA from surgically resected tumor and patient matched normal samples were extracted from formalin-fixed paraffin-embedded (FFPE) specimens. Massively parallel sequencing was performed using MSK-IMPACT™ (Memorial Sloan Kettering-Integrated Mutation Profiling of Actionable Cancer Targets) platform designed for targeted sequencing of exons and select introns of 410 cancer-related genes as previously reported [17, 18], (Supplementary Methods).

III. Functionality of somatic mutations

Potential biological significance of genetic alterations of select genes *(NOTHC1/2/3/4)* were determined using OncoKB annotation [19] and "Functional impact" as listed on www.cbioprtal.org. Mutations designated as likely/predicted oncogenic and/or probably/possibly damaging were referred to as "pathogenic".

IV. Copy number analysis (FACETS)

Copy number aberrations were identified by comparing sequence coverage of targeted regions in a tumor sample relative to a standard diploid normal sample. A minimum of 2-fold change was required to consider gene amplification or deletion. The FACETS analysis was performed in 86 (55%) samples (35 HPV-N and 51 HPV-P) with matched normal DNA and in 71 (45%) samples (48 HPV-N and 23 HPV-P) using unmatched/pool normal DNA as previously described [20]. In brief, the FACETS algorithm determines the total and allele-specific copy number from the sequence coverage and genotypes of polymorphic SNPs (single nucleotide polymorphisms) across the genome, inferred from both on- and off-target read alignments. Estimates of total copy number from FACETS analysis was used for downstream comparison of copy numbers across samples. Due to the sparsity of targeted regions in MSK-IMPACT™ assay, gains and losses were called at the arm level if at least 50% of the arm is gained or lost respectively. These are then compared across HPV status using Fisher's exact test and p-values corrected for multiple testing using false discovery rate correction [21].

V. Statistical analysis

Statistical analyses were performed using the SPSS software 24.0 (IBM Corporation, New York, NY, U.S.) and R [22]. Clinical characteristics, molecular alteration and prognosis were compared using appropriate statistical tests, i.e. Log rank test for survival analysis, Chi-square test or Fisher's exact test for nonparametric variables, and two tailed Student's t test for continuous variables. P values less than 0.05 were considered to be statistically significant. Holm-Bonferroni multiple testing correction was used for comparison of copy number changes in HPV-P and HPV-N OPSCC.

RESULTS

I. Clinicopathologic characteristics and prognosis relative to HPV status

The clinical and pathologic features of the study cohort are summarized in Table 1. In brief, among the 157 patients with OPSCC, 47% (74/157) were positive and 53% (83/157) were negative for HR-HPV. Patients diagnoses rendered over the 26 year-period showed that the proportion of smokers and heavy smokers gradually declined from 87% and 81% to 75% and 61%, respectively, while the proportion on HPV-P tumors showed a gradual temporal increase from 33% to 64% (Figure 1A). Sixty-seven tumors (43%) originated from base of tongue (BOT), 47 (30%) from palatine tonsil, and the remaining 43 (27%) from soft palate (SP). With a median follow up of 67 months overall and 182 months among living patients (range 0.2 – 291 months), 56 patients (36%) exhibited disease progression, including 31 (20%) patients with local recurrence, 19 (12%) patients with regional recurrence and 22 (14%) patients with distant metastases. Compared with HPV-N OPSCC, HPV-P tumors were associated with non-smoker status ($p=0.002$), a tumor location in the BOT and tonsil rather than SP ($p<0.001$), a higher frequency of positive neck disease ($p=0.005$). The remaining clinicopathologic characteristics did not differ significantly based on the HPV status (Table 1). The 5-year and 10-year overall survival (OS) in our cohort was 58% and 44% respectively. Patients harboring HPV-P OPSCC had significantly improved OS and progression-free survival (PFS) compared to their HPV-N counterpart (Figure 1B, 1D, Log rank test, $p<0.001$ for OS and PFS). The 5-year and 10-year OS were 42% and 23% in HPV-N patients, and 76% and 69% in HPV-P patients. In our cohort, HPV-P OPSCCs patients who smoked showed a nonsignificant trend towards poorer outcome compared with never-smokers (Log rank test, $p=0.318$ for OS, and $p=0.229$ for PFS). When comparing tumors from different subsites of the oropharynx, OPSCC from the SP had a poorer OS with OPSCC from the tonsil having the best OS (Figure 1C, 1E, Log rank test, $p<0.001$ for OS, and $p=0.002$ for PFS). Such differences in clinical outcomes were significant when comparing among any two different subsites using Log rank pairwise test (BOT vs. SP: $p=0.036$; BOT vs. tonsil: $p=0.023$, and SP vs. tonsil: $p<0.001$).

II. Genetic characteristics of OPSCC

A. Somatic mutations—The cases were sequenced at a median depth of coverage of 550-fold (range 36–4765) and 858-fold (range 52–5362) for tumor and normal DNA, respectively. Eighty-four (54%) cases were analyzed with matched normal DNA, and in the remaining cases, a pool of 10 normal DNA samples was used because the matched normal DNA was either unavailable or did not pass QC. In addition to 5 promoter (*TERT*) mutations, 1236 exonic mutations were detected in 321 of 410 sequenced genes including 998 missense, 196 protein truncating (frameshift indels, nonsense, splice site and stop-loss) variants, and 42 non-frameshift indel mutations. There was no significant difference in tumor mutation burden (TMB), in respect to the HPV status in OPSCC. The median number of mutations was 5 (range 0–18) and 4 (range 0–60) mutations per case, in HPV-N and HPV-P tumors, respectively (Figure 2A).

The most frequent and potentially significant genetic alterations in OPSCC were mutations in *TP53* (49%), *SOX2* (30%), *CDKN2A/2B* (22%), *PIK3CA* (20%), *TP63* (20%), *KMT2D*

(17%), *NOTCH1* (16%), *FAT1* (11%), and 11q13 (19%), and *FOXA1* amplifications (9%), (Figure 2B).

When comparing HPV-N tumors to HPV-P tumors the most notable genetic differences between the two subsets of OPSCC relative to the HPV/p16 status included higher frequency of mutations in *TP53* (78%, 65/83 vs. 16%, 12/74, p<0.001), *CDKN2A/2B* (39%, 33/83 vs. 4%, 7/74, p<0.001), more 11q13 gene cluster (*FGF3/FGF4/FGF19/CCND1*) amplifications (35%, 29/83 vs. 3%, 2/74, p<0.001), and more *KMT2D* mutations (23%, 19/83 vs. 9%, 7/74, Fisher's exact test, p=0.030) in HPV-N OPSCC (Figure 2). Interestingly, among HPV-P tumors the frequency of *TP53* mutations were significantly higher among cases diagnosed from 1979–1995 than among more recent cases diagnosed from 1996–2005 (25%, 10/38 vs. 6%, 2/36, Fisher's exact test, p=0.020). This correlates with the higher percentage of smokers in earlier years and explains why this study has more *TP53* mutants in HPV-P cases compared to more recent studies.

NOTCH pathway genes (*NOTCH1, NOTCH2, NOTCH3, NOTCH4, EP300, FBXW7, SPEN, KDM5A)* were mutated in 40% (33/83) and 36% (27/74) in HPV-N and HPV-P tumors, respectively. *NOTCH1* mutations alone were detected in 20% (17/83) and 11% (8/74) of HPV-N and HPV-P OPSCC, respectively (Fisher's exact test, p=0.086). *NOTCH2, NOTCH3,* and *NOTCH4* mutations were found in 5% (8/157), 10% (16/157) and 4% (6/157) OPSCC, respectively, with 88% (7/8), 50% (8/16) and 28% (2/7) of these mutations being potentially pathogenic (Supplementary Figure S1). Importantly, HPV-P *NOTCH1* mutated cases showed significantly worse OS than those with intact *NOTCH1* (Log rank test, p=0.039, Figure 3A). Details of the location of the *NOTCH1* mutations are shown in Figure 3C. Two, potentially bi-allelic mutations were detected in 4 cases; 71% (20/29) affected N-terminal EGF-like binding domain, 14% (4/29) were truncating alterations, and 76% (22/29) were likely pathogenic (i.e. likely/predicted oncogenic and/or probably/possibly damaging, Supplementary Table S1).

SOX2 amplifications were present at comparable frequencies in HPV-N and HPV-P cases (28%, 23/83 and 32%, 24/74). However, among HPV-N cases, *SOX2* amplification was found exclusively in *TP53* mutated tumors (Fisher's exact test, p=0.002), and was associated with a worse OS in comparison to *SOX2* copy number neutral cases (Log rank test, p=0.036, Figure 3B). In contrast, amplification of *SOX2* did not affect OS (Log rank test, p = 0.328) in HPV-P OPSCC.

Histone modifiers *(KMT2D, CREBBP, KMT2C, EP300, KMT2A)* were overall more frequently altered in HPV-N tumors than in HPV-P SCC (40%, 33/83 vs. 26%, 9/74, Fisher's exact test, p<0.001, Supplementary Figure S2).

NFE2L2, KEAP1, CUL3 oxidative stress genes mutations were more common in HPV-P tumors (16%,12/74 in HPV-P vs. 6%, 5/83 in HPV-N OPSCC; Fisher's exact test, p=0.069) but this did not reach statistical significance. Interestingly, the majority of these variants were detected in HPV-P tumors (71%) including all 5 *KEAP1* mutations. Four *NFE2L2* mutations, including 2 hotspot variants were detected in HPV-P never smokers (Figure 2, Supplementary Figure S2).

Targetable alterations involving *EGFR, ERBB2, and FGFR1* amplifications *and FGFR3* hotspot mutations (in R249 and S248 codons) were detected in 8% (6/74) and 14% (12/83) HPV-P and HPV-N tumors, respectively (Supplementary Figure S2). Full mutational profiles for all cases are provided in Supplementary Table S2A.

B. Somatic chromosome arm level copy number alterations—OPSCC were enriched in recurrent gains in chromosomes 1q, 3q, 22q and 20 and losses in 3p, 11q and 13q. HPV-P cases showed more frequent gains of chromosome arms 8p (p=0.005) and 8q (p=0.04) and more frequent losses of 16q (p=0.010). In contrast, HPV-N cases showed more frequent losses of 9p (p=0.006), 18q (p=0.002) and 21q (p=0.010, adjusted p-values, Holm-Bonferroni multiple testing correction), (Figure 4). No significant impact on OS was found relative to CNAs (data not shown). 11q losses and 3q gains tended to be more common in HPV-P OPSCC (43% vs 27%, p=0.03 and 62% vs 40%, p=0.007, respectively, Fisher's exact test) although no significant difference was found after Holm-Bonferroni's correction for multiple testing. Details on chromosome arms copy number changes for all cases is provided in Supplementary Table S2B.

C. Structural variants—We identified 9 structural variants with potential functional significance, with 6 being detected in HPV-N and 3 in HPV-P SCC (Figure 4C). Among HPV-N carcinomas, in two cases there were large deletions including the promoter region which suggested loss of transcriptional activity. In one such case, *FOXP1* was affected showing a large deletion involving the 5' UTR and exons 1–6, and in the other case *CDKN2A* showed a 44 Kb intragenic deletion involving the 5' UTR and exon 1 and part of exon 2. In other cases, large deletions suggested a loss of functional domain(s) and/or nearly a complete loss of functional protein. A cell cycle regulator gene *CCND1* showed 1.4 Mb intragenic deletion involving part of exon 1 and remaining downstream exons suggesting a near complete loss of the protein. A kinase encoding gene *FGFR3* showed an 18 Kb inversion with the breakpoints (1) 5 Kb upstream from exon 1 (intragenic region) and (2) within the exon 18, involving the entire protein kinase domain and suggesting a loss of functional protein. A transcription factor gene *RUNX1* showed a 3 Kb inversion involving exon 3 disrupting the RUNT domain. In one case, *NTRK2* showed multiple duplications/deletions suggestive of a processed pseudogene [23]. Among HPV positive cases, *JAK3* was affected in one case showing a 50 Kb deletion involving exon 6 and the remaining downstream exons encoding the entire kinase domain (amino acids 521–777, 822–1095) and FERM domain. In another case, *MYC* oncogene showed a 2 Kb intragenic deletion with the breakpoints (1) in the intron closely downstream to exon 1 and (2) within the exon 2. In another HPV positive case, an epigenetic modifier gene *EP300* showed a 1.2 Kb intragenic deletion of exon 18 and part of exon 19, resulting in the loss of bromodomain. Our findings are in line with previous studies suggesting that most structural variants in head and neck squamous cell carcinoma are more commonly associated with loss of function rather than with protein-coding fusion events [7].

D. Effect of smoking in HPV-P OPSCC—Our clinical outcomes data suggested patients who smoked >10 pack-years had a poorer OS compared to patients who were nonsmokers or smoked <10 pack-years (Figure 1B). Supplementary Figure S3A shows a

comparison of the somatic mutations in HPV-P tumors stratified by smoking status. HPV-P patients who smoked >10 pack-years were more likely to have tumors with a high frequency of *TP53* mutations and *MAP3K13* amplification, as well as a lower frequency of *FOXA1* amplification. However, the difference did not reach significant level due to the small sample size (Fisher's exact test, p = 0.183, 0.140 and 0.146 respectively). Among HPV-P heavy smokers, patients with *TP53*-mutated tumors tend to have worse OS and shorter PFS than *TP53*-wild types (p=0.07 and p=0.149, respectively, Supplementary Figures 3SB and S3C).

E. HPV-negative/TP53-wild type oropharyngeal squamous cell carcinomas— Eighteen patients had HPV-negative/*TP53* wild type tumors. Six (33%) tumors were found to have other known driver oncogenic alterations involving *PIK3CA, HRAS, ATM, MYC, CCND1* and *FGFR1* genes. Notably, 7 (39%) cases harbored *NOTCH1, NOTCH3* and/or *NOTCH4* mutations, and in 2 (11%) cases had *TERT* promoter mutations (Supplementary Figure S4A). Interestingly, this subset of patients showed a tendency towards poorer outcome than HPV-N/*TP53*-mutated cases although a statistical significance was not reached (Supplementary Figure S4B).

III. Pathways altered in OPSCC

Supplementary Figure S2 shows the mutations grouped by pathways in HPV-N and HPV-P tumors. The p53 signaling pathway and cell cycle control pathway were more commonly altered in HPV-N tumors whereas genes involved in oxidative stress were more commonly altered in HPV-P tumors. The PIK3/AKT/mTOR pathway was more commonly altered in HPV-P tumors (47% vs. 37%) but this was not statistically significant. NOTCH signaling was altered in 36% HPV-P tumors and 40% HPV-N tumors. As previously mentioned, mutations in *NOTCH1* were associated with poorer OS in the HPV-P patients (Figure 3A).

IV. Comparison of the genetic profile of OPSCC to other HNSCC in the literature

Supplementary Table S3 shows a comparison of mutational profiles in HNSCC. Comparisons are made only for 410 genes included in the MSK-IMPACT™ panel (Supplementary Table S4).

A. Comparison to reported genetic profiles of HPV-P OPSCC—No significant genetic differences were observed between the primary and recurrent/metastatic HPV-P tumors. Interestingly, HPV-P OPSCC showed fewer *PIK3CA* mutations in comparison to the TCGA HPV-P tumors (19% vs. 56%, Fisher's exact test, p=0.006), (Supplementary Table S3).

B. Comparison to reported genetic profiles of HPV-N SCC: OPSCC vs. oral (OSCC) vs. laryngeal SCC (LSCC)—In comparison to HPV-N OSCC and LSCC, HPV-N OPSCC had less mutations in *CDKN2A* (p=0.010 and p=0.035), *MYC* (p=0.035 and p=0.007), *EGFR* (p=0.013 and 0.053), and *KEAP1* (p=0.054 and p=0.004) respectively. HPV-N OPSCC also had less *FAT1* (p<0.001), *CASP8* (p=0.005), and *NFE2L2* (p=0.042) mutations, and more *ERBB2* (p=0.049) mutations, and more *FOXA1* (p=0.048), *SOX2* (p=0.004), and *NKX2–1* (p=0.048) amplifications than HPV-N OSCC. In addition, HPV-N

OPSCC harbored less *TP53* (p=0.013), *CUL3* (p=0.024), and *TP63* (p=0.031) than HPV-N LSCC (Supplementary Table S3).

Clinical and pathological features of 157 OPSCC are provided in Supplementary Table S5.

DISCUSSION

Here we report the genomic landscape of the largest retrospective OPSCC cohort to date with long-term outcome data which has allowed correlations to be carried out between clinical, pathological and genetic characteristics in both HPV-P and HPV-N OPSCC patients. In contrast to previously published genomic studies on SCC which report on multiple different head and neck sites, we were able to focus specifically on the oropharynx and therefore have been able to carry out a more accurate comparison of the genomic alterations in HPV-P and HPV-N tumors. We found that HPV infection, and tonsil and BOT as primary subsites emerged as favorable prognostic indicators. We found that *NOTCH1* mutations were associated with worse OS in HPV-P OPSCC, and *SOX2* amplifications were associated with worse OS in HPV-N OPSCC. Smokers with HPV-P OPSCC had a poorer OS compared to nonsmokers, harbored *TP53* mutation and *MAP3K13* amplification, and had fewer *FOXA1* amplifications. We also found that HPV-N tumors which were *TP53*-wild-type harbored frequent genetic alterations in the NOTCH pathway. In comparison to their oral and laryngeal counterparts, HPV-N OPSCC were genetically distinct and had less *CDKN2A, MYC, EGFR,* and oxidative stress genes *KEAP1* and *NFE2L2* mutations, and were relatively enriched in *SOX2, FOXA1, ERBB2* and *NKX2–1* amplifications.

Given the time period of the patients' cohort we were able to show the inverse trend of decreasing smoking and increasing HPV infection in OPSCC [1, 3, 4]. Although the reported rate of HPV-P OPSCC in the literature is variable reaching 70% [24], a 47% HPV positivity rate in our study may be explained by a high proportion of cases diagnosed prior to 1995. Even though the majority of HPV-P OPSCC arose in smokers, the significant association of HPV infection with the non-smoker status further supports the established independent oncogenic role of high-risk HPV in OPSCC [1, 4]. The presence of HPV was associated with significantly better outcome; 5-year and 10-year OS were 76% and 69%, respectively in HPV-P cases compared to 42% and 23%, respectively in HPV-N OPSCCs. A poorer outcome of SCC of the SP may be attributed to the predominance of HPV-N tumors in this subsite. Although anatomically a part of the oropharynx, SP mucosa is notably histologically distinct; it lacks a rich lymphoid stroma and specialized epithelium, which are typically seen in tonsil and considered a suitable host environment for HPV infection [25]. The distinct anatomy of SP might contribute to the lower frequency of HPV-P tumors arising in this location in comparison to the BOT and tonsil.

As expected, we found that *TP53* mutations were the most common genetic alteration in our cohort [7, 13, 26] affecting 49% of OPSCC patients and that the presence of *TP53* mutation was associated with a significantly decreased OS (p<0.001, data not shown). When sub-stratified by the HPV-status, *TP53* mutation alone did not significantly impact survival in either HPV-P or HPV-N group suggesting that the survival disadvantage of *TP53* mutation by itself is not sufficient to modify the impact of HPV on outcome in OPSCC. However, among HPV-P

heavy smokers, those with *TP53*-mutated tumors tend to fare worse than their *TP53*-wild type counterparts. This finding emphasizes the importance of the dosage to tobacco carcinogens exposure raising a question if the behavior of HPV-P/*TP53*-mutated OPSCC in heavy smokers may be more similar to that of HPV-N OPSCC and could argue against de-intensified treatment in this subset of HPV-P patients.

NOTCH1 mutations in OPSCC overall occurred at comparable frequencies to those reported on cohorts comprised of predominantly non-oropharyngeal HNSCC, and most variants were detected in the EGF-like ligand binding domain [10, 7]. Importantly, we observed poorer OS in patients with *NOTCH1*-mutated HPV-P tumors compared to their *NOTCH1*-wild type counterparts, but no such difference was seen in the HPV-N group. Clinical studies on the prognostic significance of *NOTCH1* alterations in OPSCC are limited. Tinhofer et al. studied outcome in a SCC cohort treated with concurrent chemoradiation (CXRT), including 60% OPSCC and overall 21% HPV-P tumors, and found that *NOTCH1*-mutated SCC were associated with better outcome irrespective of the HPV status [13]. They suggested that the absence of high expression and activity of NOTCH1, which is reportedly linked to chemoresistance, might explain the better outcome in CXRT-treated *NOTCH1*-mutated cases [13]. In contrast to the latter study and in line with our findings, in an OSCC cohort in Asian patients (lacking information on HPV status or postoperative treatment modality) mutant *NOTCH1* emerged as an independent prognostic indicator of decreased DFS and OS [27]. These results may support *NOTCH1* as a putative tumor suppressor gene in HNSCC. Furthermore, a recent study on a mouse model showed that inactivation of canonical Notch signaling drives head and neck carcinogenesis irrespective of the presence of HPV oncogenes. However, they also demonstrated that HPV oncogenes synergized with lost Notch signaling to induce more and higher grade cancers than seen in the HPV-N mouse model [28]. The latter data may lend support to our observation that *NOTCH1* mutated tumors may be a relatively more aggressive subset of HPV-P OPSCC. We conclude that the prognostic significance of *NOTCH1* mutations in HNSCC needs further investigation and might depend on the HPV status, treatment modality, and the tumor primary site.

Among HPV-N cases, *SOX2* emerged as a gene of prognostic significance. *SOX2* amplification was found exclusively among *TP53* mutated cases (Fisher's exact test, p=0.004), and was associated with worse OS than *SOX2* copy number-neutral cases. This could suggest that overexpressed SOX2 may act in synergy with loss of p53 function and result in a relatively more aggressive subset of HPV-N OPSCC. *SOX2* copy number status and protein expression in HNSCC relative to the HPV status are limited and controversial. While Schrock et al. reported lack of *SOX2* amplification in 31 HPV-P HNSCC along with reduced protein expression suggesting *SOX2* amplification to be mutually exclusive with HPV infection [29], other studies reported SOX2 protein overexpression in up to 92% high risk HPV-P HNSCC [30, 31]. SOX2 protein overexpression was identified as a poor prognostic indicator in HNSCC including sites other than oropharynx [31, 32]. Given the low frequency of HPV-P SCC outside the oropharynx these previously published data are in line with our data and imply that *SOX2* amplification might be a poor prognostic indicator in HPV-N OPSCC. On the other hand, in the study by Bayo et al. in a multivariate analysis on 287 HNSCC (59% from oropharynx) low SOX2 expression emerged as an independent prognostic marker for HNSCC patients overall irrespective of the tumor site or HPV status [31].

Our study also identified chromosomal and structural variant alterations in both HPV-P and HPV-N tumors. 11q losses have been reported to be frequent in HNSCC irrespective of the tumor site or HPV status [7] and commonly co-occur with 11q13 cluster amplification. Interestingly, in our study 11q loss including *ATM* gene tended to be more frequent in HPV-P tumors, which were otherwise characterized by very few 11q13 amplifications. Other recurrent chromosome arms losses implied losses of important tumor suppressor genes such as *SETD2, VHL* and *BAP1* on 3p, and *RB1* on 13q. In contrast, HPV-N tumors had a higher frequency of 9p losses consistent with frequent deletion of *CDKN2A* and *CDKN2B* genes, which is similar to prior reports on HPV-N arising in different head and neck sites [7, 12]. Interestingly, in contrast to prior studies, we also noted that HPV-N OPSCC were enriched for losses of 18q including *SMAD4*, as well as 21q losses. HPV-P had frequent chromosome 8 gains including *FGFR1* and *MYC*, and although gain of 3q including *PIK3CA, SOX2* and *TP63* was common in both subsets [12], it tended to be more common in HPV-P cases. HPV-P OPSCC also harbored more losses of 16q including *CDH1* gene. Loss of functional CDH1 has been reported to be related to tumor infiltration and metastasis in other cancers [33] and raises the question that *CDH1* loss may have a similar effect in HPV-P tumors and contribute to the significantly higher number of N positive cases in comparison to HPV-N patients.

Despite the limitations of our molecular assay to detect gene fusions, our data implied that recurrent gene fusions were not common in OPSCC. This is consistent with prior genomic studies on HNSCC. However, we did identify several novel structural variants with possible loss of functional protein involving *FOXP1, CDKN2A, CCND1, FGFR3* and *RUNX1* in HPV-N, and *JAK3, MYC* and *EP300* in HPV-P tumors. These findings warrant further studies to determine their role in OPSCC carcinogenesis.

In conclusion, our study is unique in that it is the largest genomic study on OPSCC as a single head and neck site to date. By having a cohort of tumors only on the oropharynx subsite with equal numbers of HPV-P and HPV-N tumors we have been able to identify the differences in somatic mutations, chromosomal alterations and structural variants between HPV-P and HPV-N tumors. We were able to observe the differences in respect to the oropharyngeal subsites and identify SP as the location that gives rise to predominantly HPV-N SCC with poor clinical outcome. Our data support the notion that lost Notch signaling in *NOTCH1*-mutated HPV-P tumors may co-operate with HPV oncogenes in the process of carcinogenesis and characterize a relatively more aggressive subset of HPV-P OPSCC. In addition, patients with HPV-P tumors who smoke have poorer outcome and we identify a higher incidence of *TP53* mutations in these patients. Larger studies are therefore needed to assess the risk in heavy smokers with HPV-P/*TP53*-mutant OPSCC. In HPV-N tumors, we identify the prognostic significance of *SOX2* amplification and also show that HPV-N oropharynx tumors have a different genomic profile to HPV-N tumors from other sites of the head and neck such as the oral cavity and larynx. This comparison of tumors shows that HNSCC does not merely comprise two SCC categories that can be either HPV-P or HPV-N but rather, it is a group of subsite-specific SCC that can be further subclassified based on site as well as the HPV status.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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LIST OF ABBREVIATIONS

AJCC	American Joint Committee on Cancer
AWD	alive with disease
BOT	base of tongue
damage	damaging
FFPE	formalin-fixed paraffin-embedded
HNSCC	head and neck squamous cell carcinoma
HPV	human papillomavirus
HR	high risk
HPV-N	HPV-negative
HPV-P	HPV-positive
MSK-IMPACT™	Memorial Sloan Kettering-Integrated Mutation Profiling of Actionable Cancer Targets
NA	not applicable
NED	no evidence of disease
neg	negative
onc	oncogenic
OPSCC	oropharyngeal squamous cell carcinoma
OS	overall survival
PFS	progression-free survival
pos	positive
SCC	squamous cell carcinoma

SNP	single nucleotide polymorphism
SP	soft palate
T	tonsil
unk	unknown

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Novelty & Impact Statement:

NOTCH1 mutations (11%) in HPV-positive and *SOX2* amplification (28%) in HPV-negative oropharyngeal squamous cell carcinoma are poor prognostic indicators suggesting that each group can be further risk sub-stratified based on their mutational signatures. Mutational profiles of HPV-negative squamous cell carcinoma arising in different subsites, oropharynx, larynx and oral cavity are distinct suggesting that HPV-negative squamous cell carcinoma is a genetically heterogeneous disease.

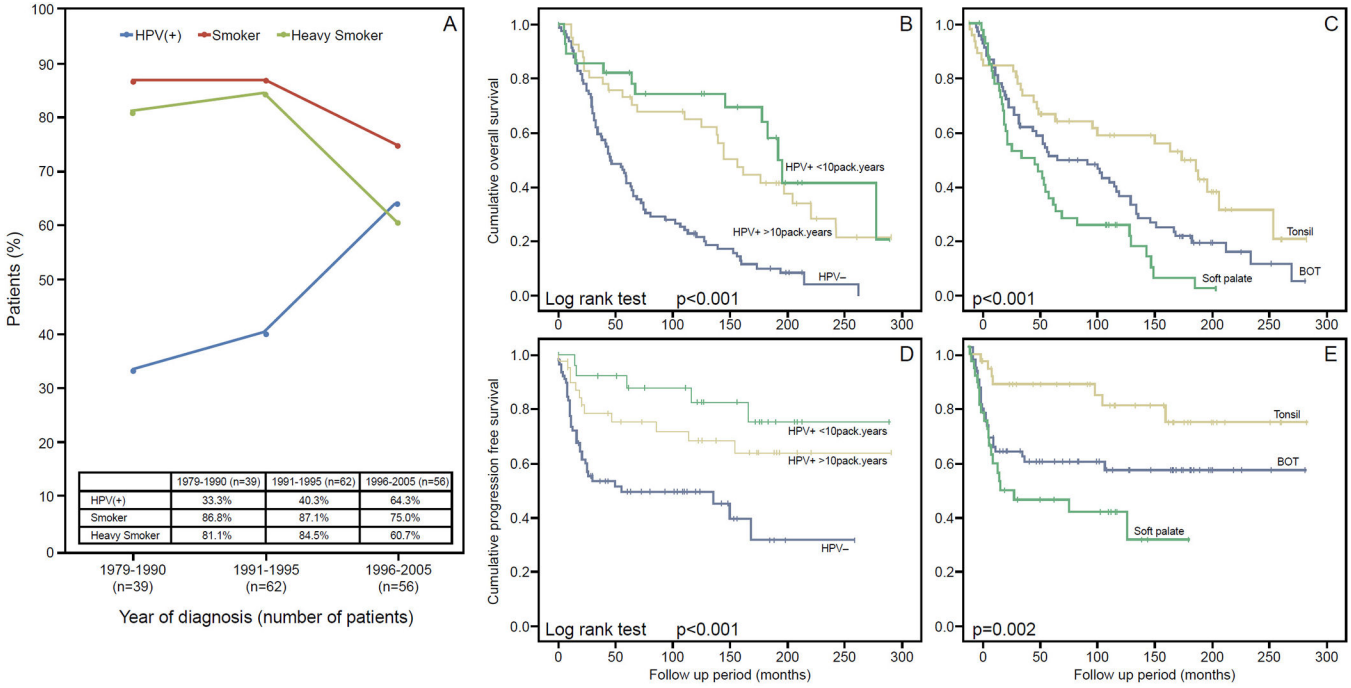

Figure 1. Epidemiology and survival impact of HPV and smoking in OPSCC.

Trend of smoking (red), heavy smoking (>10 pack/years, green) and HPV positivity (blue) in OPSCC over years in the study cohort. The rate of HPV in OPSCC increased, while the proportion of patients who smoked decreased over time (A). Kaplan Meier plots for overall survival and progression free survival according to human papillomavirus (HPV) status, smoking history (B and D) and site of origin (C and E).

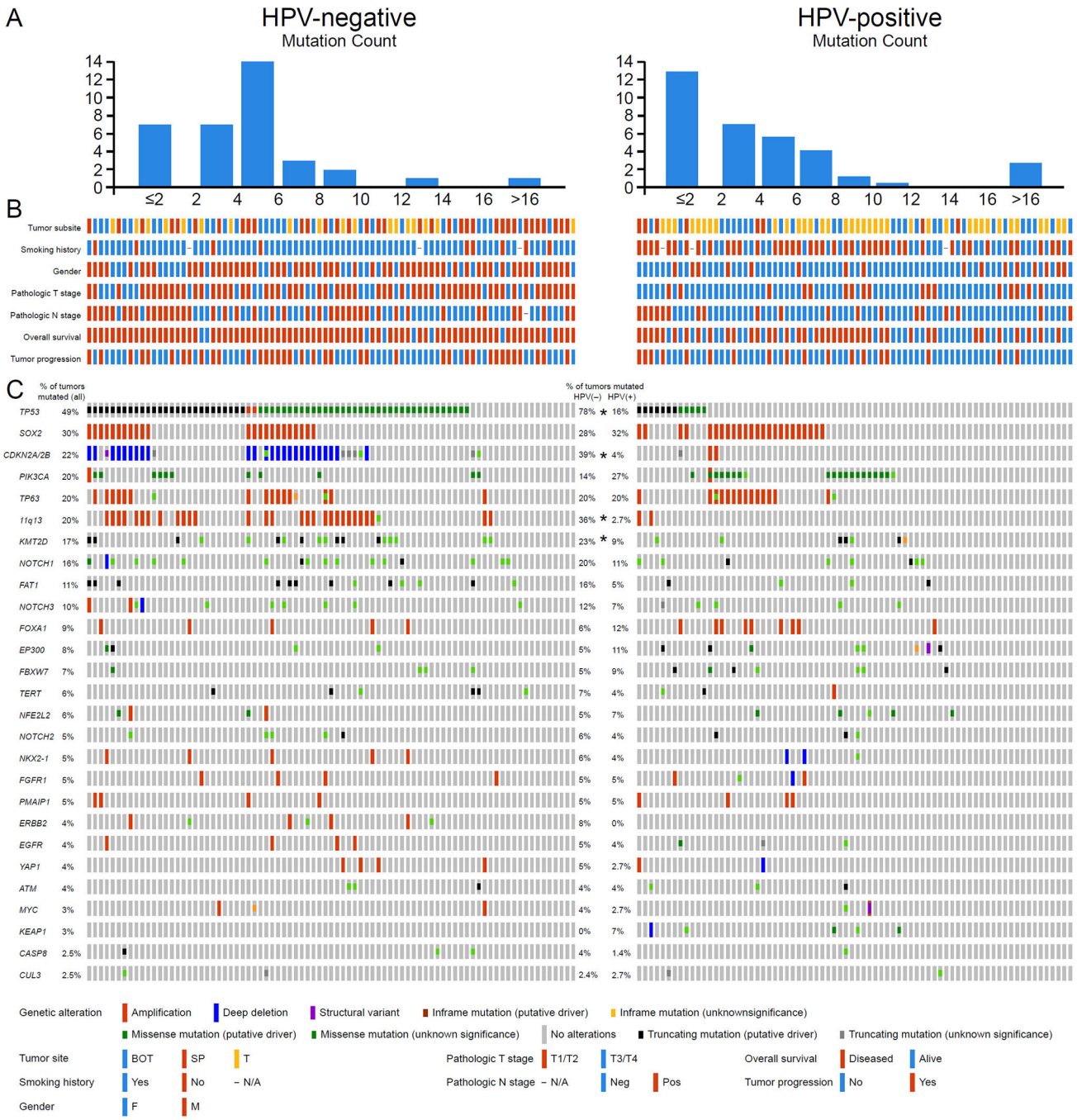

Figure 2. Common genetic alterations in OPSCC according to HPV status.

(A) Tumor mutation burden (TMB) in OPSCC relative to the HPV status. (B) Clinicopathologic features the study cohort based on HPV status of the tumors, including tumor site (SP: soft palate; BOT: base of tongue; T: tonsil), smoking history, gender, AJCC pT stage, AJCC pN stage, overall survival status and tumor progression status. Color keys are shown in the bottom panel. (C) Oncoprints of HPV-negative (left) and HPV-positive (right) OPSCCs. Middle panel shows percentage of tumors altered for each event. *p<0.05 between HPV-negative and HPV-positive OPSCCs (Fisher's exact test).

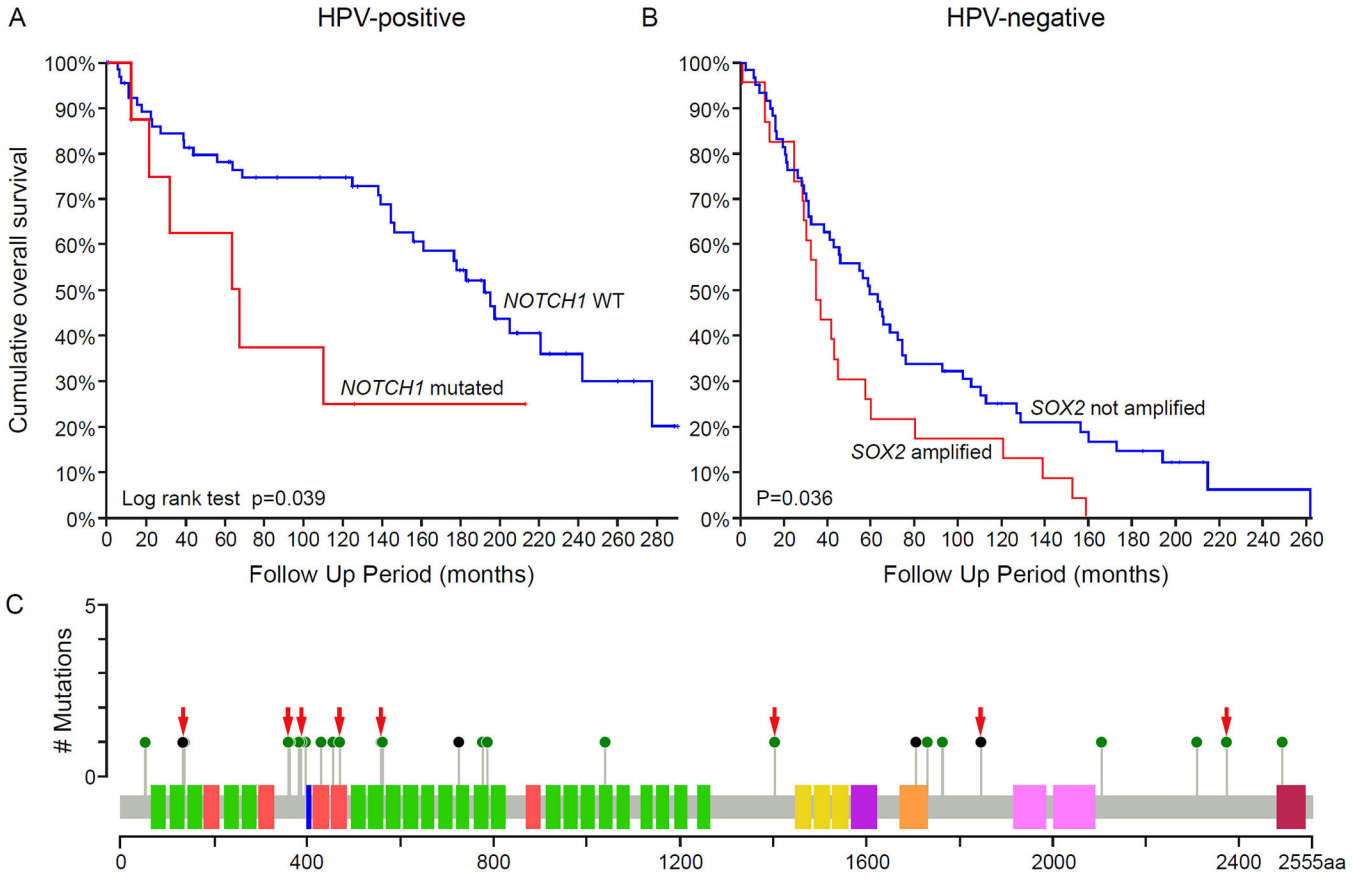

Figure 3. Prognostic molecular signatures for overall survival in OPSCC.

(A) In HPV-positive OPSCC, the presence of *NOTCH1* mutation predicts worse overall survival (OS). (B) In HPV-negative OPSCC, *SOX2* amplification is associated with worse OS. (C) *NOTCH1* mutations in OPSCC. Red arrows point to the mutations in HPV-P tumors.

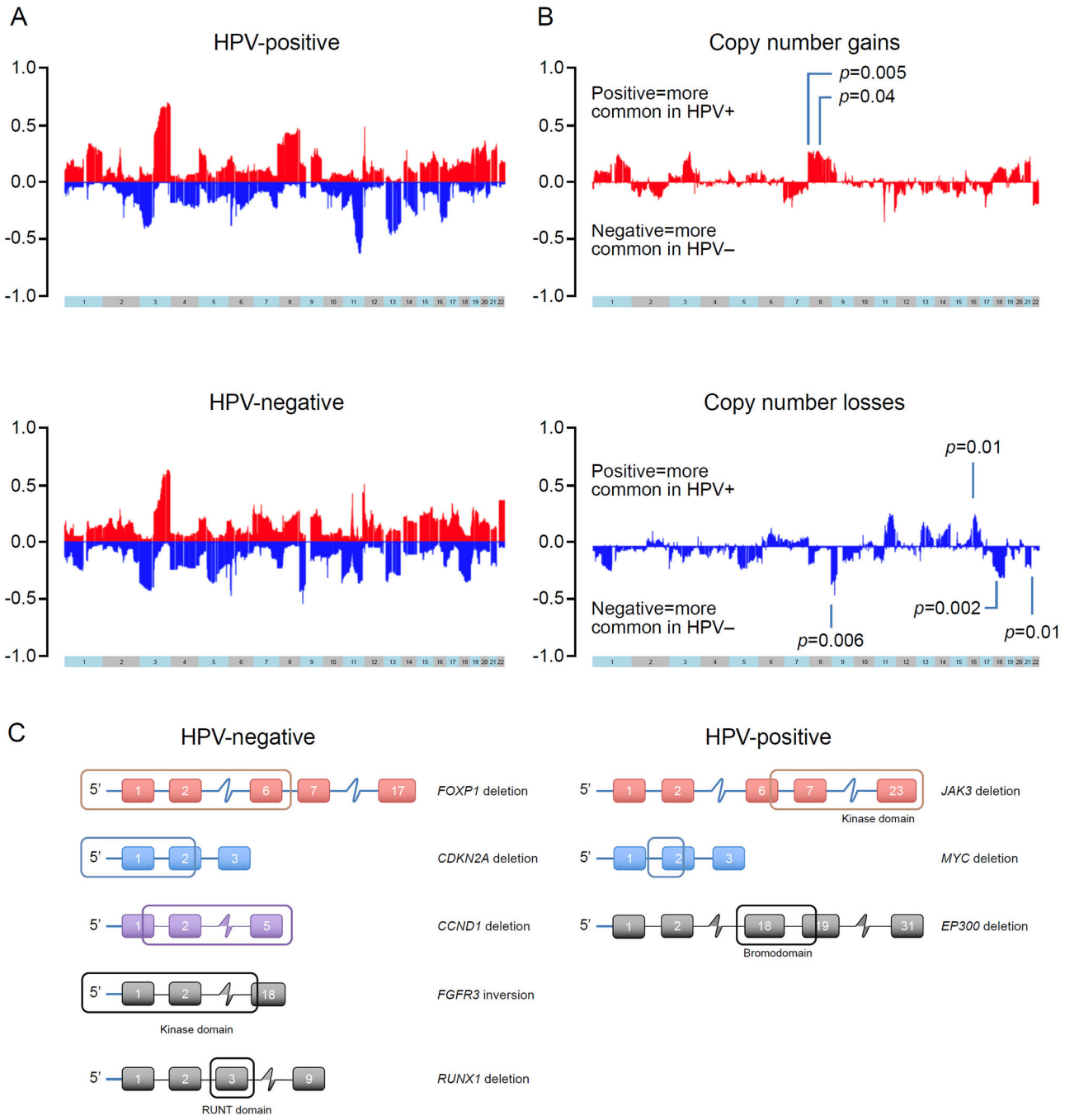

Figure 4. Somatic copy number alterations (CNA) and structural variants in OPSCC.

(A) Frequent gains (red) of 1q, 3q, chromosome 20 and 22q, and losses (blue) of 3p, 11q and 13q were detected among all cases, irrespective of the HPV status. (B) Significant differences in copy number gains (red, top) and losses (blue, bottom). A positive value means that more common in HPV-positive patients and negative means more common in HPV-negative patients. HPV positive tumors showed more frequent gains of chromosome arms 8p (p=0.005), and 8q (p=0.040), and losses of 16q (p=0.010), and HPV negative tumors were significant for more losses of 9p (p=0.006), 18q (p=0.002) and 21q (p=0.010)

(adjusted p-values, Holm-Bonferroni multiple testing correction). (C) Structural variants in OPSCC. Left side and right-side panels depict structural variants in HPV negative and HPV positive tumors, respectively. Affected regions are framed.

Table 1.

Clinicopathologic characteristics of the study cohort.

	HPV (−)	HPV (+)	P value
N	83 (53%)	74 (47%)	
Sex			0.126
Female	27 (33%)	16 (22%)	
Male	56 (67%)	58 (78%)	
Age, median (range)	61 (27 – 79)	59 (35–84)	0.445
Smoking history			0.002
Unknown	1 (1%)	0	
No	7 (8%)	20 (27%)	
Yes	75 (90%)	54 (73%)	
< 10 pack/years	2 (2%)	9 (12%)	
> 10 pack/years	71 (86%)	42 (57%)	
Unknown quantity	2 (2%)	3 (4%)	
Site of origin			< 0.001
Base of tongue	30 (36%)	37 (50%)	
Tonsil	18 (22%)	29 (39%)	
Soft palate	35 (42%)	8 (11%)	
Clinical T staging			0.183
TX	1 (1%)	0	
T1/T2	49 (59%)	52 (70%)	
T3/T4	33 (40%)	22 (30%)	
Clinical N staging			0.02
N0	38 (46%)	20 (27%)	
N+	45 (54%)	54 (73%)	
Perineural invasion			0.171
Unknown	23 (28%)	13 (18%)	
Absent	43 (52%)	49 (66%)	
Present	17 (20%)	12 (16%)	
Vascular invasion			0.264
Unknown	21 (25%)	13 (18%)	
Absent	41 (49%)	49 (66%)	
Present	17 (25%)	15 (20%)	
Surgical margin status			0.791
Unknown	1 (1%)	2 (3%)	
Negative	43 (52%)	38 (51%)	
Positive or close (<5 mm)	39 (47%)	34 (46%)	
AJCC pT staging			0.313
T1/T2	58 (70%)	57 (77%)	

	HPV (−)	HPV (+)	P value
T3/T4	25 (30%)	17 (23%)	
AJCC pN staging			0.005
Nx	1 (1%)	0	
N0	40 (48%)	19 (26%)	
N+	42 (51%)	55 (74%)	
Post-operative radiation			0.008
No	33 (40%)	15 (20%)	
Yes	50 (60%)	59 (80%)	
Follow up period, months, median (range)	153 (9 – 213)	182 (0.2 – 291)	0.626
Overall survival status			NA
Deceased	75 (90%)	40 (54%)	
Alive	8 10%)	34 (46%)	
Progression free survival status			NA
Progressed	38 (46%)	18 (24%)	
Not progressed	45 (54%)	56 (76%)	
Local recurrence			NA
Absent	60 (72%)	66 (89%)	
Present	23 (28%)	8 (11%)	
Regional recurrence			NA
Absent	71 (86%)	67 (91%)	
Present	12 (14%)	7 (9%)	
Distant metastasis			NA
Absent	68 (82%)	67 (91%)	
Present	15 (18%)	7 (9%)	

[a] P values were obtained using Fisher's Exact test or Chi-square test for nonparametric and two-tailed Student's t test for continuous variables.

[b] Deceased patients were excluded from the calculation.

Abbreviations: HPV=human papillomavirus; AJCC=American Joint Committee on Cancer, NA=not applicable.