Genomic and Molecular Profiling of Human Papillomavirus Associated Head and Neck Squamous Cell Carcinoma Treated with Immune Checkpoint Blockade Compared to Survival Outcomes

Hira Shaikh 1, Julie E. McGrath 2, Brittany Hughes 3, Joanne Xiu 2, Pavel Brodskiy 4, Ammar Sukari 5, Sourat Darabi 6, Chukwuemeka Ikpeazu 7,8, Chadi Nabhan 9, Wolfgang Michael Korn 2 and Trisha M. Wise-Draper 1,3,*

Abstract: The prognosis of recurrent and/or metastatic (R/M) head and neck squamous cell carcinoma (HNSCC) remains poor. However, human papillomavirus (HPV)-associated oropharyngeal squamous cell carcinoma (OPSCC) patients live longer than those that are negative for HPV infection. In addition, some R/M HNSCC patients respond well to immune checkpoint blockade (ICB) therapies including pembrolizumab and nivolumab, but whether HPV infection is correlated with a good response to ICB is unclear. Here we attempt to understand if ICB treatment improves survival outcomes of HPV and/or surrogate marker p16+ positive and -negative HNSCC patients. With better biomarkers, future treatment can be better tailored to individual patients to improve survival.

Cancers 2021, 13, 6309. https://doi.org/10.3390/cancers13246309
the p16− group (4.3 vs. 3.3 months, HR 0.632, p = 0.016), suggesting that p16 may be used as a prognostic biomarker in non-OP HNSCC, and further investigation through prospective clinical trials is warranted.

**Keywords:** head and neck squamous cell carcinoma; oropharyngeal cancer; p16; human papillomavirus (HPV); personalized medicine; immune checkpoint blockade; outcomes

1. **Introduction**

Head and neck squamous cell carcinoma (HNSCC) remains the sixth most common cancer worldwide despite recent advances in management, with more than 650,000 cases and 330,000 deaths annually [1]. It is predicted that by the year 2040, the worldwide incidence of HNSCC will increase by 32% and mortality by 34% [2]. Oral cavity, larynx, and hypopharynx cancers are often related to tobacco and alcohol. In contrast, most human-papillomavirus-positive (HPV+) cancers arise from the oropharynx (OP) [3,4]. HPV+ cancers usually demonstrate high p16 expression by immunohistochemistry (IHC), allowing p16 to serve as a surrogate for HPV infection [5,6]. Worldwide, approximately 25% of all HNSCCs are thought to be related to HPV [7]. In the Western world, including the United States and Europe, the incidence of HPV−associated HNSCC has risen substantially, while tobacco- and alcohol-related HNSCC has declined [4,8,9].

Previous studies have consistently shown that HPV−mediated (p16+) oropharyngeal squamous cell cancer (OPSCC) patients have better outcomes [10]. A retrospective analysis of HNSCC patients from the RTOG-0129 study, in which patients received definitive radiotherapy, demonstrated a three-year survival of 82.4% for p16+ compared to 57.1% in p16− patients [10]. The same was true in recurrent/metastatic (R/M) HNSCC. [11–13] To account for disparate outcomes between HPV+ and HPV−disease, genomic signatures have been previously explored [14]. For instance, PIK3CA mutations are common in HPV− cancers, while TP53 mutations are rare [15].

Despite improved outcomes for HPV+ disease, even in the R/M setting, the prognosis remains poor. Immune checkpoint blockade (ICB) is now approved for R/M HNSCC in the first line. However, HPV status and its role in prognosis remains unclear in R/M OPSCC upon treatment with ICB. Although not statistically significant, the overall response rate (ORR) was higher in HPV+ patients (24%) compared to HPV− patients (16%) in the KEYNOTE-012 study, which evaluated pembrolizumab in advanced, heavily pretreated HNSCC [16]. In the KEYNOTE-055 study, evaluating pembrolizumab in R/M platinum and cetuximab refractory HNSCC patients, no difference in progression-free survival (PFS) was observed between HPV+ and HPV− patients [17]. Additionally, in the CheckMate 141 trial, in which nivolumab was compared to the standard of care (SOC) in patients with platinum refractory, R/M HNSCC, a post hoc analysis demonstrated that HPV status did not confer a difference in outcome [18,19].

Given the inconsistent results, interrogation of biomarkers and genomic alterations is important to determine prognosis and potentially guide treatment paradigms in the future. Previously, high tumor mutational burden (TMB) and tumor immune infiltrate due to mutagen exposure has resulted in higher responses to immunotherapy [20–22]. In addition, HPV−related HNSCC has been shown to be enriched with tumor CD8 lymphocytes; the latter has been correlated with better outcomes with the use of ICB [23–25]. However, the significance of molecular, transcriptional, and immune signatures and the correlation with p16 expression and subsequent survival remains unclear.

Based on the above controversial data and increased projection of mortality rates, it is important to further elucidate the role of p16 and HPV in the outcomes of HNSCC patients who receive ICB. In addition, understanding molecular and transcriptional signatures in p16+ vs. p16− patients may indicate predictors of response that may better explain the
characteristics of tumors likely to respond or be resistant to ICB to further guide treatment in the future.

2. Methods

2.1. Samples

We queried the Caris Life Sciences database for p16+ and p16− HNSCC patients. Patients were considered smokers if they had >15 pack-years of tobacco use. Comprehensive molecular profiling, including whole-exome sequencing (WES), targeted Next-Generation Sequencing (NGS), whole transcriptome sequencing (WTS), and immunohistochemistry (IHC), was performed (Caris Life Sciences, Phoenix, AZ, USA).

2.2. Immunohistochemistry Analysis

p16 was determined by IHC, and a standard cut-off of 2+, >70% p16 staining was considered p16+. PD-L1 expression was assessed by the 22c3 antibody with a combined positivity score (CPS) of ≥1 being positive. CPS was determined by calculating the percentage of PD-L1-positive tumor cells, lymphocytes, and macrophages within the total number of viable cells. Mismatch repair (MMR) protein expression was tested by IHC using antibody clones (MLH1, M1 antibody; MSH2, G2191129 antibody; MSH6, 44 antibody [26]; and PMS2, EPR3947 (Ventana Medical Systems, Inc., Tucson, AZ, USA). The complete absence of protein expression of any of the 4 proteins (0+ in 100% of cells) tested was considered deficient MMR (dMMR). Microsatellite Instability Status (dMMR/MSI-H) was determined by a combination of multiple platforms to measure the MSI of MMR status of the tumor profiled, including fragment analysis (FA, Promega, Madison, WI, USA), IHC, and NGS.

2.3. Next-Generation Sequencing (NGS)

NGS was performed on genomic DNA isolated from formalin-fixed paraffin-embedded (FFPE) tumor samples using the nextSeq platform (Illumina, Inc., San Diego, CA, USA) at the Caris Life Sciences laboratory (Phoenix, AZ, USA). A custom-designed SureSelect XT assay was used to enrich 592 whole-gene targets (Agilent Technologies, Santa Clara, CA, USA). All variants were detected with >99% confidence based on allele frequency and amplicon coverage, with an average sequencing depth of coverage >500 and an analytic sensitivity of 5%. Prior to molecular testing, tumor enrichment was achieved by harvesting targeted tissue using manual microdissection techniques. Genetic variants identified were interpreted by board-certified molecular geneticists and categorized as ‘pathogenic,’ ‘likely pathogenic,’ ‘variant of unknown significance,’ ‘likely benign,’ or ‘benign’ according to the American College of Medical Genetics and Genomics (ACMG) standards. When assessing mutation frequencies of individual genes, ‘pathogenic’ and likely pathogenic’ were counted as mutations while ‘benign,’ ‘likely benign,’ and ‘variants of unknown significance’ were excluded. Tumor mutational burden (TMB) was measured by counting all non-synonymous missense, nonsense, in-frame insertion/deletion, and frameshift mutations found per tumor that had not been previously described as germline alterations in dbsNP151 and the Genome Aggregation Database (gnomAD) or as benign variants identified by Caris geneticists. The cutoff point of ≥10 mutations per MB was used based on the KEYNOTE-158 pembrolizumab trial.

2.4. Whole-Exome Sequencing (WES)

Direct Sequence analysis was performed on genomic DNA isolated from a microdissected, formalin-fixed, paraffin-embedded tumor sample using the Illumina Novaseq 6000 sequencers. A hybrid pull-down of baits designed to enrich for more than 700 clinically relevant genes at high coverage and high read-depth was used, along with another panel designed to enrich for an additional >20,000 genes at a lower depth. A 500 Mb SNP backbone panel (Agilent Technologies) was added to assist with gene amplification/deletion measurements. HPV16/18 was detected using the Caris pipeline, which
includes 39 unique baits to detect HPV16 and 50 unique baits to detect HPV18 out of a total of 2360 total pathogen baits. The threshold for positive is ≥100 reads for either HPV16 or HPV18.

2.5. Whole Transcriptome Sequencing and Immune Cell Infiltration

Qiagen RNA FFPE tissue extraction kit was used for extraction, and the RNA quality and quantity were determined using the Agilent TapeStation. Biotinylated RNA baits were hybridized to the synthesized and purified cDNA targets, and the bait–target complexes were amplified in a post-capture PCR reaction. The Illumina NovaSeq 6500 was used to sequence the whole transcriptome from patients to an average of 60 M reads. Raw data were demultiplexed by the Illumina Dragen BioIT accelerator, trimmed, counted, removed of PCR-duplicates, and aligned to human reference genome hg19 by the STAR aligner. For transcription counting, transcripts per million molecules were generated using the Salmon expression pipeline. Immune cell fraction was calculated by Quantiseq using transcriptome data [27].

2.6. Survival Analysis

Real-world overall survival (rwOS) information was obtained from insurance claims data, and Kaplan–Meier estimates were calculated from the first date of contact to the last date of contact or the first day of treatment to the last day of treatment (TOT).

2.7. Statistics

Statistical significance was determined using the Chi-Squared test and Benjamini–Hochberg correction for multiple comparisons. Kaplan–Meier estimates were calculated for molecularly defined patient cohorts. Significance was determined as p values <0.05.

3. Results

3.1. Patient Characteristics

A total of 2905 HNSCC patients were identified in the Caris database, of which 948 were OPSCC. Ages ranged from 15 to 90 years, and the median age of the cohort was 68 years (Table 1). Smoking status was available for 525 patients, and 41% of patients (215/525) were smokers. Among those who were tested for p16 and/or HPV, 32% (251/791) expressed p16 and 28% (91/326) were HPV+. The majority of p16+ tumors were OP in origin (68%, 171/251). In the OPSCC group, 41% were p16+ (171/420) and 52% were HPV positive (71/148).

Table 1. Demographics.

| Age (Years) | Gender | Smokers | P16+ | HPV+ | P16+ and HPV+ OPSCC | P16+ and HPV+ Non-OPSCC HNSCC | Primary | Recurrent/Metastatic |
|-------------|--------|---------|------|------|---------------------|-------------------------------|---------|---------------------|
| Median—68 (Range 15–90) | Male 76.4% (2219/2905) | 41 % (215/525) | 32% (251/791) | 28% (91/326) | 38% (51/134) | 5% (10/182) | 57% (1646/2905) | 43% (1259/2905) |

3.2. Genomic and Molecular Landscape between p16+ and p16−

The most common mutation in the entire cohort of HNSCC was TP53 (54%), followed by CDKN2A (17%), TP53 (33%), PIK3CA (17%), and KMT2D (10.7%) were the most common mutations identified in OPSCC (Table 2). The TP53 mutation was predominant in p16− OPSCC (49%) and non-OP HNSCC (58%) tumors in contrast to p16+ OPSCC (10%) (p < 0.0005). PIK3CA and KMT2D were the most common mutations in p16+ OPSCC (Figure 1A), while
TP53 and TERT mutations were the most common in non-OP HNSCC regardless of p16 status (Figure 1B–D). NOTCH1, CDKN2A, and TERT mutations were more prevalent in OPSCC tumors that were p16− or HPV− in contrast to OPSCC tumors that were p16+ or HPV+ (p < 0.05) (Figure 1E). When the entire cohort of HNSCC patients were analyzed (Figure 1F), there were discrepancies in commonalities of mutation frequencies between p16+ and HPV+ groups. Rb was more frequently mutated in p16+ compared to p16− but not detected in the HPV+ group, and KRAS was more likely mutated in HPV+ with lower rates in p16+ groups and not detected in p16− or HPV− groups. The most frequently identified hotspot TP53 mutations were in codons G245A, R248W, R248Q, G245F, and R248G in p16+ OPSCC and R175H, R248W, R273C, H179Y, and R273L in p16+ non-OP HNSCC.

Table 2. Molecular profiling of p16+ vs. p16− OPSCC.

| Molecular Features | All OPSCC | All Non-OP HNSCC | OPSCC p16+ | Non-OP HNSCC p16+ | OPSCC p16− | Non-OP HNSCC p16− |
|--------------------|-----------|-----------------|------------|-------------------|------------|------------------|
| PD-L1 ≥ 1 (22c3)  | 86.88% (342/394) | 87.59% (628/717) | 90% (154/171) | 86.67% (68/79) | 85% (211/247) | 91.94% (502/546) |
| TP53               | 33% (227/686) | 63.88% (888/1390) | 10% (14/140) | 32.36% (22/68) | 49% (108/219) | 58.33% (280/480) |
| TMB ≥ 10/Mb        | 10% (48/463) | 18.69% (168/999) | 13% (20/145) | 25.76% (17/66) | 14.1% (30/213) | 16.88% (81/480) |
| NOTCH1             | 9.2% (59/654) | 10.48% (237/2233) | 7.4% (9/121) | 12.9% (8/62) | 15.9% (30/189) | 12.05% (50/415) |
| CDKN2A             | 7.6% (44/576) | 22.09% (138/6218) | 0.6% (1/148) | 7.14% (5/70) | 13.2% (28/212) | 16.60% (78/470) |
| TERT               | 3.4% (10/291) | 8.58% (45/527) | 0% (0/28) | 33.33% (3/9) | 32.5% (13/40) | 33.67% (63/189) |
| PIK3CA             | 17.1% (120/702) | 11.31% (139/1405) | 17.1% (25/146) | 22.86% (16/70) | 17.1% (38/222) | 11.25% (55/489) |
| KMT2D              | 10.7% (61/572) | 12.69% (139/1095) | 11.2% (16/142) | 9.09% (6/66) | 9.8% (21/214) | 13.38% (63/471) |

3.3. ICB Biomarker Comparison in p16+ vs. p16− OPSCC

Several markers have been used to predict the response to ICB, including tumor mutational burden (TMB), microsatellite instability (MSI), programmed death-ligand 1 (PD-L1) expression, and tumor immune cell infiltration. PD-L1 positivity was 87%, and 16% had TMB (≥10Mb) for the entire HNSCC cohort. No statistical difference was detected in TMB (≥10Mb), MSI, or PD-L1 between the p16 and HPV OPSCC and non-OP HNSCC groups (Figure 3 and data not shown). However, B-cell, myeloid dendritic cells, and NK cell infiltration was enriched in p16+ versus p16− OPSCC, and neutrophil presence was reduced in p16+ tumors (Figure 4). Conversely, there was no statistically significant difference in macrophage (M1 and M2) and CD8+ T cells between the subgroups.

3.4. Survival Outcomes in p16+ and p16− Disease

Similar to previous reports, p16+ OPSCC patients had a longer survival rate compared to p16− patients with rwOS of 33 vs. 19 months (HR = 0.597, p = 0.001, respectively (Table 3 and Figure 5B). However, there was no difference in time on treatment (TOT) (4.2 vs. 2.8 months, HR 0.796, p = 0.221) between p16+ and p16− OPSCC groups treated with ICB, respectively (Table 3 and Figure 5C). For the non-OP HNSCC cohort, we also detected a longer rwOS for the p16+ group compared to the p16− group similar to OPSCC (34 vs. 17 months, HR 0.551, p = 0.0001, Table 3 and Figure 6B). Converse to the OPSCC group, when non-OP HNSCCs were stratified by treatment with ICB, TOT was higher in the p16+ group compared to the p16− group treated with ICB (4.3 vs. 3.3 months, HR 0.632, p = 0.016, Table 3 and Figure 6C).
Figure 1. Genetic landscape of p16+ and p16− OPSCC. (A–D) Whole-exome sequencing (WES), targeted Next-Generation Sequencing (NGS), and whole-transcriptome sequencing (WTS) were performed to identify the most common mutations and are graphically represented by labeled subgroups. (E,F) OPSCC and HNSCC groups were analyzed to detect the most prevalent mutations in p16 and HPV positive and negative groups. Statistical significance was determined using the chi-squared test and Benjamini–Hochberg correction. **** denotes p < 0.00005, *** denotes p < 0.0005, ** denotes p < 0.005, * denotes p < 0.05.
Figure 2. Copy Number Alterations (CNA) in p16+/− and HPV16+/− OPSCC. Copy number amplifications were detected by WES in p16+, p16−, HPV16+, HPV16− oropharyngeal tumors. Statistical significance was determined using the chi-squared test and Benjamini–Hochberg correction. *** denotes \( p < 0.0005 \) * denotes \( p < 0.05 \).

Table 2. Molecular profiling of p16+ vs. p16− OPSCC.

| Molecular Features | All OPSCC | All Non-OP HNSCC | OPSCC p16+ | Non-OP HNSCC p16− |
|-------------------|-----------|------------------|------------|-------------------|
| PD-L1 ≥ 1 (22c3)  | 86.88%    | 87.59%           | 90%        | 86.07%            |
| TP53              | 33%       | 63.88%           | 10%        | 32.36%            |
| TMB ≥ 10/Mb       | 10%       | 18.69%           | 13%        | 25.76%            |
| NOTCH1            | 9.2%      | 10.48%           | 7.4%       | 12.9%             |
| CDKN2A            | 7.6%      | 22.09%           | 0.6%       | 7.14%             |
| TERT              | 3.4%      | 8.38%            | 0%         | 33.33%            |
| PIK3CA            | 17.1%     | 11.31%           | 17.1%      | 22.86%            |
| KMT2D             | 10.7%     | 12.69%           | 11.2%      | 9.09%             |

3.3. ICB Biomarker Comparison in p16+ vs. p16− OPSCC

Several markers have been used to predict the response to ICB, including tumor mutational burden (TMB), microsatellite instability (MSI), programmed death-ligand 1 (PD-L1) expression, and tumor immune cell infiltration. PD-L1 positivity was 87%, and 16% had TMB (≥10Mb) for the entire HNSCC cohort. No statistical difference was detected in TMB (≥10Mb), MSI, or PD-L1 between the p16 and HPV OPSCC and non-OP HNSCC groups (Figure 3 and data not shown). However, B-cell, myeloid dendritic cells, and NK cell infiltration was enriched in p16+ versus p16− OPSCC, and neutrophil presence was reduced in p16+ tumors (Figure 4). Conversely, there was no statistically significant difference in macrophage (M1 and M2) and CD8+ T cells between the subgroups.

Figure 3. Markers of ICB response in OPSCC. Markers of immune checkpoint inhibitor response (PD-L1 (CPS ≥ 1), TMB ≥ 10/Mb, and dMMR/MSI-H status) were measured in p16+, p16−, HPV16+, and HPV− oropharyngeal cancers. Statistical significance was determined using the chi-squared test and Benjamini–Hochberg correction. No significance (ns) was defined as \( p > 0.05 \).
Figure 3. Markers of ICB response in OPSCC. Markers of immune checkpoint inhibitor response (PD-L1 (CPS ≥1), TMB ≥10/Mb, and dMMR/MSI-H status) were measured in p16+, p16−, HPV16+, and HPV− oropharyngeal cancers. Statistical significance was determined using the chi-squared test and Benjamini–Hochberg correction. No significance (ns) was defined as \( p > 0.05 \).

Figure 4. Immune cell infiltration in p16+ vs. p16− OPSCC. Immune cell fractions were calculated using QuanTIseq computational pipeline and RNA-seq data. The following immune cells were assessed: B cells, M1 and M2 macrophages, Monocytes, Neutrophils, NK cells, CD4+ and CD8+ T cells, Treg cells, and Myeloid dendritic cells in p16+ and p16− oropharyngeal tumors. Statistical significance was determined using the chi-squared test and Benjamini–Hochberg correction. *** denotes \( p < 0.0005 \), ** denotes \( p < 0.005 \), * denotes \( p < 0.05 \).

Table 3. rwOS and TOT (months) in p16+ vs. p16− OPSCC and non-OP HNSCC cohorts.

| rwOS and TOT                     | P16+ (Months) | P16− (Months) | HR    | \( p \) Value |
|----------------------------------|------------|-----------|------|--------------|
| Non-OP HNSCC (rwOS)              | 34         | 17        | 0.551| 0.0001       |
| OPSCC (rwOS)                     | 33.3       | 19.1      | 0.597| 0.001        |
| Non-OP HNSCC treated with ICB (TOT) | 4.3       | 3.3       | 0.632| 0.016        |
| OPSCC treated with ICB (TOT)     | 4.2        | 2.8       | 0.796| 0.221        |
Monocytes, Neutrophils, NK cells, CD4+ and CD8+ T cells, Treg cells, and Myeloid dendritic cells in p16+ and p16− oropharyngeal tumors. Statistical significance was determined using the chi-squared test and Benjamini–Hochberg correction. *** denotes $p < 0.0005$, ** denotes $p < 0.005$, * denotes $p < 0.05$.

### 3.4. Survival Outcomes in p16+ and p16− Disease

Similar to previous reports, p16+ OPSCC patients had a longer survival rate compared to p16− patients with rW OS of 33 vs. 19 months (HR = 0.597, $p = 0.001$), respectively (Table 3 and Figure 5B). However, there was no difference in time on treatment (TOT) (4.2 vs. 2.8 months, HR 0.796, $p = 0.221$) between p16+ and p16− OPSCC groups treated with ICB, respectively (Table 3 and Figure 5C). For the non-OP HNSCC cohort, we also detected a longer rW OS for the p16+ group compared to the p16− group similar to OPSCC (34 vs. 17 months, HR 0.551, $p = 0.0001$, Table 3 and Figure 6B). Conversely to the OPSCC group, when non-OP HNSCCs were stratified by treatment with ICB, TOT was higher in the p16+ group compared to the p16− group treated with ICB (4.3 vs. 3.3 months, HR 0.632, $p = 0.016$, Table 3 and Figure 6C).

**Figure 5.** Real-World Overall Survival (rwOS) in OPSCC patients. Consort diagram detailing real-world data cohorts (a) Kaplan–Meier curves representing (b) rwOS in p16+ vs. p16− OPSCC and (c) TOT in p16+ vs. p16− OPSCC treated with ICB.

**Figure 6.** Real-World Overall Survival in non-OP HNSCC patients. Consort diagram detailing real-world data cohorts (A). rwOS in non-OP HNSCC. Kaplan–Meier curves representing (B) rwOS in p16+ vs. p16− non-OP HNSCC and (C) TOT in p16+ vs. p16− non-OP HNSCC treated with ICB.

| Table 3. rwOS and TOT (months) in p16+ vs. p16− OPSCC and non-OP HNSCC cohorts. |
|---------------------------------------------------------------|
| **Non-OP HNSCC (rwOS)** | 34 17 | 0.551 | 0.0001 |
| **OPSCC (rwOS)** | 33.3 19.1 | 0.597 | 0.001 |
| **Non-OP HNSCC treated with ICB (TOT)** | 4.3 3.3 | 0.632 | 0.016 |
| **OPSCC treated with ICB (TOT)** | 4.2 2.8 | 0.796 | 0.221 |

Discussion

Our findings are consistent with previous work and confirm that TP53, NOTCH1, CDKN2A, TERT, and PIK3CA are the most frequent mutations in OPSCC [28,29]. Several of these mutations are under investigation as possible therapeutic targets. PI3K inhibitors such as BKM120 or BYL719 have been investigated alone or in combination with other agents in multiple cancers, including HNSCC [30]. However, it remains unclear if these mutations serve as independent drivers of pathogenesis and predictors of survival, necessitating further validation and pathway analysis.

The frequency of HPV+ (52%) and P16+ (41%) in the OPSCC group was lower than previously reported (~70%) in the literature [31]. However, this result could be skewed given that the majority of our cohort likely included patients who had relapsed or had metastatic HNSCC. We noted some OPSCC patients with discordant p16 and HPV status (13 p16+/HPV− and 14 p16−/HPV+ out of 125 cases (Figure S1 in Supplementary Materials)), accounting for about 22% of OPSCC cases. Lewis et al. has demonstrated that p16 serves as a superior predictor compared to HPV detection for risk stratification of...
4. Discussion

Our findings are consistent with previous work and confirm that TP53, NOTCH1, CDKN2A, TERT, and PIK3CA are the most frequent mutations in OPSCC [28,29]. Several of these mutations are under investigation as possible therapeutic targets. PI3K inhibitors such as BKM120 or BYL719 have been investigated alone or in combination with other agents in multiple cancers, including HNSCC [30]. However, it remains unclear if these mutations serve as independent drivers of pathogenesis and predictors of survival, necessitating further validation and pathway analysis.

The frequency of HPV+ (52%) and P16+ (41%) in the OPSCC group was lower than previously reported (~70%) in the literature [31]. However, this result could be skewed given that the majority of our cohort likely included patients who had relapsed or had metastatic HNSCC. We noted some OPSCC patients with discordant p16 and HPV status (13 p16+/HPV− and 14 p16−/HPV+ out of 125 cases (Figure S1 in Supplementary Materials)), accounting for about 22% of OPSCC cases. Lewis et al. has demonstrated that p16 serves as a superior predictor compared to HPV detection for risk stratification of OPSCC [6]. Comparison of outcomes between OPSCC patients that were p16+ and HPV+ versus those that were p16+ but HPV− demonstrated no difference in survival in the study [5]. Supporting p16 as a strong predictor of prognosis [32], we also detected better survival in our p16+ non-OPSCC group, whose members were more commonly HPV negative. Therefore, p16 remains a commonly used marker in most centers for risk stratification but understanding the discordance may be relevant in larger populations.

The advent of ICB has revolutionized the treatment paradigm of R/M HNSCC. Immune markers such as PD-L1 and tumor mutational burden (TMB) have emerged as predictors of immune response in various clinical trials [16,17,19,23,33]. Our data correlate with prior reports of PD-L1 positivity of ~85–98% in OPSCC [24,25]. We found no difference in PD-L1 staining in p16+ and p16− OPSCC. Therefore, PD-L1 as a biomarker and ICB response predictor is less impactful in this group. HPV (p16)-related carcinogenesis has been linked to lower rates of TMB but higher frequency of epigenetic changes leading to oligoclonal tumors that have a higher sensitivity to chemotherapy and radiation as well as ICB [34,35]. In our study, we identified no difference in rates of TMB between p16 groups, but this may be due to the small sample size of patients harboring TMB $\geq 10$/Mb (10%).

TP53 mutations were more common in p16− (49%) tumors in contrast to p16+ (10%) ($p < 0.0005$), which is concordant with what was reported by other studies [34]. The prevalence of hotspot TP53 mutations was similar to that previously reported, including TP53 missense mutations at codons R248, R273, G245, R175, R282, and H179 as the most common hotspot mutations in HNSCCs [36]. TP53 mutation has been repeatedly linked to poor outcomes in various malignancies [30], along with low response to ICB [37]. Targeting the mutation has been proposed by many to offset the poor responses to treatment, including ICB. For example, WEE1 kinase inhibitor adavosertib (AZD1775) has shown benefits in TP53 mutant HNSCC [38]. Further studies are under investigation.

Our data concur with previous reports that p16+ OPSCC patients have superior OS compared to p16− patients. However, in the OPSCC p16+ and p16− groups who received ICB, there was no statistically significant difference in the rOS or TOT [10]. These observations could be due to good outcomes in OPSCC patients regardless of the treatment type, short follow-up and/or small sample size, or possibly p16 directed alterations independent of HPV infection.

In contrast, TOT for p16+ non-OP HNSCC patients receiving ICB was longer compared to p16− patients; this finding was not reproduced in the OPSCC subgroup. While some studies have observed longer survival in p16+/HPV+ non-OP HNSCC [39], the outcomes of ICB in p16+ non-OP HNSCC have not been validated in the literature. Notably, a few studies have reported that the survival advantage of p16/HPV does not extend to the non-OP HNSCC [40]. However, many of these studies had smaller numbers, whereas ours is one of the largest cohorts reported. In addition, our cohort involved mostly R/M HNSCC, while previous reports may have had a combination of local advanced and R/M cases. Larger
cohorts studied prospectively would be required to elucidate possible genomic/molecular factors in this rare p16+ non-OP HNSCC subgroup. In addition, randomized controlled trials are required to verify the significance of p16 as a prognostic marker for ICB therapy in both OPSCC and non-OP HNSCC.

Limitations of our study include its retrospective nature, the lack of subjects’ descriptive oncology history because the data were extracted from insurance claims, and the paucity of treatment information around cases prior to obtaining tissue; patients likely received heterogeneous treatment prior to the current data analysis.

5. Conclusion

The molecular and genetic profiling of cancers may enlighten new biomarkers of response as well as potential therapeutic targets. Here, we confirm previous findings that p16+ HNSCC patients have improved survival compared to those with p16− HNSCC. Although we did not detect improved survival in p16+ OPSCC patients upon treatment with ICB, interestingly, p16+ non-OP HNSCC had longer TOT, suggesting improved response to ICB compared to those with p16− disease. In the future, these results may help guide treatment decisions and provide a rationale for further investigation. Clinical trials with large patient populations are required to assess whether p16 and other potential biomarkers can predict ICB treatment response.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/cancers13246309/s1, Figure S1: Site of origin of HNSCC in the study and p16/HPV discordance.

Author Contributions: H.S. along with T.M.W.-D. and W.M.K. conceived and designed the study. J.E.M., J.X. and P.B. collected the data and performed the analysis. B.H., H.S., T.M.W.-D. and J.E.M. wrote the paper. All authors contributed scientific input and reviewed the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: This study was conducted in accordance with the guidelines of the Declaration of Helsinki, Belmont report, and U.S. Common rule. In keeping with 45 CFR 46.101(b)(4), this study was performed utilizing retrospective, de-identified clinical data. Therefore, this study is considered institutional review board (University of Cincinnati IRB) exempt, and no patient consent was necessary from the subjects.

Informed Consent Statement: This study is considered institutional review board (University of Cincinnati IRB) exempt, and no patient consent was necessary from the subjects.

Data Availability Statement: No publicly archived datasets were used for this study.

Conflicts of Interest: H.S., B.H. and T.M.W.-D. declare no conflicts of interest. J.E.M., J.X., P.B. and W.M.K. are all employees of Caris Life Sciences. Sourat Darabi has received honoraria from Oncolens and Bayer.

References
1. Siegel, R.L.; Miller, K.D.; Fuchs, H.E.; Jemal, A. Cancer Statistics, 2021. CA A Cancer J. Clin. 2021, 71, 7–33. [CrossRef] [PubMed]
2. Estimated Number of New Cases from 2020 to 2040, Both Sexes, Age [0–85+]. Available online: https://gco.iarc.fr/tomorrow/en/dataviz/isotype?cancers=1&single_unit=50000&sexes=0 (accessed on 9 January 2021).
3. Gillison, M.L.; Koch, W.M.; Capone, R.B.; Spafford, M.; Westra, W.H.; Wu, L.; Zahurak, M.L.; Daniel, R.W.; Viglione, M.; Symer, D.E.; et al. Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. J. Natl. Cancer Inst. 2000, 92, 709–720. [CrossRef] [PubMed]
4. Gillison, M.L.; Chaturvedi, A.K.; Anderson, W.F.; Fakhry, C. Epidemiology of Human Papillomavirus-Positive Head and Neck Squamous Cell Carcinoma. J. Clin. Oncol. 2015, 33, 3235–3242. [CrossRef]
5. Shi, W.; Kato, H.; Perez-Ordonez, B.; Pintilie, M.; Huang, S.; Hui, A.; O'Sullivan, B.; Waldron, J.; Cummings, B.; Kim, J.; et al. Comparative prognostic value of HPV16 E6 mRNA compared with in situ hybridization for human oropharyngeal squamous carcinoma. J. Clin. Oncol. 2009, 27, 6213–6221. [CrossRef]
6. Lewis, J.S., Jr.; Thorstad, W.L.; Chernock, R.D.; Haughey, B.H.; Yip, J.H.; Zhang, Q.; El-Mofty, S.K. p16 positive oropharyngeal squamous cell carcinoma: an entity with a favorable prognosis regardless of tumor HPV status. Am. J. Surg. Pathol. 2010, 34, 1088–1096. [CrossRef] [PubMed]
7. D’Souza, G.; Dempsey, A. The role of HPV in head and neck cancer and review of the HPV vaccine. *Prev. Med.* **2011**, *53* (Suppl. S1), S5–S11. [CrossRef]

8. Carlander, A.F.; Gronjoh Larsen, C.; Jensen, D.H.; Garmaes, E.; Kiss, K.; Andersen, L.; Olsen, C.H.; Franzmann, M.; Høgdall, E.; Kjaer, S.K.; et al. Continuing rise in oropharyngeal cancer in a high HPV prevalence area: A Danish population-based study from 2011 to 2014. *Eur. J. Cancer* **2017**, *70*, 75–82. [CrossRef] [PubMed]

9. Louie, K.S.; Mehanna, H.; Sasiemi, P. Trends in head and neck cancers in England from 1995 to 2011 and projections up to 2025. *Oral Oncol.* **2015**, *51*, 341–348. [CrossRef]

10. Ang, K.K.; Harris, J.; Wheeler, R.; Weber, R.; Rosenthal, D.I.; Nguyen-Tan, P.F.; Westra, W.H.; Chung, C.H.; Jordan, R.C.; Lu, C.; et al. Human papillomavirus and survival of patients with oropharyngeal cancer. *N. Engl. J. Med.* **2010**, *363*, 24–35. [CrossRef]

11. Fleming, C.W.; Ward, M.C.; Woody, N.M.; Joshi, N.P.; Greskovich, J.F.; Rybicki, L.; Xiong, D.; Contrera, K.; Chute, D.J.; Milas, Z.L.; et al. Identifying an oligometric phenotype in HPV-associated oropharyngeal squamous cell cancer: Implications for clinical trial design. *Oral Oncol.* **2021**, *112*, 105046. [CrossRef]

12. Fakhry, C.; Zhang, Q.; Nguyen-Tan, P.F.; Rosenthal, D.; El-Naggar, A.; Garden, A.S.; Soulieres, D.; Troatti, A.; Avizonis, V.; Ridge, J.A.; et al. Human papillomavirus and overall survival after progression of oropharyngeal squamous cell carcinoma. *J. Clin. Oncol.* **2014**, *32*, 3365–3373. [CrossRef] [PubMed]

13. Faraji, F.; Eisele, D.W.; Fakhry, C. Emerging insights into recurrent and metastatic human papillomavirus-related oropharyngeal squamous cell carcinoma. *Laryngoscope Investig. Otolaryngol.* **2017**, *2*, 10–18. [CrossRef] [PubMed]

14. Pérez Sayáns, M.; Chamorro Petronacci, C.M.; Lorenzo Pouso, A.I.; Fadín Iruegas, E.; Blanco Carrión, A.; Suárez Peñaranda, J.M.; García García, A. Comprehensive Genomic Review of TCGA Head and Neck Squamous Cell Carcinomas (HNSCC). *J. Clin. Med.* **2019**, *8*, 1896. [CrossRef] [PubMed]

15. Zhang, Y.; Koneva, L.A.; Virani, S.; Arthur, A.E.; Virani, A.; Hall, P.B.; Warden, C.D.; Carey, T.E.; Chepeha, D.B.; Prince, M.E.; et al. Subtypes of HPV-Positive Head and Neck Cancers Are Associated with HPV Characteristics, Copy Number Alterations, PIK3CA Mutation, and Pathway Signatures. *Clin. Cancer Res.* **2016**, *22*, 4735–4745. [CrossRef] [PubMed]

16. Mehra, R.; Seiwert, T.Y.; Gupta, S.; Weiss, J.; Gluck, I.; Eder, J.P.; Burtness, B.; Tahara, M.; Keam, B.; Kang, H.; et al. Efficacy and safety of pembrolizumab in recurrent/metastatic head and neck squamous cell carcinoma: Pooled analyses after long-term follow-up in KEYNOTE-028. *Br. J. Cancer* **2018**, *119*, 153–159. [CrossRef] [PubMed]

17. Bauml, J.; Seiwert, T.Y.; Pfister, D.G.; Worden, F.; Liu, S.V.; Gilbert, J.; Saba, N.F.; Weiss, J.; Wirth, L.; Sukari, A.; et al. Pembrolizumab for Platinum- and Cetuximab-Refractory Head and Neck Cancer: Results from a Single-Arm, Phase II Study. *J. Clin. Oncol.* **2017**, *35*, 1542–1549. [CrossRef]

18. Ferris, R.L.; Blumenschein, G.R.; Fayette, J.; Guigay, J.; Colevas, A.D.; Licitra, L.F.; Harrington, K.; Kasper, S.; Vokes, E.E.; Even, C.; et al. Further evaluations of nivolumab (nivo) versus investigator’s choice (IC) chemotherapy for recurrent or metastatic (R/M) squamous cell carcinoma of the head and neck (SCCHN): CheckMate 141. *J. Clin. Oncol.* **2016**, *34*, 6009. [CrossRef]

19. Ferris, R.L.; Blumenschein, G., Jr.; Fayette, J.; Guigay, J.; Colevas, A.D.; Licitra, L.; Harrington, K.; Kasper, S.; Vokes, E.E.; Even, C.; et al. Nivolumab vs investigator’s choice in recurrent or metastatic squamous cell carcinoma of the head and neck: 2-Year long-term survival update of CheckMate 141 with analyses by tumor PD-L1 expression. *Oral Oncol.* **2018**, *81*, 45–51. [CrossRef] [PubMed]

20. Ferris, R.L.; Blumenschein, G., Jr.; Fayette, J.; Guigay, J.; Colevas, A.D.; Licitra, L.; Harrington, K.; Kasper, S.; Vokes, E.E.; Even, C.; et al. Nivolumab for Recurrent Squamous-Cell Carcinoma of the Head and Neck. *N. Engl. J. Med.* **2016**, *375*, 1856–1867. [CrossRef]

21. Desrichard, A.; Kuo, F.; Chowell, D.; Lee, K.W.; Riaz, N.; Wong, R.J.; Chan, T.A.; Morris, L.G.T. Tobacco Smoking-Associated Alterations in the Immune Microenvironment of Squamous Cell Carcinomas. *J. Natl. Cancer Inst.* **2018**, *110*, 1386–1392. [CrossRef]

22. Alexandrov, L.B.; Nik-Zainal, S.; Wedge, D.C.; Aparicio, S.A.; Behjati, S.; Biankin, A.V.; Bignell, G.R.; Bolli, N.; Borg, A.; Borresen-Dale, A.L.; et al. Signatures of mutational processes in human cancer. *Nature* **2013**, *500*, 415–421. [CrossRef]

23. Mandaal, R.; Şenbabaoğlu, Y.; Desrichard, A.; Havel, J.J.; Dalin, M.G.; Riaz, N.; Lee, K.W.; Ganly, I.; Hakimi, A.A.; Chan, T.A.; et al. The head and neck cancer immune landscape and its immunotherapeutic implications. *JCI Insight* **2016**, *1*, e98829. [CrossRef]

24. Chen, X.J.; Tan, Y.Q.; Zhang, N.; He, M.J.; Zhou, G. Expression of programmed cell death-ligand 1 in oral squamous cell carcinoma and oral leukoplaikia is associated with disease progress and CD8+ tumor-infiltrating lymphocytes. *Pathol. Res. Pract.* **2019**, *215*, 152418. [CrossRef]

25. Hong, A.M.; Ferguson, P.; Dodds, T.; Jones, D.; Li, M.; Yang, J.; Scolyer, R.A. Significant association of PD-L1 expression with human papillomavirus positivity and its prognostic impact in oropharyngeal cancer. *Oral Oncol.* **2019**, *92*, 33–39. [CrossRef] [PubMed]

26. Vanderwalde, A.; Spetzler, D.; Xiao, N.; Gatalica, Z.; Marshall, J. Microsatellite instability status determined by next-generation sequencing and compared with PD-L1 and tumor mutational burden in 11,348 patients. *Cancer Med.* **2018**, *7*, 746–756. [CrossRef]

27. Finotello, F.; Mayer, C.; Plattnier, C.; Laschober, G.; Rieder, D.; Hackl, H.; Krogsgaard, A.; Loncova, Z.; Posch, W.; Wilflingseder, D.; et al. Molecular and pharmacological modulators of the tumor immune contexture revealed by deconvolution of RNA-seq data. *Genome Med.* **2019**, *11*, 34. [CrossRef] [PubMed]
Cancers 2021, 13, 6309

28. Nakagaki, T.; Tamura, M.; Kobashi, K.; Koyama, R.; Fukushima, H.; Ohashi, T.; Idogawa, M.; Ogi, K.; Hiratsuka, H.; Tokino, T.; et al. Profiling cancer-related gene mutations in oral squamous cell carcinoma from Japanese patients by targeted amplicon sequencing. *Oncotarget* 2017, 8, 59113–59122. [CrossRef] [PubMed]

29. Stransky, N.; Egloff, A.M.; Tward, A.D.; Kostic, A.D.; Cibulskis, K.; Sivachenko, A.; Kryukov, G.V.; Lawrence, M.S.; Sougnez, C.; McKenna, A.; et al. The mutational landscape of head and neck squamous cell carcinoma. *Science* 2011, 333, 1157–1160. [CrossRef] [PubMed]

30. Tinhofer, I.; Budach, V.; Saki, M.; Konschak, R.; Niehr, F.; Jöhrens, K.; Weichert, W.; Linge, A.; Lohaus, F.; Krause, M.; et al. Targeted next-generation sequencing of locally advanced squamous cell carcinomas of the head and neck reveals druggable targets for improving adjuvant chemoradiation. *Eur. J. Cancer* 2016, 57, 78–86. [CrossRef]

31. Pan, C.; Issaeva, N.; Yarbrough, W.G. HPV-driven oropharyngeal cancer: Current knowledge of molecular biology and mechanisms of carcinogenesis. *Cancers Head Neck* 2018, 3, 12. [CrossRef]

32. Chung, C.H.; Zhang, Q.; Kong, C.S.; Harris, J.; Fertig, E.J.; Harari, P.M.; Wang, D.; Redmond, K.P.; Shenouda, G.; Troatti, A.; et al. p16 protein expression and human papillomavirus status as prognostic biomarkers of nonoropharyngeal head and neck squamous cell carcinoma. *J. Clin. Oncol.* 2014, 32, 3930–3938. [CrossRef] [PubMed]

33. Wang, J.; Sun, H.; Zeng, Q.; Guo, X.-J.; Wang, H.; Liu, H.-H.; Dong, Z.-Y. HPV-positive status associated with inflamed immune microenvironment and improved response to anti-PD-1 therapy in head and neck squamous cell carcinoma. *Sci. Rep.* 2019, 9, 13404. [CrossRef]

34. Perri, F.; Longo, F.; Caponigro, F.; Sandomenico, F.; Guida, A.; Della Vittoria Scarpati, G.; Ottaiano, A.; Muto, P.; Ionna, F. Management of HPV-Related Squamous Cell Carcinoma of the Head and Neck: Pitfalls and Caveat. *Cancers* 2020, 12, 975. [CrossRef]

35. Havel, J.J.; Chowell, D.; Chan, T.A. The evolving landscape of biomarkers for checkpoint inhibitor immunotherapy. *Nat. Rev. Cancer* 2019, 19, 133–150. [CrossRef] [PubMed]

36. Zhou, G.; Liu, Z.; Myers, J.N. TP53 Mutations in Head and Neck Squamous Cell Carcinoma and Their Impact on Disease Progression and Treatment Response. *J. Cell. Biochem.* 2016, 117, 2682–2692. [CrossRef]

37. Klimakis, A.; Rampias, T. TP53 mutational landscape of metastatic head and neck cancer reveals patterns of mutation selection. *EBioMedicine* 2020, 58, 102905. [CrossRef]

38. Kong, A.; Good, J.; Kirkham, A.; Savage, J.; Mant, R.; Llewellyn, L.; Parish, J.; Spruce, R.; Forster, M.; Schipani, S.; et al. Phase I trial of WEE1 inhibition with chemotherapy and radiotherapy as adjuvant treatment, and a window of opportunity trial with cisplatin in patients with head and neck cancer: The WISTERIA trial protocol. *BMJ Open* 2020, 10, e033009. [CrossRef] [PubMed]

39. Janecka-Widła, A.; Mucha-Malecka, A.; Majchrzyk, K.; Halaszkiewicz, K.; Przewoźnik, M.; Slonina, D.; Biesaga, B. Active HPV infection and its influence on survival in head and neck squamous-cell cancer. *J. Cancer Res. Clin. Oncol.* 2020, 146, 1677–1692. [CrossRef]

40. Clancy, K.; Hamill, C.S.; O’Neill, W.Q.; Vu, B.; Thuener, J.; Gui, S.; Li, S.; Fowler, N.; Rezaee, R.; Lavertu, P.; et al. Impact of p16 Status and Anatomical Site in Anti-PD-1 Immunotherapy-Treated Recurrent/Metastatic Head and Neck Squamous Cell Carcinoma Patients. *Cancers* 2021, 13, 4861. [CrossRef] [PubMed]