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Prognostic Impact of Genetic Mutations and HPV Viral Integration in Locally Advanced Head and Neck Squamous Cell Carcinoma Patients

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An Abstract of
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Abstract

Prognostic Impact of Genetic Mutations and HPV Viral Integration in Locally Advanced Head and Neck Squamous Cell Carcinoma Patients

By Collin V. Brummel

Background: Head and neck squamous cell carcinoma (HNSCC) is an aggressive cancer typically caused by repeated exposure to carcinogens such as alcohol or tobacco, or via infection by high-risk strains of human papillomavirus (HPV). Five-year overall survival (OS) is between 65%-95%. Locally advanced HNSCC is treated with a combination of surgery, chemotherapy, and radiation. While the treatment goal is curative, treatment profoundly impacts quality of life. Novel treatment approaches are needed to maximize curative potential while improving patient quality of life post-treatment.

Objective: This study validates whether variables previously reported to have prognostic significance in HNSCC, TP53 or NOTCH1 genomic alterations, impact survival outcomes in a cohort of HNSCC tumors. Additionally, this study investigates whether HPV viral load within a tumor affects 5-year OS within a subset of these patients.

Methods: A de-identified dataset containing clinical, demographic, genomic, and viral data on 250 patients treated at the University of Michigan between 2000-2022 was analyzed. The primary outcome of interest was 5-year (OS). Patients were stratified by variables of interest and survival analyses were performed via the Kaplan-Meier method. Survival distributions were compared using the Mantel-Cox log-rank test, with putative predictor variables for OS further analyzed via univariate Cox proportional hazards regression analysis.

Results: Patients with TP53 mutated tumors had significantly worse 5-year OS (29.5% vs 72.8%, $p < 0.0001$) compared to those with TP53 wildtype tumors. NOTCH1 and HPV viral read count did not have a significant effect on OS. Cox proportional hazards analysis of baseline differences between the TP53 mutated and wildtype cohorts failed to identify an alternative predictor of OS.

Conclusion: TP53 mutation status, but not NOTCH1 mutation status or HPV viral read count quartile, significantly impacted 5-year OS in 231 locally advanced HNSCC patients. NOTCH1 and HPV viral read count were not recapitulated. This cohort may be useful in further assessing 1) the prognostic relevance of NOTCH1 mutations and HPV viral read count in HNSCC and 2) identifying other biomarkers that could optimize existing treatment approaches for HNC to improve survival and quality of life outcomes for patients affected by this highly morbid cancer type.

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Chapter 1: Introduction

Head and neck squamous cell carcinoma (HNSCC) is the 7th most common cancer worldwide, affecting over 800,000 people annually (Chow, 2020). The three major subsites for HNSCC tumor growth include the oral cavity, oropharynx, and larynx/hypopharynx. Historically, HNSCC etiology has been associated with heavy alcohol and tobacco exposure. However, more recently, human papillomavirus (HPV) infection has been identified as a distinct causative agent of HNSCC, particularly among tumors originating in the oropharynx. HPV-associated HNSCC (HPV+ HNSCC) is one of the few cancers recently identified to be increasing in incidence in the United States (Siegel et al., 2023).

Five-year survival for non-HPV driven HNSCC (HPV- HNSCC) is around 65% (Cohen et al., 2018). Survival for HPV+ HNSCC is higher: between 85-95% depending on other risk factors (Leeman et al., 2017). However, in those who develop recurrent disease, treatment options are limited and prognosis is poor (Wang et al., 2022).

Treatment options for both HPV+ and HPV- HNSCC may involve surgery, chemoradiation, targeted immunotherapies, or some combination of the three (Leeman et al., 2017). Genomic and/or viral signatures may help stratify which patients require higher intensity treatment from those who may benefit from reduced exposure to radiation and/or chemoimmunotherapy, particularly in HPV+ HNSCC where survival rates are high but treatment-related quality of life is often diminished (Wang et al., 2022). Identifying genomic drivers of HNSCC may also help determine which targeted immunotherapies may be most impactful, both in the upfront and metastatic treatment settings (Solomon et al., 2018).

For patients with HPV+ cancer, disease is caused by a few high-risk HPV subtypes; in HNSCC, subtype 16 (HPV16) is the most prevalent high-risk strain and is estimated to cause 80 - 90% of HPV+ HNSCC tumors (Gillison et al., 2015). While presence of HPV16 is well-documented in HPV+ HNSCC, whether the amount of HPV16 integration within these tumors is associated with disease prognosis is understudied.

Mutations or loss of the tumor suppressor gene *TP53* are found in many cancers. In almost every cancer where they are observed, loss of *TP53* function is associated with poor prognosis (Olivier et al., 2010; Petitjean et al., 2007); prior studies have demonstrated that mutation/loss of *TP53* in HNSCC is also associated with poor prognosis (Nathan et al., 2022; Solomon et al., 2018).

After *TP53*, *NOTCH1* is the second most mutated gene in head and neck cancers (Fukusumi & Califano, 2018). Both HPV and non-HPV associated HNSCCs may be marked by a mutation in the *NOTCH1* gene. HNSCC tumors with *NOTCH1* mutations have been shown to confer worse prognosis compared with wildtype *NOTCH1* (Fukusumi & Califano, 2018).

The aim of this study is twofold: 1) to determine whether HPV16 viral load within HPV+ HNSCC tumors impacts prognosis/survival, and 2) to validate whether *TP53* and *NOTCH1* mutations also confer poor prognosis in a large cohort of patients treated at the University of Michigan. This pilot dataset is intended to discover trends and associations in HNSCC genomics and viral signatures that may serve to guide future inquiries. As previously described, data suggests that HNSCC tumors with *NOTCH1* and *TP53* mutations confer a worse prognosis compared with wildtype; determining if this observation is also true in the UM HNSCC cohort is an important first step in validating if this cohort recapitulates relatively well-established clinical outcomes previously observed at other cancer centers. In addition, we hope to elucidate the

relationship between HPV16 viral load and survival outcomes in HPV+ HNSCC, which has been minimally investigated and could add to the prognostic understanding of this disease.

Chapter 2: Review of the HNSCC Literature

Head and neck squamous cell carcinoma (HNSCC) is a cancer that arises in the mucosal lining of the mouth, back of throat, hypopharynx, and larynx. It is estimated that 54,000 new head and neck cancers were diagnosed in the United States in 2022, comprising 3% of all malignancies (Barsouk et al., 2023). In HPV- HNSCC, alcohol and tobacco consumption (often working synergistically) are the main HNSCC carcinogens in the United States (Johnson et al., 2020). Beginning in the 1990s, it became clear that a growing proportion of oropharyngeal HNSCCs were associated with high-risk strains of the HPV virus (Haraf et al., 1996; Snijders et al., 1992; Wilczynski et al., 1998). Since 2010, mortality rates for oropharyngeal HNSCCs, 70% of which are HPV+, increased at a 2% annual rate in men and 1% annual rate in women, making it one of only six cancers to be increasing in incidence in the United States in the last decade (Siegel et al., 2023).

The median age of patients with HPV- HNSCC is 66 years, while the median age for patients with HPV+ HNSCC is 58 years (Windon et al., 2018); both subgroups are 2-4 times more likely to be men, due to increased rates of smoking, alcohol consumption, and sexual practices within the demographic (D'Souza et al., 2016; Johnson et al., 2020).

One study looking at 10-year overall survival (OS) rates in 581 HNSCC cases found that HPV+ oropharyngeal patients had the highest survival at 87%, this is contrasted with a 56% 10 year OS in HPV- oropharyngeal patients. For other non-HPV associated HNSCCs, 10-year OS was 69% for oral cavity, 67% for larynx, and 51% for hypopharynx (Das et al., 2015). Another study found 5 year OS for all HPV- HNSCC subgroups combined was 65% from 2007-2013 (Cohen et al., 2018), and increasing to 68.5% between 2013-2019 (2013-2019, n.d.). While a marked

survival difference exists between locally advanced primary HPV+ and HPV- HNSCC disease, if the cancer fails to respond to initial treatment and metastasis develops, prospects in both subsets of patients are equally grim, with most patients dying within a year of distantly metastatic diagnosis (Leeman et al., 2017).

Treatment for locally advanced HNSCC depends on the tumor subsite. In oral cavity malignancies, surgical excision (and usually reconstructive surgery) followed by adjuvant chemoradiation is the typical treatment course. In oropharyngeal cancers, patients are often initially treated with seven weeks of daily radiation combined with concurrent weekly chemotherapy. Patients with HNSCC of the larynx or hypopharynx either undergo 1) combination chemoradiation therapy in an effort to preserve the larynx, or 2) total/partial surgical resection of the larynx (Johnson et al., 2020).

Complications from HNSCC, both from the disease itself and its treatment, profoundly impact patients' quality of life (QOL). Patients typically report, during treatment and afterwards, problems with saliva production, swallowing, eating, speaking, loss of taste and smell, persistent pain, disfigurement, and stress (Bschorer et al., 2022; Pezdirec et al., 2019; Sherman & Simonton, 2010). Unsurprisingly, these challenges can significantly affect social interactions and relationships even with close friends and family. In one QOL study of 109 post-treatment HNSCC patients, persistent problems swallowing affected over 41% of respondents, greatly impacting their social life, particularly their ability to comfortably eat in public (Pezdirec et al., 2019). Studies of caregivers of patients with HNSCC show persistent trouble sleeping and stress (Rigoni et al., 2016), highlighting this disease burden not only on patients but on their close relations. Unfortunately, studies have shown a two-fold increased suicide rate in survivors of

HNSCC compared with other cancer type survivors, and four-fold increased rates over the general population; the authors of this study stated that “the disease has been described as the most emotionally traumatic and psychologically distressing of all cancers” (Osazuwa-Peters et al., 2018).

Due to the sometimes severe impact on a patient’s life, even if their disease is “cured”, there has been a recent push to de-escalate therapies and/or find more precise cellular targets in an attempt to minimize treatment side effects and preserve higher QOL measures. Newer treatment modalities, such as immunotherapy or targeted therapies, as well as chemoradiation de-escalation strategies, have increasingly utilized in the clinic and in clinical trials and in practice to maintain similar or better survival outcomes while reducing treatment-related toxicity. Identifying new biomarkers to help stratify which patients may potentially benefit from these novel treatment approaches is an ongoing focus in HNSCC research where more data is needed (Dietz et al., 2017; Rosenberg & Vokes, 2021).

HPV in HNSCC

Human papillomavirus (HPV) is the most common sexually transmitted infection in the United States, with an estimated 24 million new infections and a total prevalence of 77.3 million infected persons aged 18 - 59 years old in 2018 (Lewis et al., 2021). While most people are able to clear HPV on their own within two years of initial infection, a subset of patients do not clear HPV which typically remains latent in their body for many years (Gillison et al., 2015). Although asymptomatic, latent HPV infections have the capacity to cause cancer decades after the initial infection; approximately 37,000 new HPV-associated cancer cases are diagnosed each year in the USA (CDC, 2023).

Within the body, HPV-associated cancers tend to arise in epithelial mucosal tissues in areas involved with sexual activity, including as the cervix, vagina, vulva, penis, anus, and oropharynx (back of the throat, including the tonsils and base of the tongue) (Gillison et al., 2015; Roman & Aragones, 2021). From a global perspective the most common HPV-associated malignancy is cervical cancer, especially in lower income countries. However, in the United States and other high income countries, head and neck squamous cell carcinoma of the oropharynx has surpassed cervical cancer as the most frequent HPV-associated cancer. Corresponding with this etiological shift, the patient population for HPV-associated cancers in the USA has shifted from 35 - 50 year old women to white males aged 50 years and older (Roman & Aragones, 2021; Sabatini & Chiocca, 2020).

The prevailing theory behind the sex discrepancy is that one of the most significant risk factors for developing HPV+ HNSCC is the lifetime number of oral sexual partners (Lechner et al., 2022). One prospective study of 409 individuals looked at HPV in saliva samples and found significant differences in viral presence between men (15.4%) and women (5.6%) (D'Souza et al., 2016). This difference is likely due to differing sexual practices between the two groups.

The HPV virus itself is small, non-enveloped, and contains an approximately 8 KB double stranded DNA genome (Gao & Smith, 2016; Szymonowicz & Chen, 2020). Like other viruses, HPV viral proteins manipulate normal cellular pathways to induce viral genome replication/amplification and synthesis of viral proteins (Vonsky et al., 2019). HPV is dependent on the host cell for replication, and therefore requires access to actively dividing epithelial cells; these cells are found at the basement membrane underneath the surface layers of epithelium and are accessed most commonly via small tears in epithelial tissue (Graham, 2017; Stanley, 2010). Once inside the cell, the virus travels to the nucleus, where the capsid disassembles and releases

its proteins to initiate viral transcription (Graham, 2017). In those with persistent HPV infection, the virus can remain as either distinct, circular episomes in the cytoplasm or integrate into the host genome. Integrated HPV is associated with worse prognosis, likely due to vast downstream effects on gene expression and immune response (Koneva et al., 2018). Integration of viral DNA into the host genome is an area of intense interest in HPV cancer research, but how it occurs and its direct role in oncogenesis is not well understood (Parfenov et al., 2014; Pinatti et al., 2018).

HPV contains 6 early transcribed genes: E1, E2, E4, E5, E6, and E7 and 2 late transcribed genes: L1 and L2, with an additional non-coding region (Gao & Smith, 2016). HPV genes E6 and E7 have been deemed oncoproteins, binding to tumor suppressor proteins p53 and pRB, respectively, and causing their degradation (Gao & Smith, 2016); numerous studies have demonstrated expression of E6 and E7 in HPV+ HNSCCs (Avincsal et al., 2021; Kahue et al., 2018; Pinatti et al., 2018).

To date, over 450 HPV virus subtypes have been identified (Yu et al., 2022), 170 of which have the potential to infect human epithelial tissue (Sabatini & Chiocca, 2020). These infectious viral subtypes are characterized as low-risk (eg. HPV-6, HPV-11) or high-risk (eg. HPV-16, HPV-18, HPV-33, HPV-58, among others) depending on their carcinogenic potential (Gao & Smith, 2016). Recent estimates suggest that roughly 70% of new oropharyngeal HNSCC tumors in the USA are secondary to infection with high-risk HPV subtypes (CDC, 2023). Of the high-risk HPV subtypes capable of causing HNSCC, the most commonly detected variant is HPV16 with recent reports estimating that approximately 80 - 90% of HPV+ HNSCC are caused by this subtype alone (Gillison et al., 2015; Ndiaye et al., 2014; Pinatti et al., 2018; Ziai et al., 2021).

Clinically, tissue is tested for HPV either directly using PCR or in-situ hybridization for E6 or E7, using HPV type specific probes (e.g. HPV16 and HPV18) or indirectly using immunohistochemistry (IHC) staining for the p16 protein. P16 is overexpressed in cells due to the downregulation of pRB by E7 (Marur et al., 2010; Wasylyk et al., 2013). Due to its ease and cost-effectiveness, p16 IHC has become the accepted surrogate marker for determining if oropharyngeal tumors are HPV+ (Marur et al., 2010). However, it is well documented that there are tumors that may show expression of p16 while actually being HPV-negative. One large review of studies in Europe and Canada found that 9% of oropharyngeal tumors were p16+ while being HPV- ; for HNSCC tumors outside of the oropharynx, the discordance rate was nearly 30% (Mehanna et al., 2023). This study found that survival outcomes for p16+/HPV- patients were worse than for p16+/HPV+ patients, but better than for p16-/HPV- patients (Mehanna et al., 2023).

While it is established that patients with HPV+ oropharyngeal tumors have a better prognosis compared with their HPV- counterparts, it is less understood whether the viral load of HPV within tumor cells confers a better or worse prognosis within the subset of patients with HPV+ disease. A recent Japanese study of 89 tumors (44 HPV- and 45 HPV16+) found that, after categorizing into 4 quartiles (none, low= <10, medium= 10-2600, and high >2600) of HPV16 copies per cell, there was significantly worse progression-free and overall survival in the low copy number group compared with the medium and high copy number groups (Hashida et al., 2021). Interestingly, this study found no difference in overall survival between the HPV+ and HPV- groups, perhaps suggesting limitations in follow up time and/or lack of power in their small sample size. More research is needed to replicate these findings, and to determine if they are applicable to other populations.

TP53 in HNSCC

Tumor suppressor gene *TP53* is among the most commonly mutated or deleted genes across all cancers (Aubrey et al., 2016). Despite its ubiquity, and being one of the most studied genes in oncogenomics, the precise mechanism of action is not fully defined (Aubrey et al., 2016).

When cells are damaged or begin to grow abnormally, the p53 network, otherwise kept in an "off" state, activates and then suppresses the proliferation of these cells (Aubrey et al., 2016; Vogelstein et al., 2000). Almost any mutation in the *TP53* gene results in its inability to transcribe the p53 protein, therefore suppressing the anti-tumor effects of the gene (Vogelstein et al., 2000). From a clinical standpoint, *TP53* mutated HNSCC tumors have been shown to be more resistant to radiation therapy (Nathan et al., 2022). Numerous studies suggest *TP53* may be an important prognostic marker in HNSCC, and could potentially serve as a biomarker to stratify treatment. However, further research is needed in this area (Nathan et al., 2022)

TP53 mutations are very common in HPV- tumors, while being somewhat rare in HPV+ HNSCC (Solomon et al., 2018). In HPV+ HNSCC, *TP53* is inactivated, though not mutated, by the E6 protein, causing unregulated cellular proliferation (Nathan et al., 2022). To summarize, while both HPV- and HPV+ HNSCCs are likely impacted by the inactivation of *TP53*, in a genomic mutational analysis, somatic mutations are most likely to be found in HPV- disease.

One study of 420 HNSCC tumors, which did not differentiate by subsite or HPV status, showed a *TP53* mutation rate of 53.3%; the authors found that patients with *TP53* mutated tumors had a 70% increased risk of dying compared with those without *TP53* mutation (Poeta et al., 2007). A more recent analysis of The Cancer Genome Atlas (TCGA) data of 415 HNSCC tumors found a

TP53 mutation rate of 68.9%; this study found a similar 61% increased risk of dying compared to those without *TP53* mutation (Caponio et al., 2020). Importantly, this TCGA study further differentiated by subsite, showing drastic differences in survival *only in oropharyngeal tumors* with and without *TP53* mutation (HR 11.657; 95% CI: 2.668 - 50.929; P=.001) (Caponio et al., 2020).

A third study, also conducted with TCGA data, found in an analysis of 239 HNSCC patients with available genomic and clinical information that only 31.8% of tumors contained *TP53* mutations. In a comparison between the mutated and wild type *TP53* groups, this study found no difference in age, sex, t-stage, lymph node involvement, or clinical grade, suggesting that *TP53* mutation alone may be a prognostic variable (Kong et al., 2022). However, neither subsite nor HPV status were clearly differentiated, making potential clinical implication uncertain.

In summary, studies demonstrate *TP53* mutation prevalence between 31-69% of HNSCC tumors. In those with a *TP53* mutation, studies have shown a 61-70% increased chance of dying compared to those without mutation of *TP53*. These statistics may be skewed by the number of HPV+ oropharyngeal cases included in the respective data sets, as most HPV+ tumors do not contain somatic *TP53* mutations and survival rates are significantly better in this subset of HNSCC patients.

NOTCH1 in HNSCC

The *Notch* gene was first phenotypically observed as notches in fruit fly wings in the early 1900s (Leong & Karsan, 2006). In mammals, there are four members of the Notch family: *NOTCH1*-NOTCH4; due to its involvement with cell differentiation, *Notch* signaling plays a significant

role in embryogenesis, and throughout the lifespan continues to regulate proper tissue development (Leong & Karsan, 2006; Shah et al., 2020).

Atypical *NOTCH1* expression has been observed in both hematologic and solid malignancies (Leong & Karsan, 2006). In certain heme-related cancers, aberrant *NOTCH1* is a well-studied causative oncogenic agent. Less concrete causative data exists for solid tumor malignancies, though many studies demonstrating correlations between *NOTCH1* mutations and cancer are highly suggestive that inactivating mutation in *NOTCH1* plays a role in tumorigenesis (Leong & Karsan, 2006). In solid tumors, *NOTCH1* mutations likely contribute to inhibiting apoptosis and increasing cellular proliferation (Leong & Karsan, 2006).

In HNSCC, there is evidence that inactivation of *NOTCH1* can play an important role in tumorigenesis. A collaborative study between MD Anderson Cancer Center and Johns Hopkins University Medical Center conducted whole exome sequencing (WES) on 32 HNSCC tumors with matched normal DNA. In this "discovery cohort" 15% of HNSCC tumors were found to have *NOTCH1* mutations, making it the second-most mutated gene (after *TP53*) observed in these patients (Agrawal et al., 2011). Building on this work, the investigators sequenced an additional cohort of 88 HNSCC samples; of the entire 120 samples analyzed, 21 (17.5%) showed mutations in *NOTCH1* (Agrawal et al., 2011). A similar WES study conducted by a separate group at around the same time found that *NOTCH1* was either mutated or deleted in 10/74 tumors, for a prevalence of 13.5% (Stransky et al., 2011).

Notably, both above studies used tumor tissue from all major HNSCC subsites, including HPV and non-HPV associated malignancies. Shah et. al. reported in their 2020 review paper that

NOTCH1 was mutated in approximately 10% of HPV+ HNSCCs, about half the rate of *NOTCH1* mutations in HPV- HNSCCs (Shah et al., 2020).

One study found that those with HNSCC tumors with high *NOTCH1* expression had higher disease-specific and overall survival compared with those with none or low expression (Wirth et al., 2018). This finding was replicated in a similar European study (Grilli et al., 2020), suggesting that *NOTCH1* plays a tumor-suppressive role, and that its mutation (ie. inactivation) may contribute to tumorigenesis (Liu et al., 2016). Indeed, in a study of 128 patients with HNSCC, those with a somatic *NOTCH1* mutation had 4.5 higher odds [95% CI: 1.4-14.1] of developing recurrence and 5.8 higher odds [95% CI: 1.5-23] of death in the 13 years of follow - up compared to patients with wild type *NOTCH1* (Liu et al., 2016).

Summary

Despite improvements in surgical techniques, chemotherapy utilization, radiation targeting, and addition of novel immunotherapies to the arsenal of available treatment options, survival outcomes for many HNSCC patients, especially once the disease has recurred, remain poor. Greater understanding of HNSCC disease biology is needed in order to 1) develop risk-stratified treatment approaches for existing therapies that balance survival outcomes with potential treatment-related toxicities, and 2) identify biomarkers to help improve usage of newer treatment approaches (eg. immunotherapy) or potentially identify novel targets for the development of new therapies. This analysis is intended to contribute to this important body of knowledge, specifically investigating whether HPV16 viral load within HPV+ HNSCC tumors impacts prognosis/survival, and additionally validating whether *TP53* and *NOTCH1* mutations also confer poor prognosis in a large cohort of patients treated at the University of Michigan.

Chapter 3: Methodology

Analysis of this de-identified dataset was deemed non-human subject research by the Emory IRB.

HNSCC Dataset Overview

Targeted next-generation genomic sequencing was performed on 250 locally-advanced HNSCC tumors of the larynx/hypopharynx, oral cavity, or oropharynx from patients treated at the University of Michigan between 2000 - 2022. Clinical and demographic information was collected on each patient via retrospective chart review of the electronic health record.

Formalin-fixed, paraffin embedded (FFPE) tissue was obtained from the University of Michigan Department of Pathology archival tissue bank (IRB #HUM00080561). If UM FFPE was not available, FFPE tissue was obtained from the original treating institution with patient written consent (IRB #HUM00042189; #HUM00085888). A representative hematoxylin and eosin (H&E) stained slide was reviewed for tumor content by a board-certified pathologist. Tumor tissue was taken directly from the FFPE, or unstained slides were scraped with a sterile scalpel, aligning with the tumor content on the H&E slide. DNA was isolated from the tumor tissue (Qiagen, catalog #80234). When possible, patient-matched non-cancerous "normal" DNA was purified from banked whole blood (Qiagen, catalog #51185). If blood was not available, non-neoplastic cells from the archival FFPE were obtained and isolated for normal DNA (Qiagen, catalog #80234).

DNA was submitted to the University of Michigan Genomic Sequencing Core for targeted capture sequencing. The custom targeted capture panel included 194 genes known or suspected to be involved in carcinogenesis, including *NOTCH1* and *TP53*, as well as 2 oncoviruses,

including HPV. To call mutation status most accurately in *NOTCH1* and *TP53*, only tumors with matched normal DNA for comparison were included in the gene analysis. In the HPV analysis, matching normal DNA was not required as viral integration and copy number can be assessed from tumor DNA alone. From the 250 original patients, only patients with p16+ oropharyngeal HNSCC were included in the HPV analysis (Figure 1).

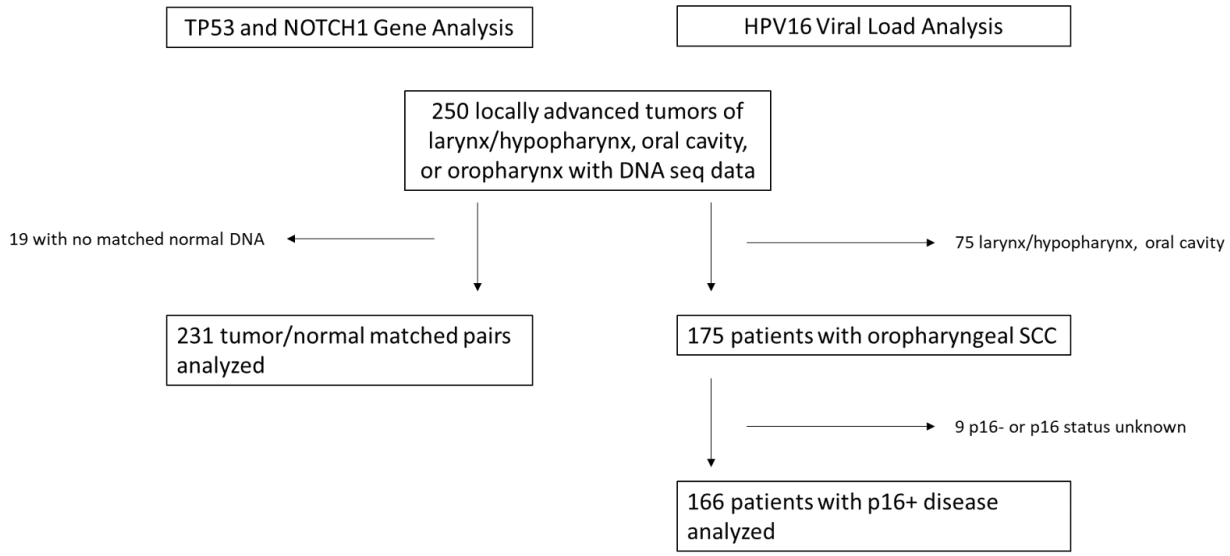

Figure 1. Overview of study cohorts and data analysis inclusion criteria**.**

Patient Stratification and Study Outcomes

For the HPV16 read count survival analysis, 166 patients were divided into quartiles based on HPV16 reads in their tumor sample; 7 patients in this cohort (7/166, 4.2%) had no HPV16 reads identified. This is likely indicative either of infection with a different high-risk HPV subtype or of a true discordant p16+/HPV- patient. These patients were included in the analysis. For the gene mutation survival analysis, 231 HNSCC patients were stratified by whether they harbored *TP53* mutations or *NOTCH1* mutations.

The primary outcome of interest in both analysis groups was 5-year overall survival (OS). Other variables collected included sex, age at diagnosis, smoking history, smoking pack years, HNSCC subsite, p16 status, tumor stage (T-stage), and time (in days) from diagnosis to death or time (in days) from diagnosis to date of last follow up as of 12/31/2023. Patients who were still alive or lost to follow-up were censored on the date of their last documented contact with the healthcare system as of 12/31/2023.

Statistical Analysis

Baseline characteristics for patients in both datasets are reported as frequencies (number and percentages) for categorical variables and median (range) for continuous variables. Categorical variables were analyzed using the chi-squared test or Fisher's exact test, as appropriate based on sample size. Nonparametric continuous variables were analyzed using the Mann-Whitney U (Wilcoxian rank-sum) test. Survival analyses were performed using the Kaplan-Meier method, with survival distributions compared using the Mantel-Cox log-rank test. To identify predictor variables for overall survival, univariate Cox proportional hazards regression analysis was performed on the *TP53* cohort. Univariate variables with a p value < 0.1 would subsequently be included in multivariate analysis (if applicable). A two-sided significance level (alpha) of less than 0.05 was considered statistically significant for all analyses. Statistical analyses were performed using the SAS statistical software package (SAS 9.4).

Chapter 4: Results

Cohort Baseline Characteristics

p16+ oropharynx tumor samples (n=166) were included in the HPV analysis (Table 1). Patients were stratified into quartiles based on HPV16 read count in their tumor: Q1) 0-40,035 (n=41), Q2) 42,858-475,895 (n=42), Q3) 461,187-1,900,062 (n=41) and Q4) 2,012,300-27,232,607 (n=42). Across the four quartiles (Q1-Q4, respectively), patients were predominately male (92.7%, 81.0%, 82.9%, 90.5%) and over age 50 at the time of diagnosis (90.1%, 85.7%. 87.8%, 78.6%). Smoking history (never vs. current/former) was roughly equivalent within each quartile, with median pack years smoked (6 patients with missing data) ranging from 13 (Q2) to 25 (Q1) across all four quartiles. The majority of patients in each quartile had smaller tumor burden at time of diagnosis (T-stage 1 or 2): 68.3%, 59.5%, 78.0% and 69.0% in Q1-Q4 respectively. There were 28 deaths (28/166; 16.9%) observed in this cohort (Q1=7, 4.2%; Q2=10, 6.0%; Q3=6, 3.6%; Q4=5, 3.0%). No significant baseline differences were noted between the quartiles for any of the variables assessed, including: sex, age at diagnosis, smoking history, or T-stage (Table 1).

Table 1. Baseline characteristics of the HPV+ cohort (n = 166). *Pack years of former or current smokers; **time to death from initial/primary diagnosis

		HPV16 Read Count Quartiles (n=166)				
Variable	**Description**	**Q1 (n=41)**	**Q2 (n=42)**	**Q3 (n=41)**	**Q4 (n=42)**	**p value**
HPV16 read count	median (range)	7362 (0-40035)	156003.5 (42858-457895)	1122586 (461187-1900062)	4023754 (2012300-27232607)	N/A
Sex	Male	38 (92.7%)	34 (81.0%)	34 (82.9%)	38 (90.5%)	0.3414
	Female	3 (7.3%)	8 (19.0%)	7 (17.1%)	4 (9.5%)	
Age at diagnosis	median (range)	64 (40-80)	59 (39-79)	57 (41-73)	58 (39-90)	0.0549
Smoking history	Never smoker	17 (41.5%)	22 (52.4%)	23 (56.1%)	20 (47.6%)	0.6203
	Former or current smoker	24 (58.5%)	19 (45.2%) (missing data =1)	18 (43.9%)	21 (50.0%) (missing data =1)	
Pack years*	median (range)	25 (2-80) (missing data n=3)	13 (1-126) (missing data n=1)	17.5 (.25-60)	15 (.36-90) (missing data n=2)	0.4575
T-stage	1	17 (41.5%)	15 (35.7%)	18 (43.9%)	10 (23.8%)	0.2374
	2	11 (26.8%)	10 (23.8%)	14 (34.1%)	19 (45.2%)	
	3	3 (7.3%)	7 (16.7%)	2 (4.9%)	5 (11.9%)	
	4	10 (24.4%)	10 (23.8%)	7 (17.1%)	8 (19.0%)	
Time to death**	median (range)	1070 (362-4806)	893 (287-4712)	526 (279-2306)	509 (61-2328)	0.2415

HNSCC patients (n=231) with matching tumor and normal DNA were sequenced using a targeted capture method and included for gene analysis (Table 2). Within the overall cohort, 49 patients (21.2%) had a *TP53* mutation while 13 (5.6%) had a *NOTCH1* mutation. The majority of patients (189/231; 81.8%) in the overall cohort were over age 50 at the time of diagnosis; nearly half (109/231; 47.2%) were older than 60 years old. The majority of patients in the overall cohort were male (181/231; 78.4%), had p16+ primary tumors (151/231; 65.4%), and were either current or former cigarette smokers (128/231; 55.4%).

Table 2. Baseline characteristics of the entire cohort (n = 231), stratified by *TP53* or *NOTCH1* mutation status. *Pack years of former or current smokers; **time to death from initial/primary diagnosis; ***significant at p=.05

		TP53 Gene Analysis (n=231)			NOTCH1 Gene Analysis (n=231)		
Variable	**Description**	**TP53 Wildtype (n= 182, 78.8%)**	**TP53 Mutated (n=49, 21.2%)**	**p value**	**NOTCH1 Wildtype (n=218, 94.4%)**	**NOTCH1 Mutated (n=13, 5.6%)**	**p value**
HNSCC subsite	Larynx/hypopharynx	4 (2.3%)	7 (14.3%)	<.0001***	11 (5.0%)	0 (0%)	0.6626
	Oral cavity	29 (15.9%)	34 (69.4%)		58 (26.6%)	5 (38.5%)	
	Oropharynx	149 (81.9%)	8 (16.3%)		149 (68.3%)	8 (61.5%)	
p16 Status	P16+	145 (79.7%)	6 (12.2%)	<.0001***	145 (66.5%)	6 (46.2%)	0.1762
	p16-	13 (7.1%)	8 (16.3%)		18 (8.3%)	3 (23.1%)	
	p16 unknown/not tested	28 (5.8%)	35 (71.4%)		55 (25.3%)	4 (30.8%)	
Sex	Male	148 (81.3%)	33 (67.3%)	0.035***	171 (78.4%)	10 (76.9%)	1.000
	Female	34 (18.7%)	15 (30.6%)		47 (21.6%)	3 (23.1%)	
Age at diagnosis	20-29	0 (0%)	2 (4.1%)	0.1835	2 (1.0%)	0 (0%)	0.8265
	30-39	4 (2.2%)	2 (4.1%)		6 (2.8%)	0 (0%)	
	40-49	26 (14.3%)	8 (16.3%)		32 (14.7%)	2 (15.4%)	
	50-59	66 (36.3%)	14 (28.6%)		74 (33.9%)	6 (46.2%)	
	60-69	63 (34.6%)	18 (36.7%)		78 (35.8%)	3 (23.1%)	
	70-79	21 (11.5%)	4 (8.2%)		23 (10.6%)	2 (15.4%)	
	>80	2 (1.1%)	1 (2.0%)		3 (1.4%)	0 (0%)	
Smoking history	Never smoker	90 (49.5%)	9 (18.4%)	0.0003***	92 (42.2%)	7 (53.8%)	0.6571
	Former or current smoker	90 (49.5%) (missing data n=2)	38 (77.6%) (missing data n=2)		122 (56.0%) (missing data n=4)	6 (46.2%)	
Pack years*	median (range)	25 (.25 - 120) (missing data n=9)	37.3 (7 - 126)	0.0164***	30 (.25 - 126) (missing data n=9)	34.8 (8 -110)	0.4599
T-stage	1	61 (33.5%)	8 (16.3%)	0.0463***	68 (31.2%)	1 (7.7%)	0.1701
	2	57 (31.3%)	14 (35.9%)		65 (29.8%)	6 (46.2%)	
	3	24 (13.2%)	9 (18.4%)		32 (14.7%)	1 (7.7%)	
	4	40 (22.0%)	18 (36.7%)		53 (24.3%)	5 (38.5%)	
Time to death (days) **	median (range)	1013 (61 - 4712)	503 (176 -4806)	0.1941	811 (61 - 4806)	428 (362 -498)	0.3205

When *TP53* mutation status was used to stratify the overall cohort, several significant baseline differences were noted between patients with tumors expressing wildtype *TP53* compared to those with *TP53* mutated tumors (Table 2). First, the HNSCC primary tumor subsite varied significantly ($p < 0.0001$); most *TP53* mutated tumors were from the oral cavity (34/49; 69.4%) with the remaining *TP53* mutated samples originating from the larynx/hypopharynx (7/49; 14.3%) and oropharynx (8/49; 16.3%) in almost equal proportion. In contrast, the vast majority of *TP53* wildtype HNSCC tumors were from the oropharynx (149/182; 81.9%) with relatively few primary tumors originating from the oral cavity (29/182; 15.9%) or larynx/hypopharynx (4/182; 2.3%). Significant differences between the *TP53* wildtype vs mutated cohorts were also noted for the proportion of p16+ tumors (79.7% vs 12.2%; $p < 0.0001$), the proportion of former/current cigarette smokers (49.5% vs 77.6%; $p = 0.0003$), and the median number of pack years smoked (25 vs 37.3; $p = 0.0164$). A significant difference ($p = 0.0463$) was also noted in the T-stage of the primary resected tumor between the cohorts, with a higher proportion of *TP53* mutated patients presenting with more advanced tumors (i.e. stage 3 or 4) compared to those in the *TP53* wildtype group (55.1% vs 35.2%). Finally, a statistically significant difference ($p = 0.035$) was also noted in the sex distribution of patients in each cohort, although in both the *TP53* wildtype and *TP53* mutated groups the majority of patients were male (81.3% vs 67.3%).

No significant baseline differences were noted between the *NOTCH1* wildtype and *NOTCH1* mutated tumors for any of the variables assessed (Table 2).

Survival Outcomes

When divided into quartiles based on HPV16 viral read count, no survival difference was noted among patients with p16+ HNSCC of the oropharynx (Figure 2, p = 0.5501).

In the *TP53* stratified cohort, patients with HNSCC tumors that had a *TP53* mutation had significantly lower 5-year overall survival (Figure 3 [29.5% vs 72.8%%; p < 0.0001]) compared to those with wildtype *TP53* tumors. In contrast, no significant difference in 5-year overall survival was noted for patients with NOTCH 1 mutations (Figure 4 [76.9% vs 61.5%; p = 0.578]).

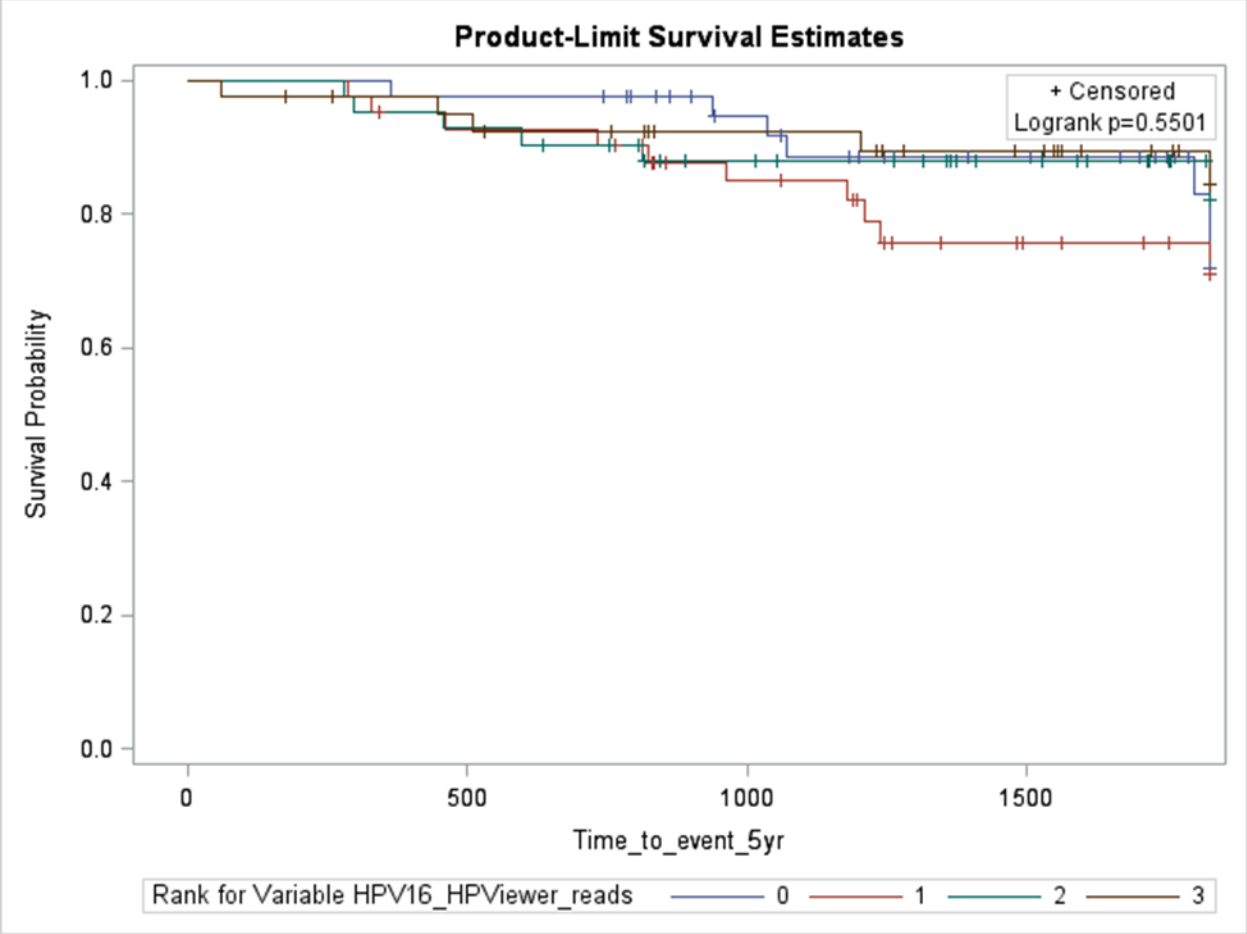

Figure 2. Overall survival outcomes for the HPV+ cohort (n = 166), grouped into quartiles based on HPV viral load (copies/cell). 0 = 1st quartile (lowest viral load), 1 = 2nd quartile, 2 = 3rd quartile, 3 = 4th quartile (highest viral load)

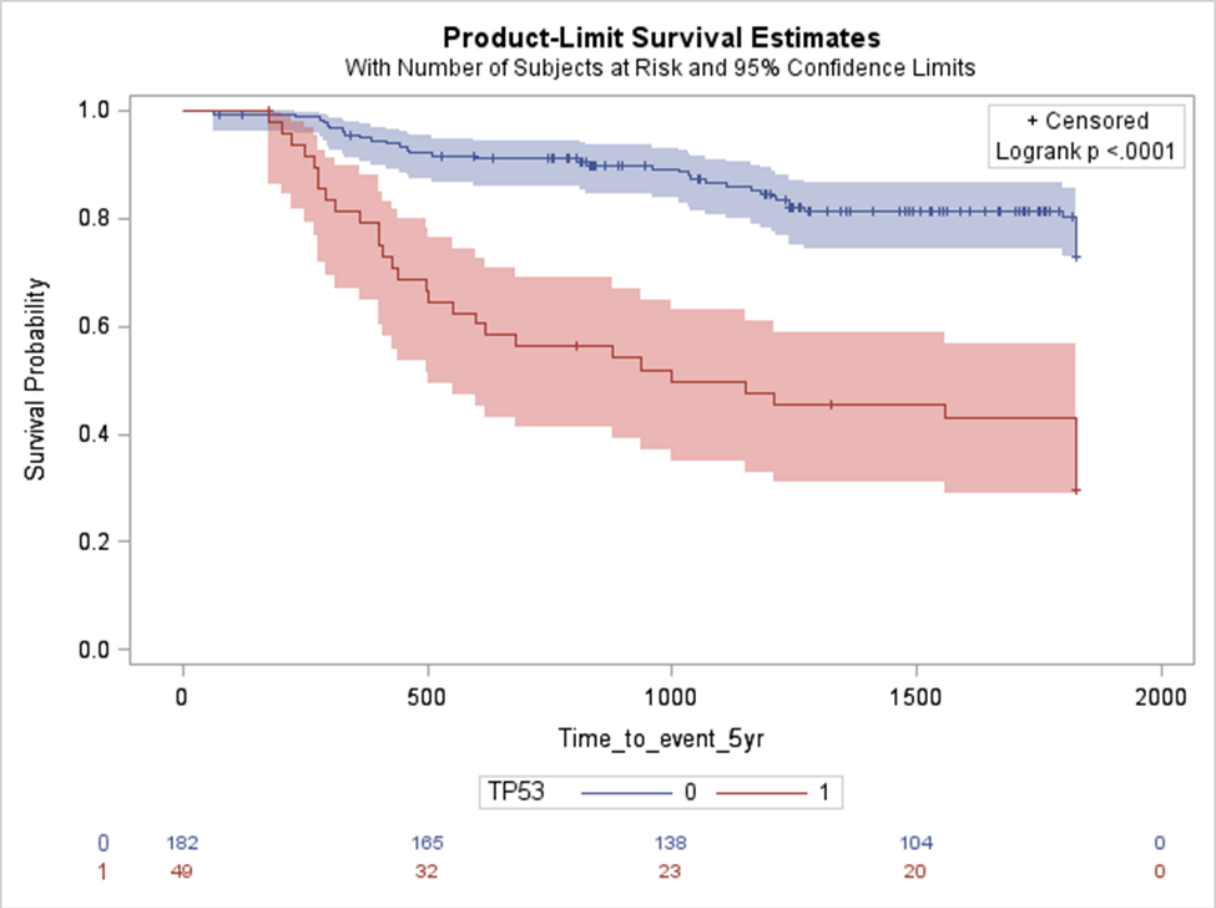

Figure 3. Overall survival outcomes with 95% confidence interval bands for the entire cohort (n= 231), stratified by *TP53* mutation status. 0 = *TP53* wildtype, 1 = *TP53* mutated

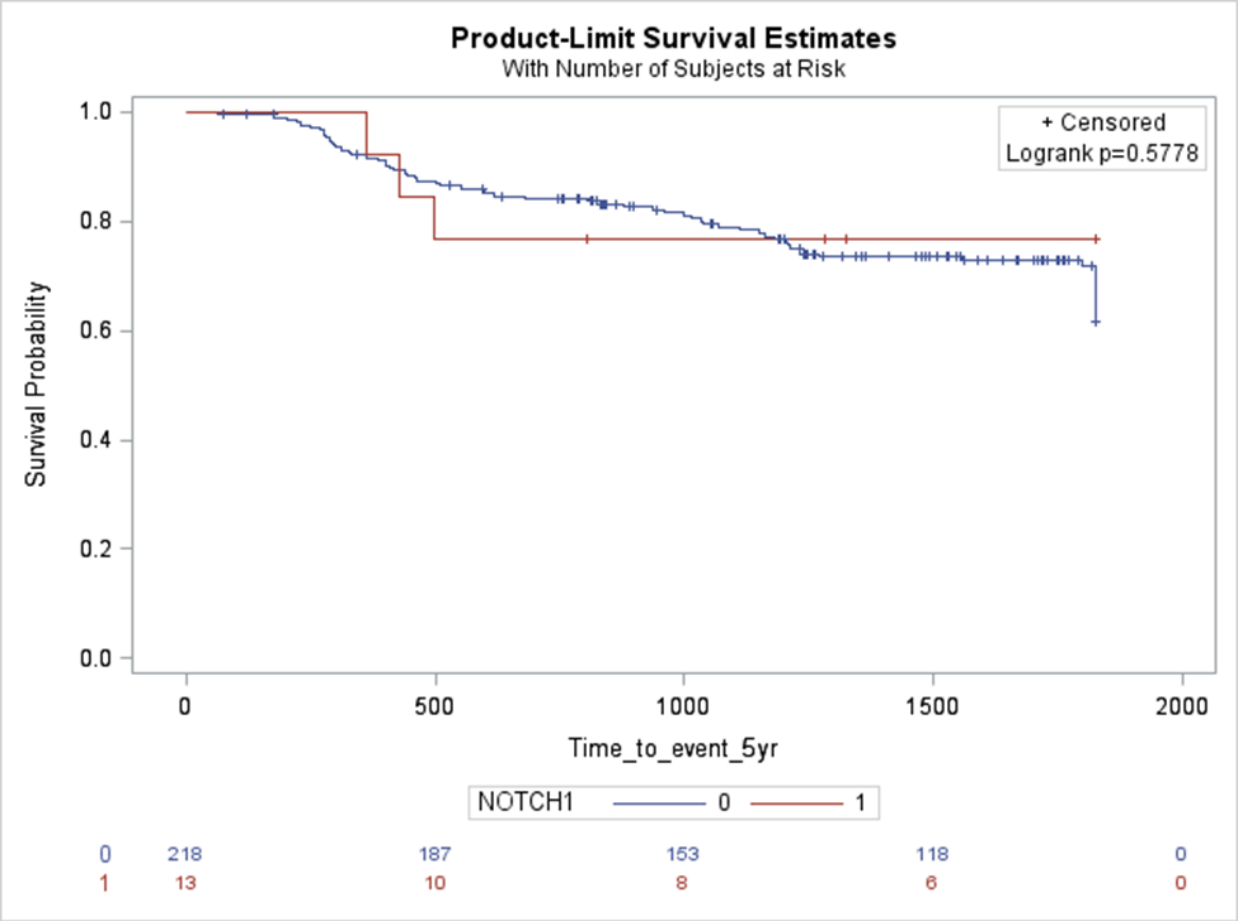

Figure 4. Overall survival outcomes for the entire cohort (n = 231), stratified by *NOTCH1* mutation status. 0 = *NOTCH1* wildtype, 1 = *NOTCH1* mutated

Given the baseline differences between the *TP53* stratified cohorts, Cox proportional hazards analysis was performed to identify characteristics aside from the presence of a *TP53* mutation that may also predict overall survival (Table 3). Univariate Cox proportional hazards analyses were performed for the following variables that differed between the cohorts at baseline: HNSCC subsite, p16 status, sex, smoking history (never vs. former/current cigarette smoker), smoking pack years, and T-stage of the primary tumor. None of these baseline differences between the *TP53* cohorts were found to be statistically significant after univariate Cox proportional hazards analysis, and therefore no multivariate analysis was done.

Table 3. Cox proportional hazard univariate analyses for the *TP53* stratified cohort (n = 231).

Variable	**Hazard Ratio (95% CI)**	**P value**
HNSCC subsite	0.987 (0.587 – 1.650)	0.960
p16 Status	1.111 (0.658 – 1.876)	0.694
Sex	1.143 (0.704 – 1.858)	0.589
Smoking history	1.221 (0.711 – 2.099)	0.470
Pack years	0.453 (0.099 – 2.069)	0.306
T-stage	1.165 (0.720 – 1.885)	0.538

Chapter 5: Conclusions, Implications, and Recommendations

Summary of Study

Despite recent treatment advancements, outcomes for many HNSCC patients remain poor and work is required to improve survival outcomes in this group of cancer patients. Even if treatment works as intended and the cancer does not recur, quality of life for HNSCC patients is among the lowest for all cancer subtypes. Greater understanding of HNSCC at the genomic level is needed to help develop risk-stratified treatment approaches for utilizing existing therapies such as chemotherapy, radiation, or surgery in order to optimally balance survival outcomes with treatment-related toxicities. In addition, genomic data may also identify new biomarkers to help identify new targets for treatment and/or improve the use of newer HNSCC treatment approaches such as immunotherapy, or potentially identify novel targets for the development of targeted therapies that may exhibit more favorable therapeutic indexes.

This study investigates whether viral load of HPV16 is associated with 5-year OS. This aim was more exploratory, as there are few published studies assessing this relationship in the literature. Stratifying patients with HNSCC oropharyngeal tumors that were p16+ (the accepted clinical surrogate for HPV positivity) into quartiles based on HPV16 viral read count reveals no difference in 5-year OS. Other clinical characteristics of this subgroup closely match those of other published cohorts, adding to our confidence in our findings.

Additionally, this study examines whether a cohort of HNSCC tumors from patients treated at the University of Michigan recapitulates prognostic data identified in HNSCC patient cohorts treated at other centers, specifically by examining whether *TP53* and *NOTCH1* mutations have a significant impact on 5-year overall survival (OS). This preliminary assessment of the dataset is

a critical first step, as it determines whether the University of Michigan dataset matches well-accepted prognostic variables previously identified in the medical literature. Analysis of the University of Michigan HNSCC dataset reveals that *TP53* is an independent predictor of worse 5-year OS, while mutation status of the *NOTCH1* gene did not impact 5-year OS.

Discussion of Key Results

In the University of Michigan HNSCC cohort, we find patients with *TP53* mutations have significantly worse 5-year OS compared to those with wildtype *TP53*. This observation agrees with previous reports analyzing the impact of *TP53* on survival in HNSCC, but in our cohort highlights several important observations that warrant more discussion. First, the prevalence of *TP53* mutations in our cohort was only 21.2%. This is somewhat lower than in other published reports, where the prevalence of *TP53* mutations ranges from 31 - 69% depending on the study (Caponio et al., 2020; Kong et al., 2022; Poeta et al., 2007). One explanation for why our cohort's *TP53* mutation rate is lower than others in the literature is the preponderance of HPV+ tumors (65.4%) in our cohort as *TP53* prevalence is known to be lower in p16+ HNSCC tumors (Solomon et al., 2018). Despite its lower prevalence, we still find *TP53* mutation to be an independent predictor of OS in our cohort even when controlling for other variables including tumor subsite, p16 status, sex, smoking status, and tumor size (T-stage). This finding is interesting, as having fewer *TP53* mutations in our overall cohort should make it more difficult to detect an OS effect. The fact that we still easily detect this difference infers that *TP53* must have a very strong effect on survival in HNSCC. However, several significant baseline differences were apparent between those in our cohort with *TP53* mutated tumors compared to those with wildtype *TP53* which could also potentially impact OS. To confirm whether another variable could be driving the survival difference observed in our study, Cox proportional hazards

analysis was performed. Any baseline characteristic that exhibited a statistically significant difference at baseline between the *TP53* mutated and wildtype groups was included as a variable of interest in univariate Cox proportional hazards testing. After univariate analysis, none of the other baseline differences were found to have a significant effect on overall survival, supporting the assertion that the presence or absence of a *TP53* mutation is the most important clinical feature affecting survival in our cohort.

With regard to *NOTCH1* mutations, we also note a lower prevalence of these mutations in our cohort compared to other previously published reports. In our cohort, we identify *NOTCH1* mutations in 5.3% of tumors sequenced; previous reports in the literature suggest that *NOTCH1* mutations are prevalent in 10-20% of HNSCC tumors (Agrawal et al., 2011; Stransky et al., 2011). This again may be skewed by the preponderance of HPV-associated cancers in our cohort, as *NOTCH1* mutation prevalence has been reported lower in this subgroup (closer to 10%) compared with other HNSCC sites (closer to 20%). When compared to wildtype *NOTCH1* tumors, we did not find a significant difference in 5-year OS in patients with mutated *NOTCH1*. This differs from an earlier report in the literature which found that *NOTCH1* mutations conferred a higher likelihood of tumor recurrence and mortality in HNSCC patients (Liu et al., 2016). It is likely that baseline differences between the patient population included in our cohort compared to those included in previous reports could explain this difference, although our ability to discern this is limited because p16+ status was not reported in the prior study cohort. *NOTCH1* somatic mutations are less frequently seen in HPV+ HNSCC, so if a difference does exist our cohort may be underpowered to detect a survival difference for this variable relative to other studies that may have a higher proportion of HPV- tumors. Alternatively, it may be that

NOTCH1 is not as robust of a biomarker as previously reported, as this University of Michigan cohort is one of the larger sequencing cohorts to date.

Additionally, this study adds a novel element to the medical literature by examining whether the number of HPV16 copies within HNSCC tumors affects overall survival in p16+ oropharyngeal patient subset. This question has not been well studied previously in the literature; a single prior report in Japanese HPV+ HNSCC patients suggests those with low HPV viral loads (defined as HPV copy number < 10 copies/cell) have significantly worse PFS and OS compared with those with “medium” or “high” viral load; in addition, this study suggests that the PFS and OS of patients with low HPV viral load is prognostically comparable to patients with HPV- tumors (Hashida et al., 2021). In this cohort, we analyze p16+ oropharyngeal tumors and divide these patients into quartiles of equivalent size based on HPV viral read count, which is a surrogate for copy number. Unlike the previous Japanese report, we fail to detect a difference in overall survival between quartiles, although there are differences between the studies that deserve further discussion. First, our study has a substantially larger sample size (n = 166, with ~40 patients per quartile) than the prior Japanese study (n = 45, ~15 per group) which should reduce the impact that any single data point has on the endpoints of interest. However, differences with how the different groups were defined in each study make it hard to directly compare findings, and in fact could explain the discrepancy in results between the studies. In the Japanese study, “low copy number" was defined as having fewer than 10 HPV copies/cell, but in our study we did not define a hard cutoff for “low read count" and simply divided our cohort up into 4 equal quartiles. The read count in our lowest quartile ranges from 0 copies to up to 40,035 copies/cell, which on the high end encompasses patients who were defined as “medium” (10 - 2600 copies/cell) and “high” (>2600 copies/cell) expression in the Japanese report. The differences in

read count observed in the previous study may be obscured because of this major difference; it may be worth exploring if using the same cutoffs from the Japanese study still results in discrepant survival outcomes. In addition to this major difference, there may also be inherent differences between HNSCC in our cohort compared to a Japanese population that could also complicate comparing outcomes between cohorts. More work is required to definitively determine if prognostic differences based on HPV viral load do exist in HPV+ HNSCC.

Limitations and Strengths

This study has several limitations. The retrospective, single center study design makes it possible that this University of Michigan cohort is not representative of HNSCC at large. As previously mentioned, this cohort is enriched for HPV-associated oropharyngeal cancers and therefore inferences about *TP53* and *NOTCH1* mutations should be interpreted cautiously as the prevalence of these alterations in HPV+ HNSCC is lower compared to HPV- tumors which tend to arise from other subsites. This limitation is particularly apparent in our assessment of *NOTCH1* mutated tumors, where only 3 deaths among 13 patients were observed. In addition, we note a number of baseline differences between subgroups in our cohort, particularly in the analysis of *TP53* mutations. To account for the impact any of these baseline differences may have had on outcomes, Cox proportional hazards analysis was performed which failed to detect any significant effect of these baseline differences on survival.

Additionally, our study did not differentiate between mutation type (eg. deletion, missense, stop-gain, etc.), simply categorizing mutation status as a binary wildtype vs. mutant. This dataset is likely underpowered to detect differences at mutation-type level, but may be an avenue for future directions if additional analysis on a greater number of patients is performed.

Despite the aforementioned limitations, our study also has a number of strengths. Compared to prior reports, this study includes a relatively large sample size of HNSCC patients. While we acknowledge the cohort is skewed heavily with regard to tumor subsite (oropharyngeal) and HPV+ disease, we were nevertheless able to recapitulate the known negative effect of *TP53* on survival in the overall cohort, which is a well-established prognostic variable in HNSCC. The high proportion of patients with HPV+ disease in our cohort also provides the opportunity to more directly investigate potential prognostic variables, such as HPV viral load, that are specific to this HNSCC subset. Finally, our decision to utilize 5-year overall survival as our main outcome in this cohort is a strength as survival is the most important endpoint for clinicians and patients.

Implications

To our knowledge, this dataset is one of the larger single-institution clinical and genomic databases assembled to date. Understanding if this dataset is representative of HNSCC nationally is an important step in determining its applicability of further analyses and the degree of confidence in conclusions. We were able to demonstrate a significant effect of *TP53* on survival; this infers our cohort may be a good representative sample of HNSCC more generally as this finding has been observed in many previous studies in this setting. As our cohort is enriched for HPV+ disease, it is a robust dataset to use for any future analysis of this subgroup because the clinical characteristics are congruent with prior reports in the literature. This is useful from a public health standpoint, as the incidence of HPV+ HNSCC in the United States is rising (Chaturvedi et al., 2011; Gillison et al., 2015). While treatment outcomes for HPV+ HNSCC are very good, treatment-related toxicity is significant for HNSCC patients and therefore the field is

studying how to de-escalate therapy to better balance the efficacy/toxicity ratio in this HNSCC subgroup. Identification of better biomarkers is a major research focus to distinguish which patients can de-escalate therapy, and what the optimal treatment duration is based on patient-specific factors.

Recommendations

Our study demonstrated that a large cohort of HNSCC patients treated at the University of Michigan exhibit survival outcomes similar to those reported in the literature at other centers, in particular with regard to those with *TP53* mutations. This cohort was enriched for HPV+ oropharyngeal tumors, which provides an opportunity to utilize this cohort to further explore putative biomarkers of treatment response with the aim of de-escalating therapy in this patient population. As a first step, we divided our HPV+ cohort into quartiles to explore whether HPV viral load is predictive of poor outcomes in a subset of HPV+ tumors; although we did not identify a survival difference in our initial quartile analysis, another smaller study did identify a difference in outcomes between low expression and medium/high expression (Hashida et al., 2021). Future analyses in our cohort should examine whether or not using similar cutoffs (i.e. < 10 copies/cell) to this other study identifies a survival difference. We also recommend that further work be done to identify other biomarker candidates to help determine 1) who can de-escalate therapy in HPV+ HNSCC and 2) if there are patient-specific factors that can be tracked to assess response to treatment and personalize the decision to de-escalate therapy.

As other candidate biomarkers are identified, having a robust HPV+ HNSCC cohort that is known to recapitulate expected clinical outcomes will be useful in validating these biomarker candidates in future studies.

Conclusion

In this study, we were able to demonstrate the well-established observation that *TP53* mutations conferred worse overall survival in HNSCC patients treated at the University of Michigan and validate that this cohort reflects similar baseline characteristics and clinical outcomes compared to previous research. We did not observe worse survival outcomes in patients with *NOTCH1* mutations which has also been previously reported in the literature, although this result should be carefully considered given our cohort's high proportion of HPV+ tumors which resulted in a relatively small sample of *NOTCH1* mutations. We also observe that HPV viral load (assessed via quartiles) in HPV+ tumors did not appear to impact survival outcomes as previously reported in the literature, although this result should also be cautiously assessed due to differences in the definition of HPV viral expression in our study vs a prior report. Our cohort may be useful for future biomarker analyses in HNSCC, particularly in HPV+ disease, that could optimize use of existing treatment strategies and potentially identify new therapeutic targets for this increasingly prevalent and highly morbid cancer subtype.

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