Abstract (230)

Objectives: To explore whether HPV-related biomarkers predict oropharyngeal squamous cell cancer (OPSCC) survival similarly across different global regions, and to explore their prognostic utility among non-oropharyngeal (non-OP) head and neck cancers.

Methods: Data from 1362 head and neck SCC (HNSCC) diagnosed 2002-2011 was used from epidemiologic studies in: Brazil (GENCAPO study, n=388), U.S. (CHANCE study, n=472), and Europe (ARCAGE study, n=502). Tumors were centrally tested for $p16^{INK4a}$ and HPV16 DNA (by PCR). Risk of mortality was examined using Cox proportional hazard models.

Results: There were 517 OPSCC and 845 non-OP HNSCC. Cases were primarily male (81%), ever smokers (91%), with median age of 58 years and median follow-up of 3.1 years (IQR=1.4- 5.9). Among OPSCC, the risk of mortality was significantly lower among 184 HPV-related (i.e., p16+/HPV16+) compared to 333 HPV-unrelated (p16- and/or HPV16-) cases (HR=0.25, 95%CI=0.18- 0.34). Mortality was reduced among HPV-related OPSCC cases from the U.S., Europe, and Brazil (each $p \leq 0.01$) and after adjustment, remained significantly reduced (aHR=0.34, 95%CI=0.24- 0.49). Among non-OP HNSCC, neither p16 (aHR=0.83, 95%CI=0.60-1.14), HPV16 DNA (aHR=1.20, 95%CI=0.89-1.63), or p16+/HPV16+ (aHR=0.59, 95%CI=0.32-1.08) was a significantly predictor of mortality. When interaction was tested, the effect of HPV16/p16 was significantly different in OPSCC than non-OP HNSCC (p-interaction=0.02).

Conclusion: HPV-related OPSCCs had similar survival benefits across these three regions. Prognostic utility of HPV among non-OP HNSCC is limited so tumor HPV/p16 testing should not be routinely done among non-OP HNSCC.

Keywords: oral HPV, p16, non-OP, prognostic, risk factors, Brazil, Europe

Introduction

Head and neck squamous cell carcinoma cancer (HNSCC) has an estimated global annual burden of more than 500 000 incident cases and 300 000 deaths.[1] The incidence of HNSCC caused by human papillomavirus (HPV) is increasing in many high-income countries, but not in other areas.[2] Questions remain regarding the role of geographic differences in HPV associated HNSCC.[3]

Several recent studies suggest HPV-related biomarkers have utility in predicting HNSCC survival.[4–10] Due to the improved survival in these cases, different staging for HPV-related oropharyngeal cancers has even been proposed,[11,12] and several HPV-OPSCC de-escalation trials are being conducted.[13,14] However, recurrence remains an issue for HPV-related OPSCC patients, and it is unclear which patients may benefit from de-escalated therapy or de-intensified follow-up. Many questions remain, including whether HPV-related biomarkers also have utility in identifying better survival among non-oropharyngeal HNSCC. In addition, given the global geographic variation in the role of HPV, it is not known whether these biomarkers have consistent prognostic utility across different regions. We therefore performed a large international study to rigorously evaluate the ability of biological and behavioral markers to predict HNSCC survival.

Materials and Methods

<u>Study Population and design</u>

This analysis is based on cases derived from case-control studies of HNSCCs in three distinct regions[15]. Studies included: the Brazilian Head and Neck Genome Project (GENCAPO; Southern Brazil – São Paulo)[16], the Carolina Head and Neck Cancer Study (CHANCE, North Carolina, U.S.A.)[17] and the Alcohol Related Cancers and Genetic Susceptibility in Europe study (ARCAGE).[18] Data from ARCAGE included cases from Germany (36%, n=181), Italy (52%, n=259), and U.K (12%, n=62). All cases were enrolled into their respective studies between 2002 and 2011 (US cases enrolled 2002-2006) and prognostic data was collected prospectively by each cohort. The design was for each study to contribute 400-500 HNSCC cases, including at least 110 OPSCC. For each case, studies provided paraffin embedded tumor tissue, risk factor survey data, and cancer characteristics, as well as prognosis and recurrence (obtained through medical record abstraction and cancer registry matching). Data from each study was harmonized by the study datacenter (G.D., D.B., and D.A), which compared all study surveys and centralized data into comparable categories. Detail on data collection and harmonization were previously reported.[15].

Tumor HPV and p16 testing

Tumors were tested centrally at IARC. $p16^{INK4a}$ expression (called p16 hereafter) was tested using immunhistochemistry, according to the protocol provided with the CINtec Histology p16 Kit (9511, mtmlabs). Expression was scored using a composite score based on the percentage of stained cells (0%=negative[0]; 1-10%[1], 11-50%[2], and 51-80%[3], 81-100%[4]=diffusely positive) and the intensity of the nuclear or cytoplasmic staining (no[0], weak[1], moderate[2], or strong[3] staining). The final score was determined by multiplying the extent of positivity

scores of stained cells (0-4) with the intensity scores (0-3); scores of 4 or greater (moderate positivity in more than 10% of cells) were considered positive for p16 expression.

DNA was extracted from paraffin embedded tumor tissues manually and HPV genotyping was performed using the type specific E7 polymerase chain reaction bead-based multiplex method (E7-MPG, IARC, Lyon, France).[19,20] This assay detects all 18 high-risk HPV types, but as HPV16 causes the vast majority of HPV-related OPSCC[15], only HPV16 is considered in this paper (prevalence of other HPV types detected has been reported elsewhere).[15] Five percent of tumors were randomly included as genotyping duplicates. Agreement between duplicates was 97%. All samples were processed together and blinded.

Survey Data

Data collected by the CHANCE, ARCAGE, GENCAPO studies is referred to as data from the "U.S.", "Europe", and "Brazil" hereafter. Tobacco use was measured as, "ever smoke cigarettes, cigars, and/or pipes regularly", with ever regular chewing tobacco use also included in the definition of every tobacco use for the U.S. study only. Regular use was defined in these studies as: ever 100 cigarettes or equivalent of other tobacco product (U.S.), at least once a week for a year (Europe), and daily for a year (Brazil). Cumulative tobacco use was calculated as pack-years of cigarette use plus cigar-years (where 1 cigar equaled 5 cigarettes), plus pipe-years (where 1 pipe equaled 4 cigarettes) plus in the Brazilian study hand-rolled cigarettes (where 1 hand-rolled cigarette equaled 5 store-bought cigarettes).

Regular alcohol use was measured as “ever drink alcoholic beverages regularly”, where regularly was defined as: ever (U.S. and Europe), and at least once a month (Brazil). Cumulative alcohol use was calculated as the sum of drink-years for beer, wine, and liquor drinking. One drink of alcohol was defined as 330mL (12oz) for beer, 125mL (4oz) for wine, and 50mL or one shot for liquor. A drink-year was considered two drinks per day for one year. The drink-years for beer, wine, and liquor were summed for the cumulative alcohol “drink-year” measure.

Cancer characteristics and survival

Tumor sub-site was classified as oropharynx (ICDO C01.9, 02.4, 09.0-10.9), hypopharynx (ICDO C13.0-13.2, 13.8), larynx (C32.0-23.2, 32.8-32.9), or oral cavity (C00.0-00.9, 02.0-02.3, 02.8-03.1, 03.9-04.1, 04.8-05.0, 05.8-06.2, 06.8-06.9). All other sites were excluded. All non-OP HNSCCs that were HPV16-positive had sub-site reconfirmed by local site re-review of all records. Tumor stage was classified using TNM grouping based on the AJCC cancer staging handbook and atlas sixth edition[21], and was available in 89% of cases including 96% of U.S., 75% of Europe and 98% of Brazil cases. Missing stage data in the European study was because the ARCAGE study was initially designed with a focus on risk factors for cancer occurrence, and comprehensive collection of clinical data was not emphasized during the initial years of the study.

Overall survival and disease-specific survival were collected by each study. Recurrence was collected by the European and Brazilian study but not the U.S. study. Follow-up was obtained from each cohort as the date of last confirmed contact, vital status at censor, and date of

death (if applicable). Linkage with national death files was performed by all three studies (in the Brazilian study this matching was only done for participants with missing or unconfirmed information). In addition, linkage with cancer registry data was performed by all three studies (except the German site of the ARCAGE study, which relied on medical record abstraction for cancer confirmation and details).

Statistical Analysis

Characteristics of cases were compared between region/studies using chi-squared for categorical and test of medians for continuous variables. The primary analysis defined cases as HPV-related if they were both p16-positive and HPV16 DNA-positive, and defined cases as HPV-unrelated if they were p16-negative and/or HPV16 DNA-negative. We also examined associations with survival when considering tumor p16 status alone (negative/positive) or tumor HPV16 DNA status alone (negative/positive).

Predictors of overall survival (all-cause mortality) were evaluated using Cox proportional hazard models. Predictors were explored for all HNSCC, and when stratified by sub-site (OPSCC, non-OP HNSCC), and by region/cohort. Mortality was also explored using Kaplan Meier curves stratified by sub-site (oral cavity, oropharynx, hypopharynx, larynx), region/cohort (Brazil, Europe, U.S.), and tumor p16/HPV16 status. The joint effects of HPV and tobacco use on survival were also considered by combining tumor HPV/p16 and tobacco pack-years (pkyr); as survival was similar among smokers with <20 and those with ≥20 pkyr these groups were further combined into ever smokers (≥1 pkyr), within each HPV strata.

Results

Characteristics of cases

There were 1362 incident HNSCCs included in this analysis, all diagnosed between 2002-2011. This included cases from the U.S. (n=472), Europe (n=502), and Brazil (n=388). There were 517 oropharyngeal, 397 laryngeal, 382 oral cavity, and 66 hypopharyngeal SCC. Cases were primarily male (81%), ever smokers (91%), ever drinkers (90%) and had a median age at diagnosis of 58 years (Table 1).

There were many similarities in the characteristics of cases in each of the three regions. In each region, most cases were male, ever smokers with a high median pack-year, and were primarily 50-69 years of age at diagnosis (Table 1). However, there were some notable differences in case characteristics between regions. For example, a larger proportion of OPSCC cases in the study were from the U.S. (47%) than from Europe (22%) or Brazil (31%, $p<0.001$). Cases from Brazil were significantly more likely to be male, stage IV, to have 40 or more pack-years, 40 or more drink-years, and to have died from HNSCC, compared to cases from Europe and the U.S. (Table 1).

As previously reported, p16/HPV16 positivity was higher among OPSCC than non-OP HNSCC cases (35% vs 4%, $p<0.001$).[15] The proportion of OPSCC that were HPV-related varied significantly by region from 4.1% in Brazilian and 31% in European to 59% of U.S. OPSCCs ($p<0.001$).[15]

Case Follow-up

Median follow-up after HNSCC diagnosis was 3.1 years (IQR= 1.4- 5.9). There were 653 deaths observed during 5600 total person-years of follow-up. There were 179 recurrences among 3242 person-years of follow-up in Brazil & Europe (U.S. cohort did not have recurrence data). Median survival was significantly lower among hypopharyngeal cases (3.5 years), than oropharyngeal (5.7 years), oral cavity (5.6 years), and laryngeal (7.3 years) cases (p=0.005); Supplementary Fig. S1. These survival differences, in part reflected differences in stage at diagnoses between case sites, as only 11% of hypopharyngeal cases were early stage (stage 1-2), compared with 14% of oropharyngeal, 42% of oral cavity, and 38% of laryngeal cases (p<0.001).

There were 517 OPSCC followed for 2310 person-years, including 269 deaths. Median OPSCC survival was 5.7 years and causes of death included death from HNSCC (n=162, 60%), from other cancer (n=68, 25%), from a non-cancer cause (n=32, 12%), and 7 (3%) deaths for whom cause was not known. Among 845 non-OP HNSCC followed for 3290 person-years, there were 384 deaths. Median survival for non-OP HNSCC was 6.2 years, and causes of death included deaths from HNSCC (n=207, 54%) from other cancer (n=78, 20%) from a non-cancer cause (n=73, 20%), and 26 (7%) deaths for whom cause was not known.

Predictors of OPSCC Survival

As described in Table 2, among OPSCCs there was a significantly lower risk of death among 184 HPV-related (i.e. p16+/HPV16+) compared to 333 HPV-unrelated (i.e. p16- and/or HPV16-) cases (HR=0.25, 95%CI=0.18-0.34). Similar differences were observed when considering tumor p16-positivity alone (HR=0.32, 95%CI=0.25-0.42; Supplementary Fig. S2) or tumor HPV16 DNA positivity alone (HR=0.36 95%CI=0.27-0.46). Three year overall survival was 82% among HPV-related OPSCCs compared to only 45% among HPV-unrelated OPSCC (p<0.001). This reduction in risk of death among HPV-related OPSCC cases was consistently observed in the U.S. (p<0.001), Europe (p=0.01), and Brazil (p=0.02), (Figure 1; Table 2).

In unadjusted analysis among OPSCC, the risk of death was higher in Europe (HR=1.89, 95%CI=1.36-2.64) and in Brazil (HR=2.97, 95%CI=2.23-3.96) than in the U.S. (Table 3). After adjusting for HPV, age, stage, sex, tobacco and alcohol use, risk of death was similar in Europe and the U.S. but remained elevated among cases from Brazil (aHR=1.68, 95%CI=1.21-2.35). Other significant risk factors for increased OPSCC mortality included higher cancer stage (stage 4 vs 1 aHR=2.05, 95%CI=1.03- 4.06), ever regular tobacco use (aHR=3.20, 95%CI=1.47- 6.96), and older age (per 10-year increase, aHR=1.15, 95%CI 1.00-1.33), Table 3. Risk of death remained significantly *lower* among HPV-related compared to HPV-unrelated OPSCC (aHR=0.34, 95%CI=0.24- 0.49). As shown in Figure 2, three year survival was lowest among HPV-unrelated ever smokers (44%), moderate among both HPV-unrelated never smokers (74%) and HPV-related ever smokers (78%) and highest in HPV-related never smokers (93%), p<0.001. When examining all cases of HNSCC, risk factors for mortality were similar to those for OPSCC (Table 3).

We subsequently explored survival among HPV-related OPSCC cases, using recently proposed prognostic risk groupings that included both stage, age, and smoking pack-years.**[22]** Among HPV-related OPSCCs in this study, overall survival at 3 years was high among stage 1-3 cases whether they had ≤ 20 pkyr (group I) or >20 pkyr (group II), 90% and 91%, respectively. Survival among stage 4 HPV-related OPSCCs was 80% and 55% among those ≤ 70 years old (group III) and >70 years old (group IV), respectively. Survival was lower among HPV-unrelated OPSCC than HPV-related OPSCC within each of these groups (each $p<0.001$).

Predictors of non-OP HNSCC Survival

The predictive utility of HPV16 and p16 among non-OP HNSCC was less clear than that among OPSCC (Figure 3). In univariate analysis among non-OP HNSCC, HPV-related cases had significantly lower risk of death compared to HPV-unrelated cases (HR=0.55, 95%CI=0.31-0.97). This difference was observed among non-OP HNSCC cases from both the U.S. (HR=0.61, 95%CI=0.28-1.32) and Europe (HR=0.56, 95%CI=0.23-1.35); no HPV16+/p16+ non-OP HNSCC cases were detected in Brazil. When considering only p16 tumor status (HR=0.74, 95%CI=0.57-0.96, Table 2) the effect on survival was attenuated and when considering only tumor HPV16 DNA (HR=0.93, 95%CI=0.72-1.20) there was no association (Table 2).

After adjusting for other risk factors, neither p16 alone (aHR=0.83, 95%CI=0.60-1.14), or HPV16 DNA alone (aHR=1.20, 95%CI=0.89-1.63) was a significantly predictor of risk of death among non-OP HNSCC. Risk of death was reduced, although no longer statistically significant, when

using the more specific combined HPV16+/p16+ marker for an HPV-related compared to an HPV-unrelated non-OP HNSCC cases (aHR=0.59, 95%CI=0.32-1.08). Results were similar when disease-free survival was considered (results not shown). Risk factors for survival among non-OP HNSCC included a significant independent effect of older age (per 10-year increase, aHR=1.22, 95%CI=1.09-1.37), higher stage (stage 4 vs 1, aHR=1.93, 95%CI=1.40-2.66) and ever regular alcohol use (aHR=1.68, 95%CI=1.08-2.59). Patients with laryngeal tumor site had significantly lower risk of death compared to those with oral cavity cancers (aHR=0.72, 95% CI=0.57-0.91). Risk of death among non-OP HNSCCs was higher among Brazilian than U.S. (or European) cases in unadjusted analysis (HR=1.33, 95%CI=1.09-1.89), but after controlling for other risk factors, survival was similar across regions (Table 3).

When interaction between tumor site and HPV16+/p16+ was formally tested, a significantly different effect of HPV16+/p16+ on survival was observed among OPSCC than non-OP HNSCC (p-interaction=0.01). After adjusting for other factors, this difference remained, with a significant reduction in mortality observed among HPV16+/p16+ OPSCCs (aHR=0.29, 95%CI=0.08-1.06) but not among HPV16+/p16+non-OP HNSCC (aHR=0.68, 95%CI=0.37-1.24) cases (p-interaction=0.02).

Recurrence

Recurrence was explored among the European and Brazilian cases, as the U.S. cases did not have recurrence information. There were 179 recurrences observed, including 74 recurrences among 271 OPSCC and 105 recurrence among 576 non-OP HNSCC. Risk was lower in HPV-

related than HPV-unrelated OPSCC for both recurrence (2-year recurrence 12% vs. 32%, 5-year recurrence 15% vs 36%, p=0.01) and disease-free survival (DFS; 3-years: 88% vs 66%, p=0.01; aHR=0.53 95%CI=0.19-1.43). Among non-OP HNSCC this difference in recurrence for HPV-related and HPV-unrelated was less clear (2-year recurrence 0% vs. 14%, 5-year recurrence 6% vs 21%, p=0.21).

Discussion

This is one of the first large studies to examine the prognostic utility of HPV biomarkers among HNSCC across continents, using centralized testing and controlling for other risk factors. While the proportion of OPSCC caused by HPV varied widely by region, HPV-related OPSCC cases had similar survival benefits across these different continents. Tumor p16 and HPV16 DNA positivity both were strong biomarkers for improved survival among OPSCC, but their prognostic utility was not as clear among non-OP HNSCC.

The utility of tumor HPV16 and p16 as diagnostic markers among OPSCCs is now well established.[9,23,24] The reduction of risk of death among HPV-related OPSCCs in this study was stronger than some previous studies,[5,25] but was similar in magnitude to some other studies.[6,26,27] Given better survival among HPV-related OPSCC, treatment de-intensification has been suggested and is currently being investigated.[23] This study suggests that HPV-related markers had similar prognostic utility among OPSCC across different countries and continents.

This study suggests that tumor p16 and HPV16 positivity do not have as much prognostic utility among HNSCC outside of the oropharynx, especially after accounting for other risk factors. This is consistent with several previous studies suggesting p16 and/or HPV are not predictors of survival among laryngeal[11,12] or hypopharyngeal[12] SCC. In contrast, an RTOG study did report significant prognostic utility of p16 among non-OP HNSCC (which included a group of 80 oral cavity, 181 laryngeal, and 61 hypopharyngeal cases).[28] However, similar to our study, this RTOG study also reported significant interaction of p16 with tumor site (i.e. that the effect of HPV was significantly stronger among OPSCC than non-OP HNSCC), and found no prognostic effect of HPV ISH among non-OP HNSCC.

Taken together, this research suggests that p16 and HPVPCR likely do not have prognostic use when used alone among non-OP HNSCC. In our study, when a more specific definition requiring both p16-positivity and HPV16-positivity was used, cases with this dual positivity appeared possible have improved survival, but survival was not statistically significantly different (similar to findings in the RTOG trial for dual positivity).[28] This is consistent with a recent study of 142 hypopharyngeal cases which reported prognostic utility when using dual HPV16DNA/P16 positivity.[29] The lack of an association between tumor HPV16 DNA detection and risk of death among non-OP HNSCC underscores that the prognostic utility of this testing is limited to OPSCC. Other risk factors among non-OP HNSCC were consistent with previous research, highlighting the importance of stage, age, tobacco and alcohol use. Alcohol use and

intensity were significant independent predictor of non-OP HNSCC survival across all three regions.

Median survival was notably longer among US than European or Brazilian cases. This is in part explained by the higher proportion of HPV-related OPSCC cases in the U.S. and lower prevalence of co-factors such as tobacco use in the U.S. In multivariate models, region was not a significant predictor of mortality among non-OP HNSCC, but mortality did remain higher among Brazilian OPSCC. This suggests there may be other diagnostic or treatment related factors among OPSCC contributing to OPSCC survival differences.

This study had several limitations and strengths. First, information on tobacco and alcohol use was collected with different survey instruments in each of the three studies. However, each study collected in-depth information on these risk factors directly from the participants (not through medical record abstraction) with clear definitions which could then be harmonized by the datacenter. There was variation in the number of cases with each tumor site from each study. While we cannot exclude the possibility that misclassification of some case sites could have occurred and might have influenced the proportion of HPV-related OPSCC cases if it occurred all tumor sites, designations were carefully reviewed by each study, with a second review of tumor site for all HPV-related non-OP HNSCC cases. All tumor samples were tested centrally and blinded to subsite in the same laboratory. We could not differentiate between stage IV A, B, and C or evaluate treatment in this study and thus can not exclude that some of

the variation in survival observed between regions is explained by differences in the proportion of stage IVC cancers and/or in therapy.

This is one of the first large epidemiologic studies with centralized testing to compare the effect of HPV on OPSCC and non-OP HNSCC survival across continents. We found similar reductions in mortality for HPV-related OPSCC across each region. p16 and HPV16 DNA each showed utility in predicting mortality when used alone or in combination, particularly for OPSCC. The limited prognostic utility of p16 and HPV16 DNA status when used alone for non-OP HNSCC may suggest they should not be used for non-OP HNSCC. Although the impact of HPV on HNSCC varies by region, with large differences in the proportion of OPSCC cases caused by HPV, this study suggests there are HPV-related OPSCC cases in each region with similarly high survival.

Figure Legends

Figure 1: Overall survival among oropharyngeal squamous cell patients with HPV16 DNA positive/p16 positive (HPV-related) compared to HPV16 or P16 negative (HPV-unrelated) tumors, for the US (panel A), Europe (panel B), and Brazil (panel C).

Median survival was significantly higher among HPV-related than HPV-unrelated OPSCC in the U.S (n=242; 3.4 years vs. median not reached, p<0.001), Europe (n=112; 2.2 years vs. median not reached, p=0.01), and Brazil (n=163; 1.5 years vs. median not reached, p=0.02).

Figure 2: Survival of 514 oropharyngeal squamous cell cancers by tumor HPV status and history of tobacco use.

Figure 3: Overall survival among patients with HPV16 DNA positive/p16 positive (HPV-related) tumors compared to those that are HPV16 or p16 negative (HPV-unrelated), for all 1362 head and neck squamous cell cancers(panel A), 517 oropharyngeal squamous cell cancers only (panel B), and 845 non-oropharyngeal head and neck squamous cell cancers only (panel C).

Supplementary Figure S1: Overall survival of 1474 head and neck squamous cell cancers, by tumor site

Supplementary Figure S2: Survival by tumor p16 status among 523 oropharyngeal squamous cell cancer (panel A) and 942 non- oropharyngeal head and neck squamous cell cancer (panel B).

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