ABSTRACT

Objective: To determine the prevalence and describe the clinical characteristics of high risk HPV among patients with oropharyngeal squamous cell carcinomas in our institution utilizing p16 and HPV DNA in-situ hybridization testing and to determine the factors associated with high risk HPV positivity.

Methods:

Design: Retrospective Cohort Review
Setting: Tertiary Private Training Hospital
Participants: 29

Results: A total of 29 primary oropharyngeal squamous cell carcinomas were diagnosed during the 11-year study period (January 2010 to December 2021). Based on the HPV in-situ hybridization status, the prevalence of high risk HPV oropharyngeal squamous cell carcinoma in our institution was 52%. Majority of these cases were males (87.5%) with a median age of ≤55 years old (60%) who are non-smokers (88.2%) and non-drinkers of alcoholic beverages (80%). There was no statistically significant association between age group, sex, smoking status, alcohol intake, lymph node status and high risk HPV infection. The most common tumor site involved in HPV-positive oropharyngeal squamous cell carcinoma was the tonsil (87%). Majority demonstrated a non-keratinizing histology (73%) with positive lymph node status (67%) upon clinical presentation. Fifteen (83%) of the 18 p16 positive squamous cell carcinomas were positive for high risk HPV-DNA. Of note, 3 (17%) out of the 18 p16 positive squamous cell carcinomas turned out to have negative HPV DNA-ISH status.

Conclusion: Although no statistically significant correlation between any clinical characteristic with viral status was established, HPV-mediated oropharyngeal squamous cell carcinoma in this institution was commonly seen among males aged 54 years old and below who are non-smokers and non-drinkers of alcoholic beverages with the palatine tonsil as the most common
Original Articles

Methods

Following approval of the Institutional Review Ethics Committee (RPC-024-03-17), surgical pathology files from January 2010 to December 2021 were reviewed and histologically diagnosed cases of primary squamous cell carcinoma of the oropharynx with available paraffin blocks were included in the study cohort. Baseline demographic and clinical data were extracted from the patients’ medical records and clinical charts. The histopathology samples were taken from tissue biopsies performed before surgery (resection) and treatment (chemotherapy and radiotherapy) were performed. Excluded were records of patients with multiple primary carcinomas, cases with previously diagnosed carcinomas regardless of anatomical site or histopathology, other forms of cancer presenting in the oropharynx (i.e. salivary gland carcinomas and adenocarcinomas), squamous cell carcinomas of adjacent head and neck subsites presenting as extension to the oropharynx and patients with previous oncologic and/or radiation management.

Tissue sections

Formalin-fixed paraffin-embedded tissue blocks were retrieved from the Institute of Pathology. A histopathology review by one of the principal investigators (MVC) was performed on all of the hematoxylin and eosin stained sections. The confirmed tumor areas were then mapped for p16 immunohistochemical staining and subsequent HPV in-situ hybridization testing.

p16 Immunohistochemical staining

One micron tissue sections were stained for p16 using Optiview DAB IHC Detection kit (Roche Diagnostics 6396500001, Basel, Switzerland) on a Ventana Benchmark X instrument (Tissue Diagnostics, ADRIAMED Ltd. Skopje, Macedonia). The sections were stained using hematoxylin II and a bluing reagent. Expression of p16 was assessed using a mouse monoclonal antibody against p16 (E6H4 clone, CINtec™ Histology, Ventana Medical Systems, Tucson, AZ, USA). A cervical biopsy sample with high p16 expression was used as a positive control. A positive p16 immunostain result was characterized by a strong and diffuse nuclear and cytoplasmic staining in more than 70% of the tumor cells. All other staining patterns were considered negative.

HPV-DNA In Situ Hybridization (HPV DNA-ISH)

The in situ hybridization was performed on routinely processed, formalin fixed, paraffin embedded tissues on a Ventana Benchmark XT instrument. It employs a Ventana Inform HPV III Family 16 Probe (B) to detect HPV. It has a probe cocktail with positive hybridization to the following high risk HPV genotypes: 16, 18, 31, 33, 35, 45, 52, 56, 58 and 66.
ORIGINAL ARTICLES

The ISH/iView Blue Plus Detection Kit (Roche Diagnostics 05278856001, Basel, Switzerland) detects DNP labeled probe and antibody bound to the antigen in a paraffin embedded tissue section. The labeled probe or antibody is located by an anti-DNP antibody and biotin-conjugated secondary antibody. A Streptavidine-Alkaline Phosphatase enzyme conjugate is added and the formed complex is visualized by Bromo-4-Chloro-Indolyl Phosphate and Nitroblue Tetrazolium chromogen. The sample tissue that was used as a positive control was a cervical tissue biopsy with a histopathologic diagnosis of squamous cell carcinoma and with a p16 positive immunostain result. A negative control was also used to monitor unintended probe and antibody cross reactivity to cellular components. The same sample tissue was utilized for the positive control per manual insert recommendation.

Interpretation was done using light microscopy. A positive reaction is indicated by the presence of a diffuse or punctate staining pattern. The diffuse pattern appears as large, uniform, globular dark blue precipitate within the epithelial cell nucleus. The punctate pattern is a discreet, stippled dark blue nuclear pattern.

Assay validation was done prior to the initial use of the reagent. The performance was verified by testing it on a series of specimens with a histopathologic diagnosis of squamous cell carcinoma with known diffuse positivity for p16 immunohistochemical stain.

Data Analysis

Sample size was calculated based on the incidence of HPV among oropharyngeal cancer patients assumed to be 70% with a maximum allowable error of 9% and a reliability of 80%. The sample size required was 44.

Descriptive statistics were used to determine the overall frequency of HPV positive cases of oropharyngeal squamous cell carcinoma as well as the proportion of HPV-positive oropharyngeal squamous cell carcinoma by age, sex, tobacco usage, alcohol consumption, drug use, primary tumor subsite (tonsil, base of tongue, soft palate), lymph node status, lymphovascular invasion and tumor histology (non-keratinizing or keratinizing).

Associations between categorical data were tested with Fisher’s exact test. Data was encoded in MS Excel 2010 for Windows version 14.0.7193.5000 (Microsoft Corp., Redmond, WA, USA) and analyzed using IBM SPSS version 14 (IBM Corp., Armonk, NY, USA).

RESULTS

There were 29 participants included in the study. The mean age (± standard deviation) and median age were 57.41 ± 13.88 and 55 years old respectively with a range of 20 to 85 years old. Twenty three (79%) were males and 6 (21%) were females. Twenty seven of the 29 patients had a known smoking status with 21 (72.4%) non-smokers and 6 (20.7%) smokers. There were also 26 patients with known alcohol intake status comprised of 10 (34.5%) with history of alcohol intake, and 16 (55.2%) with no history of alcohol intake. Nineteen (65.5%) of the 29 participants had no history of drug use. There were 26 (89.7%) who received both radio- and chemotherapy and 3 (10.3%) participants had radiotherapy alone. Twenty five of the 29 patients had known lymph node status, 16 (64%) of whom had positive lymph nodes. Unfortunately, none of the 29 patients with oropharyngeal squamous cell carcinoma had a record of their HPV vaccine status in their charts.

Nineteen of the 29 patients had the tonsil (65.5%) as their primary tumor site while 10 (35.5%) patients presented with tumor located at the base of tongue. Eighteen patients (62%) exhibited a non-keratinizing histology. Lymphovascular invasion was appreciated in 34.5% of tumor samples.

Immunohistochemical staining with p16 conducted on the 29 samples revealed 18 (62%) positive cases and 11 (38%) negative cases. As per College of American Pathologists guidelines, HPV-DNA in situ hybridization was performed only on the p16 positive cases. Fifteen (83%) of the 18 p16 positive squamous cell carcinomas were positive for high risk HPV-DNA. Of note, 3 (17%) out of 18 p16 positive squamous cell carcinomas turned out to have negative HPV DNA-ISH status.

Using the median age to categorize the patients into 2-tiered groups, 9 (60%) of 15 participants less than 54 years old were positive for high risk HPV squamous cell carcinoma. Six (43%) of the 14 patients 54 years old and above were positive for high risk HPV squamous cell carcinoma. In terms of sex, 1 (17%) of the 6 female participants was positive for high risk HPV squamous cell carcinoma. Fourteen (61%) of 23 male participants were positive for high risk HPV squamous cell carcinoma. Overall, no association with age (Fisher exact probability test, p = .0063) and sex (Fisher exact probability test, p = .454) was identified.

Of those with known smoking status, 14 (70%) of 20 participants who do not smoke were positive for high risk HPV squamous cell carcinoma. Of the six participants who smoke, only one was tested for high risk HPV and was negative while the rest did not warrant high risk HPV testing due to their negative p16 status. This data failed to show association of smoking history with high risk HPV squamous cell carcinoma (Fisher exact probability test, p = .508).

Eight (50%) of the 16 participants who never had alcohol intake were positive for high risk HPV squamous cell carcinoma. Of the 10 patients who had a history of alcohol intake, 6 were positive for high risk HPV and one was negative for high risk HPV squamous cell carcinoma while the remaining three did not warrant testing with high risk HPV due to their negative p16 status. No statistically significant association was
seen between alcohol intake status and high risk HPV squamous cell carcinoma (Fisher exact probability test, \( p = .039 \)).

Ten (62.5%) of the 16 participants with positive lymph node status were positive for high risk HPV squamous cell carcinoma. This data did not show association between lymph node status and high risk HPV squamous cell carcinoma (Fisher exact probability test, \( p = .633 \)).

Two (20.0%) of the 10 participants with tumor in the base of the tongue had positive HPV-ISH results. Thirteen (68.4%) of the 19 participants with tumor in the tonsil had positive HPV-ISH results. No association between anatomic subsite and high risk HPV status was seen (Fisher exact probability test, \( p = .365 \)).

Four (36.4%) of 11 patients with keratinizing histology had a positive HPV-ISH result. Eleven (61%) of the 18 participants with non-keratinizing histology had positive HPV-ISH results. There was no statistical association between histology and high risk HPV status (Fisher exact probability test, \( p = .256 \)).

Eight (80%) of the 10 participants with lymphovascular invasion had positive HPV-ISH result. This data failed to show statistically significant association between lymphovascular invasion and high risk HPV status (Fisher exact probability test, \( p = .063 \)).

The patient demographics and pathologic characteristics in relation to HPV status are summarized in Tables 1 and 2.

### DISCUSSION

During the 11-year study period (January 2010 to December 2021), a total of 29 primary oropharyngeal squamous cell carcinomas were biopsied and diagnosed histologically in St. Luke’s Medical Center Quezon City and Global City. Of these cases, 15 HPV-positive oropharyngeal squamous cell carcinomas were proven by HPV-ISH method. In this institution, HPV-mediated oropharyngeal squamous cell carcinoma has a prevalence of 51.72%.

Among Asian countries, South East Asia has the highest prevalence (48.61%) of head and neck squamous cell carcinoma followed by East Asia (42.84%). However, in this meta-analysis, no data on HPV-mediated oropharyngeal squamous cell carcinoma was available from South East Asia. Based on the same meta-analysis, the average age of affected patients is 57.3 years old overall. In the United States, the increase in HPV-mediated oropharyngeal squamous cell carcinoma is highest among the middle-aged (40 – 59 years old) population. These findings are congruent with our study since patients with HPV-associated oropharyngeal squamous cell carcinoma have a median age of 55 years old.

In terms of sex predilection, a higher prevalence among the male population is noted to be similar to the findings of other studies. This can be attributed to the fact that men are more likely than women to have an oral HPV infection. Based on this particular study, this may be due to the higher HPV viral load in the female genital mucosa than that of the male genital mucosa, hence men giving oral sex to women have higher viral dose exposure. In the current study, no association regarding the sexual practice and behavior of the patients can be made since all of the HPV-positive cases had no available data regarding this.

Similar to the social demographics in Western countries, our study showed that the majority of patients with HPV-mediated oropharyngeal squamous cell carcinomas were non-smokers (88%) and non-alcoholic.

### Table 1. Clinical-Demographic Characteristics and High Risk HPV Infection

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>High Risk HPV Status</th>
<th>( p ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Age group</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤55 years old</td>
<td>9 (90.0)</td>
<td>1 (10.0)</td>
</tr>
<tr>
<td>&gt;55 years old</td>
<td>6 (75.0)</td>
<td>2 (25.0)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>1 (50.0)</td>
<td>1 (50.0)</td>
</tr>
<tr>
<td>Male</td>
<td>14 (87.5)</td>
<td>2 (12.5)</td>
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<tr>
<td>Smoking status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>0</td>
<td>1 (100.0)</td>
</tr>
<tr>
<td>No</td>
<td>15 (88.2)</td>
<td>2 (11.8)</td>
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<tr>
<td>Alcohol intake</td>
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<td></td>
</tr>
<tr>
<td>Ever</td>
<td>6 (85.7)</td>
<td>1 (14.3)</td>
</tr>
<tr>
<td>Never</td>
<td>8 (80.0)</td>
<td>2 (20.0)</td>
</tr>
<tr>
<td>Lymph Node status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>6 (50.0)</td>
<td>6 (50.0)</td>
</tr>
<tr>
<td>Negative</td>
<td>2 (28.6)</td>
<td>5 (71.4)</td>
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### Table 2. Pathologic Characteristics and HPV Infection

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>HPV Status</th>
<th>( p ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Tumor site</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Base of tongue</td>
<td>2 (100.0)</td>
<td>0</td>
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<tr>
<td>Tonsil</td>
<td>13 (81.25)</td>
<td>3 (18.75)</td>
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<tr>
<td>Histology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Keratinizing</td>
<td>4 (100)</td>
<td>0</td>
</tr>
<tr>
<td>Non-keratinizing</td>
<td>11 (78.6)</td>
<td>3 (21.4)</td>
</tr>
<tr>
<td>Lymphovascular invasion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>8 (100)</td>
<td>0</td>
</tr>
<tr>
<td>Absent</td>
<td>7 (70)</td>
<td>3 (30)</td>
</tr>
</tbody>
</table>
beverage drinkers (80%). In a single-institution retrospective study done at the University of Texas MD Anderson Cancer Center, majority of newly diagnosed patients with oropharyngeal squamous cell carcinoma were non-smokers and non-drinkers of alcoholic beverages.\textsuperscript{14} Cases with HPV-positive oropharyngeal squamous cell carcinoma are also less likely to have a history of smoking, with most of the series consisting of 30% non-smokers in the HPV-positive group compared with less than 5% in the HPV-negative group.\textsuperscript{16} Several studies have shown that HPV-associated squamous cell carcinoma is incited by exposure and subsequent infection with high-risk HPV (HPV 16) and such carcinoma can progress independently of alcohol and smoking status.\textsuperscript{17,18} Likewise, HPV-positive squamous cell carcinoma has wild-type p53 and high levels of p16. On the other hand, HPV-negative squamous cell carcinoma exhibits mutation in p53 and is caused by long standing exposure to alcohol and smoking products.\textsuperscript{18} Several studies have shown strong association between HPV-mediated oropharyngeal squamous cell carcinoma in the absence of tobacco use.\textsuperscript{19,20,21} A recent study also reported that non-smokers are 6.1 times more likely to be infected with high risk HPV.\textsuperscript{22} However, this statistical association between non-smokers and HPV-positive squamous cell carcinoma was not observed in our study population.

In a 2017 study done in the northwestern region of the Philippines, the prevalence of HPV-mediated oropharyngeal squamous cell carcinoma was 13.4%.\textsuperscript{23} In the same study which included other head and neck areas, the oral cavity (53.7%) surprisingly comprised the majority of HPV-mediated head and neck squamous cell carcinomas. The median age at diagnosis was 60 years old and in terms of risk factors, the majority were smokers (70.3%) and alcoholic beverage drinkers (47.3%).\textsuperscript{22} In contrast, the prevalence of HPV-mediated oropharyngeal squamous cell carcinoma in our study was 36.3%. The difference in the site prevalence, median age and smoking association from our study could possibly be attributed to the contrasting population included in the study cohort and differences in sexual practices of Filipinos between the two geographically distinct regions. A local study in 2004 noted that only a minority of those surveyed practiced oral sex and these were mostly young Filipinos residing in urban or metropolitan areas.\textsuperscript{24} However, the population of the northwestern study mostly comprised an older age group who were living in rural areas where such sexual practices are usually not observed.\textsuperscript{23} In one study, HPV-mediated oropharyngeal squamous cell carcinoma was highest among Caucasians followed by Hispanics, with African Americans having the lowest prevalence.\textsuperscript{25} However, African American patients of high socioeconomic status were more likely to have HPV positive carcinomas than were those of low socioeconomic status.\textsuperscript{24} Though socioeconomic status may play a role, its extent of association could not be established across the local studies as such data were not extensively explored. On the other hand, the higher incidence of oral cavity squamous cell carcinoma in the northwestern region can be attributed to the habit of betel nut chewing and tobacco chewing, both of which are not commonly practiced in the metropolitan areas.\textsuperscript{26} Another possible cause could be the difference in employed testing methods. The northwest region of the Philippines study utilized Polymerase Chain Reaction (PCR) for HPV DNA and HPV mRNA detection while this study used p16 immunostaining and HPV DNA in-situ hybridization.

According to Sturgis et al., the rate of HPV-positive squamous cell carcinoma has consistently been highest in the oropharynx compared with the oral cavity, larynx or hypopharynx.\textsuperscript{12} In oropharyngeal squamous cell carcinoma, the tumor sites which are most strongly associated with HPV are the tonsil (41.4%) and base of the tongue (44.3%).\textsuperscript{14} This is congruent with our study since the majority of the HPV-mediated oropharyngeal carcinomas also involved the tonsil (75%) and base of tongue (25%). A study in 2000 showed that oropharyngeal tumor specimens were 7.7 times as likely to have HPV DNA.\textsuperscript{18} The preference of HPV for the oropharyngeal area is related to the presence of transitional mucosa in the oropharynx which is predominantly found in the tonsillar area.\textsuperscript{27,28,29} The invagination of the mucosal surface of the tonsil can also facilitate virus capture and promote access to the basal cells which are the cells preferentially infected by HPV.\textsuperscript{30} These aforementioned findings support the higher proportion of HPV-mediated squamous cell carcinoma in the tonsillar area.

Majority of the HPV-positive oropharyngeal samples demonstrated a non-keratinizing histology and a positive lymph node status (n=7; 88%). More than half (n=5; 63%) did not present with lymphovascular invasion. Studies have shown that HPV-mediated oropharyngeal squamous cell carcinomas are more likely to present with higher N stage\textsuperscript{31,32} and demonstrate a non-keratinizing histology.\textsuperscript{18,33,34} HPV-mediated oropharyngeal squamous cell carcinoma is more likely to present with small primary tumors hence patients are more likely as well to be asymptomatic. As a result, these patients do not seek medical treatment until a symptomatic nodal disease occurs.\textsuperscript{35} Due to the increasing incidence of oropharyngeal squamous cell carcinoma and its definite association with high risk HPV infection, there is now an emphasis on the classification of oropharyngeal squamous cell carcinoma based on the presence or absence of high risk HPV. In terms of prognosis, HPV-mediated oropharyngeal squamous cell carcinoma carries a favorable prognosis compared to HPV-negative tumors. The five-year survival rates for patients with advanced stage HPV mediated oropharyngeal squamous cell carcinoma are 75-80% compared to survival rate of less than 50% among patients with HPV negative carcinoma.\textsuperscript{35} In terms of diagnosis, the National Comprehensive
Cancer Network Clinical Practice Guidelines in Oncology directs that oropharyngeal squamous cell carcinoma be tested for HPV by p16 immunohistochemistry.\textsuperscript{36} Immunohistochemical staining with p16 has been widely adopted since it is cost effective, reliable and is able to examine paraffin embedded tissue. Utilization of p16 immunostain is a good screening test for HPV positive oropharyngeal squamous cell carcinoma that is in keeping with general HPV testing recommendations. This is because the p16 protein is overexpressed in HPV positive cases and lost in HPV negative cases.\textsuperscript{37} However, as sensitive as it is as a surrogate marker for HPV, its overexpression does not always translate to the presence of high risk HPV infection among HPV-ISH samples. The discordance between the two methods can be attributed to the presence of high risk HPV subtypes which are not detected by the probe, the presence of p16 promoter hypermethylation or p16 overexpression that occurs independently of HPV gene expression.\textsuperscript{38,39}

As a result, multimodality HPV testing is now being utilized to definitively identify HPV-mediated oropharyngeal squamous cell carcinoma. For example, the National Institute for Health and Care Excellence (NICE Guidelines) recommends reflex testing for high risk HPV DNA or RNA ISH in all p16 positive oropharyngeal squamous cell carcinoma.\textsuperscript{40,41} In one study, HPV DNA ISH was noted to have a specificity of 100% and sensitivity of 83%.\textsuperscript{42} Likewise, DNA ISH (88%) has a similar specificity with DNA PCR (87%).\textsuperscript{42} Another advantage of HPV DNA ISH is that it can also be utilized in paraffin embedded tissue samples and even in fine needle aspirate specimens. However, it should be noted that in some practice, almost all of their p16 positive but HPV DNA-ish negative patients were actually positive for HR-HPV by mRNA in-situ hybridization. Thus, many consider the detection of HR-HPV E6 and E7 messenger RNA by ISH as the gold standard.\textsuperscript{43} Nevertheless, given the higher specificity of ISH compared to p16 immunostain, the use of both tests results in a higher sensitivity and specificity for detecting high risk HPV compared to single modality testing.

Since the prevalence rate of HPV-mediated oropharyngeal squamous cell carcinoma varies depending on the study population, the current data of this study is limited to the population catered by the private tertiary hospital in the metropolitan area. Our study is limited by its small sample size of 29 that did not meet the computed required sample size of 44, and our results have to be taken in this light. A longer study period and/or a larger sample size are recommended to further establish the sought after association with the other clinical and pathologic factors. In terms of diagnosis, all oropharyngeal squamous cell carcinomas particularly those in the tonsillar area should be routinely tested for HPV viral status. Given the established high sensitivity and low specificity of p16 immunostain in detecting high risk HPV infection, a second diagnostic test such as HPV DNA-ISH which is highly specific may be performed in conjunction with the p16 stain in order to assess and confirm the high risk HPV status of an oropharyngeal squamous cell carcinoma for better prognostication. Again, a higher number of cases may elucidate this association better. We also recommend that future studies consider alternative testing protocols that will be made available as more testing methods come in to our country to validate data from Western counterparts.

In summary, our study suggests that institution-wise, HPV-mediated oropharyngeal squamous cell carcinomas are commonly seen among middle-aged males (median age of 55) who are non-smokers and non-drinkers of alcoholic beverages. In terms of pathologic features, majority of HPV-mediated oropharyngeal squamous cell carcinoma exhibit a non-keratinizing histology. The use of p16 immunostain as a surrogate marker for HPV status correlates well with HPV in-situ hybridization studies. From a clinical standpoint, attending physicians should be more discerning when a patient presents with a tonsillar mass, taking into consideration non-traditional epidemiologic factors associated with HPV-mediated oropharyngeal squamous cell carcinomas and thus should align their queries to pertinent sexual practices and vaccination status.
REFERENCES


