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Preliminary Study on Prevalence of P16-Positive Squamous Cell Carcinoma of the Oral Cavity, Oropharynx and Larynx in Rizal Medical Center and its Histomorphologic Correlation

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ABSTRACT

Background. A considerably large portion of the cases of cancer, particularly squamous cell carcinoma (SCC) involving the head and the neck may be due to consumption of tobacco and alcohol. However, its increase in occurrence at specific sites of the head and neck may indicate the possibility of other etiological factors. One of which is infection by certain high-risk human papillomavirus (HPV). P16 immunochemistry serves as a very good surrogate marker of active HPV in these tumors. The detection of HPV-related head and neck cancers have relevance in clinical practice because of its prognostic implications.

Objectives. The general objective of this study is to determine the prevalence of p16-positive SCC in the oral cavity, oropharynx, and larynx in Rizal Medical Center from January 2019 to December 2019. The specific objective is to compare the Hematoxylin and Eosin (H&E) stain histomorphology (keratinization and mitotic activity) of p16-positive versus p16-negative specimens.

Methodology. This is a cross-sectional study which included all routine histopathology specimens coming from the oral cavity, oropharynx and larynx in Rizal Medical Center for the year 2019 with a diagnosis of squamous cell carcinoma. The tissue specimens considered were those that measured at least 1 cm in diameter, or aggregate diameter if tissue is fragmented. The slides of all eligible cases were reviewed and immunohistochemically stained for p16. The p16 IHC slides were read as either positive or negative, while the mitotic activity and keratinization were observed in the H&E-stained slides. The interpretation of the diplomate pathologists for each of the slides were documented and corresponding statistical analyses were performed.

Results. P16 IHC showed twenty-one (88%) p16 negative cases and three (12%) p16 positive cases. In terms of mitosis, ten cases have mitosis falling within the 1-10 per high power field (HPF) range (42%), six within 11-20 per HPF (25%), and 8 cases have ≥21 mitosis per HPF (33%). In terms of keratinization, three cases are non-keratinizing (12%) and twenty-one cases are keratinizing (88%). There is significant difference in the keratinization histology ($p<0.05$) of the p16-positive versus the P16-negative cases. On the other hand, no significant difference in the mitotic activity ($p>0.05$) was noted.

Conclusion. There is a low prevalence of HPV-related SCC of the oral cavity, oropharynx, and larynx in Rizal Medical Center. The histomorphologic findings confirm that keratinization, significantly predicts HPV status in oropharyngeal SCC. Mitotic activity may not be reliable in predicting the HPV status or p16 IHC reactivity of a case. Keratinization in oropharyngeal SCCs may provide valuable information in certain instances, particularly when HPV testing is not immediately available, although the combined tumor morphology and p16 IHC is more ideal.

Key words: papillomavirus infections, squamous cell carcinoma of head and neck, mouth, larynx

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INTRODUCTION

A considerably large portion of the cases of cancer involving the head and the neck is by higher-than-normal consumption of tobacco and alcohol. However, the noticeably increased occurrence of the said phenomenon at specific sites indicates the possibility that other etiological factors are involved. In certain localities, it has been reported that infection by certain high-risk types of high-risk human papillomavirus (HPV) are implicated in cases of head and neck cancers – the most notorious of which is oropharyngeal cancer. Evidence suggested by current studies identify HPV16 as being associated in cancers of the tonsils, the base of the tongue, and other sites in the oropharynx.[1] This is not to say that HPV16

is solely responsible for the development of these cancers since several risk factors have also been found to protract their prevalence and worsen their effects such as changing sexual behaviors, involvement in oral sex, high turnover rates in terms of sexual partners, and involvement in sexual intercourse with someone of the same sex.[2,3]

In the United States, HPV-associated cancer rates increased for oropharyngeal cancer from year 1999 to 2015.[3] In the Philippines, Bruni et al., mentioned that there is an increased incidence of oropharyngeal cancer in the year 2018. The annual number of new cancer cases for males is 311 and 118 for females.[3] Currently, there is no available local data on the prevalence of p16 positivity for head and neck squamous cell carcinoma (SCC).

Because of the prognostic implications of HPV-related oropharyngeal SCC, histomorphology and p16 immunohistochemistry (IHC) may help physicians in their clinical judgement and treatment approaches. P16 IHC may be utilized as an alternative means to indicate the presence of high-risk HPV[4] and it offers a more cost-effective, more manageable and convenient alternative to HPV-specific testing.

The detection of HPV in oropharyngeal SCC has relevance in clinical practice because of its prognostic implications. Several studies in the past have indicated better prognostic outlooks and improved disease-free survival for patients diagnosed with HPV-positive tumors in the oral cavity. Some medical researchers opined that the significantly better clinical outlook for HPV-positive tumors is attributed to their radiosensitivity and focality. In fact, the National Comprehensive Cancer Network (NCCN) guidelines have prescribed separate treatment algorithms for p16-positive and p16-negative oropharyngeal SCCs.[5] The actual prevalence of HPV in oral SCC must also be elucidated because it has implications to public health. For instance, said statistics will provide an input for health agencies to determine whether or not there is a need to allocate resources for HPV vaccination or prevention. The determination of morphologic features such as keratinization status and mitotic index as predictors of HPV status may be of value in resource-limited settings when p16 IHC is not readily available.

OBJECTIVES

General objective

To determine the prevalence of p16-positive SCC in the oral cavity, oropharynx, and larynx in Rizal Medical Center from January 2019 to December 2019.

Specific objective

To compare the Hematoxylin and Eosin (H&E) stain histomorphology (keratinization and mitotic activity) of p16-positive versus p16-negative specimens.

REVIEW OF RELATED LITERATURE

Head and neck SCC usually manifest in the larynx, oropharynx, nasopharynx, hypopharynx, paranasal sinuses, oral cavity, and salivary glands.[6] They have been listed as among the commonly occurring cancers in the world with estimated figures of around 600,000 new cases and approximately 320,000 deaths yearly, indicating an increasing trend from the figures obtained in the previous years.[7] Large scale epidemiological researches have shown that head and neck cancers are more common among men than women. Moreover, about 90% of head and neck cancers are SCC, rendering said histologic type as the most commonly occurring.[6] The presence of HPV in these tumors as identified in several studies suggests the etiological implication of HPV in tumorigenesis. Initially, HPV was believed to account for at least 23% of oropharyngeal cancer.[8] In more recent studies, however, it has been shown that at least 70% of oropharyngeal cancer incidence in the US in the last three decades may be casually linked to HPV.[9] These may be conferred by the fact that unlike cervical cancer which affects only women, oropharyngeal cancer affects both men and women.

Worldwide data on the prevalence and type distribution of HPV in head and neck SCC have been systemically reviewed and subjected to meta-analysis with results showing HPV DNA to be less prevalent in the in the oral cavity (24.2%) and in the larynx/hypopharynx (22.1%) than in the oropharynx (45.8%). HPV16 is implicated in 82.2% of all HPV DNA-positive cases, thereby indexing it as genotype most frequently found in head and neck SCC.[10]

The relative prevalence of HPV-associated oropharyngeal SCC varies among geographic regions, the highest (29%-93%) cases having been observed from economically developed countries.[11] HPV has been identified as the primary and essential etiologic agent of cervical cancer due to its susceptibility of being transmitted through sexual intercourse. In like manner, HPV-positive oropharyngeal SCC has also been associated with sexual behavior.[12]

In the Philippines, a 2013 study estimated that the annual incidence (per 100,000 population) of cancer in the oral cavity is 2.4, while laryngeal and oropharyngeal cancers had an annual incidence of 1.5 and 1.3, respectively.[7] In terms of incidence among women, cancers of the lip/oral cavity rank 7th, cancers in the larynx rank 10th, and that in the pharynx ranks 13th.[7] Excessive alcohol and tobacco consumption have been listed as the main risk factors in head and neck SCC. In response, campaigns against alcohol and tobacco use in several western countries over the recent decades have significantly reduced the incidence of cancers in the oral cavity and the larynx. Unfortunately, said measures seem to have failed where oropharyngeal cancer is concerned.[11,13] In fact, it has been observed that there remains to be a steady increase in cases of SCC in the oropharynx and this is especially true in countries with more developed economies.

HPV types are either low-risk or high-risk. Carcinomas are usually implicated with high-risk serotypes of HPV, 16 and 18.[14] In cases of carcinogenesis involving HPV, molecular activity is characterized by the papillomaviral DNA integrating with the host DNA. HPV E6 binds with the gene product p53, which is a tumor-suppressor, and inactivates it. At the same time, the HPV E7 does the same thing with the retinoblastoma tumor-suppressor protein (pRb), effectively deactivating the tumor-suppressing function of the retinoblastoma gene which results to cell

cycle progression.[15] The inactivation of the retinoblastoma protein by E7 causes a feedback loop that increases the activity of the P16 cyclin-dependent kinase which upregulates cell cycle. This marked upregulation of P16 has been observed in cases of head and neck SCC caused by high-risk HPV because this molecular activity leads to a paradoxical overexpression of the tumor-suppressor protein p16, which is consistently overexpressed in the nuclei and cytoplasm of tumors with transcriptionally active high-risk HPV.[16] P16 IHC thus serves as a very good surrogate marker of active HPV in these tumors.

The prognostic value of p16 positivity in head and neck SCC has been documented by several medical researchers. In particular, studies that looked into the better clinical outcome for patients with p16-positive oropharyngeal SCC corroborate that patients with HPV-related tumors in the head and neck region have better clinical outlooks than HPV-negative patients.[17-19] The importance of determining pl6-positivity for better prognosis holds true for patients treated with radiotherapy alone as well as for those who received treatment that combined systemic treatment and radiotherapy.

The prognosis for HPV-related SCC shows markedly better outlooks than for HPV-negative carcinomas notwithstanding the tendency of HPV-related SCCs to metastasize to neck lymph nodes early in the course of disease. In fact, risk of death is 30-50% lower for HPV-related oropharyngeal SCC than for HPV-negative SCC. However, patients with history of excessive tobacco use and/or are currently (heavy) smokers may not benefit from the aforesaid improved prognosis. HPV-related oropharyngeal SCC responds better to both primary chemoradiation and surgical treatment. This may be conferred by the fact that these tumors have lower mutation rates and are less genetically complex than HPV-negative cancers. There are now definitive prospective studies showing that the prognosis of HPV-related OPSCC patients has improved such that the head and neck oncology community is essentially united in the concept that all new patients should be tested for high-risk HPV.[20]

According to Chernock (2012), most HPV-unrelated tumors have keratinizing characteristics while most HPV-related SCCs in the oropharynx appear usually as non-keratinizing. Microscopically, HPV-related non-keratinizing tumors have the characteristic of aggregating into large nests with borders that push against each other due to the absence of adequate stromal response. These tumors tend to undergo frequent mitoses and is characterized by an accumulation of dead cells in a central location, otherwise known as central comedonecrosis. The shape of the tumor cells varies; some are ovoid while other are spindle-shaped. The cell borders are often indistinct. These tumor cells are also characterized by hyperchromatic nuclei with no distinct nucleoli. There is either very minimal squamous maturation or none at all. Alternately, HPV-related tumors have also been described as basaloid, basal-like, poorly-differentiated, or non-keratinizing.[21] According to Bishop et al. (2015, 2017), HPV-SCCs are characterized by elevated mitotic rates and frequent tumor necrosis. In contrast, non-HPV-related keratinizing SCCs are typically composed of infiltrative nests with prominent stromal desmoplasia. The tumor cells are polygonal with distinct cell borders and have more abundant, eosinophilic cytoplasm. Squamous maturation is diffuse.[22,23]

METHODOLOGY

This is a cross-sectional study that involves routine histopathology specimens coming from the oral cavity, oropharynx and larynx in Rizal Medical Center for the year 2019 diagnosed as SCC. This study was reviewed and granted approval by the Institutional Review Board (IRB) of Rizal Medical Center.

Inclusion and exclusion criteria

This study involves routine histopathology specimens coming from the oral cavity, oropharynx and larynx in Rizal Medical Center for the year 2019 diagnosed as SCC. The tissue specimens must fulfill the following conditions: the surgical procedure done to the specimen was at least an incision, wedge, excision or resection; for incision or wedge biopsies, the tissue on the slide must be at least 1 cm; and no prior IHC was done on the specimen. Any of the following criteria excluded a specimen from this study: other carcinomas of the sites of interest aside from SCC, tissue size less than 1 cm in diameter or aggregate diameter; and cases for which the microscopic slides and/or paraffin blocks could not be retrieved (slide reviews, missing blocks). The researchers focused on tissue specimens with greater than or equal to 1 cm aggregate/greatest tissue area on the slide to ensure adequate tissue area for study and better assessment of mitotic activity and keratinization.

Study sample

A total of twenty-four (24) tissue specimens from the oral cavity, oropharynx and larynx, with a diagnosis of SCC obtained as per inclusion and exclusion criteria, were included in this study.

P16 IHC staining

A specific slide and block was chosen for p16 IHC staining and sent to Providence Hospital Laboratory for processing. Heat-Induced Epitope Retrieval (HIER) was used as the antigen retrieval method. Slides were stained by mouse monoclonal p16 IHC stain (Clone name: E6H4™) by CINtec®, using the Ventana Benchmark XT machine.

Hematoxylin & Eosin stain and Immunohistochemical stain interpretation

The labels of slides stained with H&E and p16 IHC were covered and were instead assigned with numbers one to twenty-four (1 to 24). Three (3) board certified anatomic pathologists were blinded and asked to interpret the stained slides. The p16 IHC slides were read as either positive or negative, while the mitotic activity and keratinization were observed in the H&E-stained slides. The interpretation of the pathologists for each of the slides were tallied and recorded. Concordance of interpretation is achieved when two of the three pathologists have the same reading for p16 IHC, keratinization, and mitotic index. The microscope used was the Olympus CX23 light microscope.

P16 IHC

AJCC criteria for p16 immunopositivity is diffuse (≥75%) tumor expression, with at least moderate staining intensity.

Overexpression of p16 is usually localized to tumor cell nuclei and cytoplasm, so that p16 staining that is localized only to the cytoplasm is deemed non-specific and, thus, not diagnostic (negative)[24] (Figures 1 and 2).

Keratinization

Keratinizing SCCs appear pink on low power due to abundant keratinizing cytoplasm, keratin pearls, and prominent intercellular bridges. They are characterized by their growth in irregular cords and nests with associated stromal desmoplasia. In contrast, features indicative of non-keratinizing SCC include growth in circumscribed sheets and nests of cells without associated desmoplasia; a blue appearance on low power due to high nuclear-cytoplasmic ratio; tumor infiltrating lymphocytes and lack of keratin; plentiful tumor necrosis; and lack of intercellular bridges[23] (Figures 3 and 4).

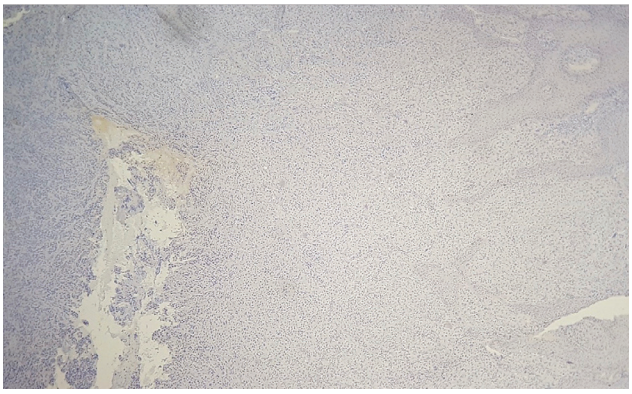

Figure 1. P16 negative IHC staining (P16 IHC, 40x).

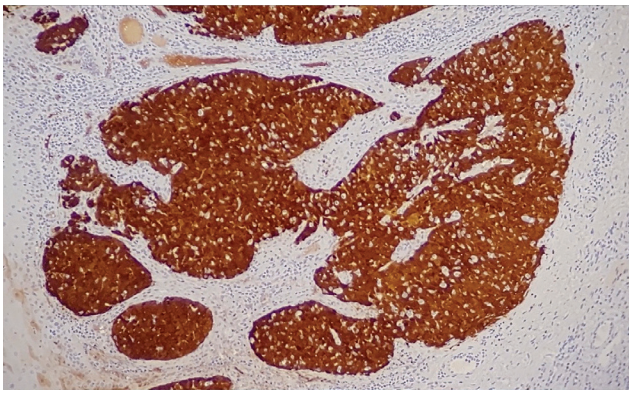

Figure 2. P16 positive IHC staining. (P16 IHC, 100x).

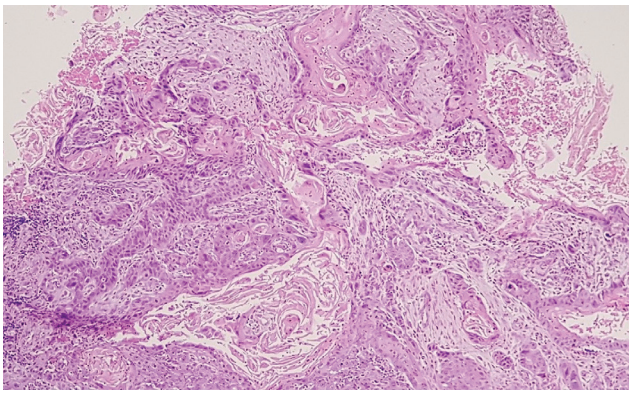

Figure 3. Keratinizing SCC (H&E, 100x).

Mitosis

Mitotically active cells were counted in a total of 10 high power fields (HPF) (Figure 5).

Data handling and analysis

Obtained data was encoded in MS Excel and summarized using descriptive statistics. Categorical data were presented as frequencies and percentages. The Shapiro-Wilk Test was used to test the normality of the dataset in the three variables, namely, IHC staining, mitosis activity, and keratinization. Shapiro-Wilk Test of normality provides that the dataset is normal only if the obtained *Sig.*-value is greater than 0.05 ($p>0.05$). Shapiro-Wilk Test results for IHC staining, mitosis activity, and keratinization all yielded a *Sig*-value of 0.000 which did not satisfy normal distribution. Hence, the use of nonparametric tests in treating all three datasets.

Test for significant differences between p16 IHC staining and the variables mitotic activity and keratinization were determined using Mann-Whitney U test at 0.05 significance level. For easy and effective visualization, stacked or clustered bar charts was created. Interrater agreement was assessed by computing Fleis Multirater Kappa coefficient (K). The results are as follows: mitosis, K=0.250, keratinization, K=0.343, and for p16 Prevalence, K=0.379 which all signify fair strength of agreement using the K value interpretation by Altman.

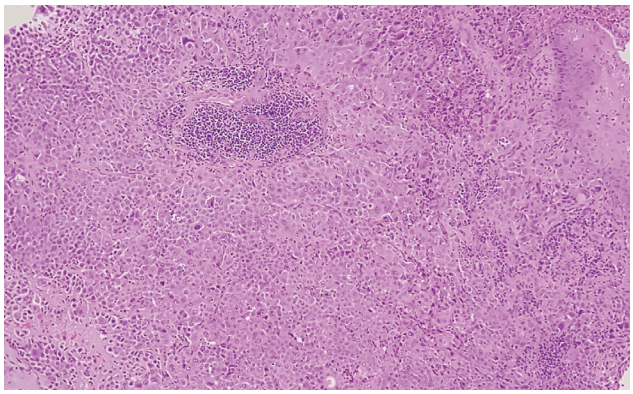

Figure 4. Non-keratinizing SCC (H&E, 100x).

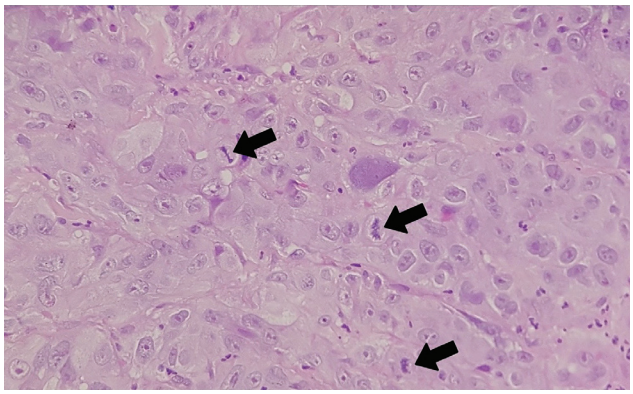

Figure 5. Mitotically active cells *(black arrows)* (H&E, 400x).

RESULTS

Out of the twenty-four slides submitted for p16 IHC stain, three (12%) turned out to be p16 positive, and 21 (88%) were p16 negative. All of the three p16 positive cases were from subjects within the age bracket 60-73 years. Two of the positive cases were from male subjects and one from a female. The sources of the p16 positive specimens were the following: oropharyngeal mass, alveolar ridge mass, and tonsillar mass.

On the other hand, p16 negative cases had an age bracket of forty-eight to eighty-two years (48-82), with thirteen (13) males and eight (8) females. Tissues of the p16 negative cases were from the tongue, larynx, alveolar ridge and oropharyngeal areas (Table 1).

The H&E slides were then evaluated for mitotic activity and keratinization. Of the 24 cases, ten cases have mitosis falling within the 1-10 per high power field (HPF) range (42%), six within 11-20 per HPF (25%), and 8 cases have ≥21 mitosis per HPF (33%). In terms of keratinization, three cases (12%) are non-keratinizing and twenty-one cases (88%) are keratinizing (Table 2).

The three p16 positive cases fell under different mitotic count ranges. In the p16 negative cases, there were nine (38%) that fell under the mitotic range of 1-10, five (21%) fell under 11-20, seven (29%) fell under ≥21/HPF (Table 3).

For the p16 positive cases, two have non-keratinizing histology (75%) and one has a keratinizing histology (8%). For p16 negative group there were eighteen (75%) cases that are keratinizing and three (13%) that are non-keratinizing (Table 4).

The p16 positive group had a higher mitotic activity by 0.77 than the p16 negative group as reflected in the mean ranks (positive=13.17, negative=12.40) (Table 4). Mann-Whitney U test was utilized to compare the difference of these 2 groups in terms of mitotic activity given that the distribution of observations for the aforesaid variables were not approximately normally distributed. Results show that the slight difference in the mean ranks between p16 positive and p16 negative is not significant (U= 29.5, p=0.852) at 0.05 significance level.

Regarding keratinization, the p16 positive group and the p16 negative group had a mean rank difference of 6.29 which was found to be significant (U=15.000, p=0.041) at 0.05 significance level.

Table 1. Demographics and stratification of P16 positive and P16 negative cases

	p16-positive (n=3)	p16-negative (n=21)
Age	60 - 73	48 - 82
Gender	Male – 2 Female – 1	Male – 13 Female – 8
Specimen sources	Oropharynx – 1 Alveolar ridge – 1 Tonsil – 1	Tongue – 10 Larynx – 9 Oropharynx – 1 Alveolar ridge – 1

Table 2. Profile in terms of p16 IHC stain, mitosis, and keratinization

p16 IHC stain	n (%)
Negative	21 (88%)
Positive	3 (12%)
Mitosis / high power field	
1-10	10 (42%)
11-20	6 (25%)
≥21	8 (33%)
Keratinization	
Non-keratinizing	3 (12%)
Keratinizing	21 (88%)

Table 3. Mitotic activity of p16 negative and p16 positive cases

p16 IHC staining	Mitosis / high power field n (%)		
	1 - 10	11 - 20	≥ 21
Negative	9 (38%)	5 (21%)	7 (29%)
Positive	1 (4%)	1 (4%)	1 (4%)

Table 4. Keratinization of p16 negative and p16 positive cases

p16 IHC staining	Keratinization n (%)	
	Keratinizing	Non-keratinizing
Negative	18 (75%)	3 (13%)
Positive	1 (4%)	2 (8%)

DISCUSSION

In this study, three out of the twenty-four included cases turned out to be p16 positive, and belonging to the older population in the 60-73 years age bracket. This is in contrast to other western studies which show higher HPV-association in younger individuals. The prevalence of HPV-related cancers in the Philippines and Southeast Asia in general, is not well established. Most publications regarding HPV-associated oropharyngeal SCC are from western countries and are noted to have a high incidence and cases are still rising. This indicates the need for better diagnostics regarding HPV-related cases of the oral cavity, oropharynx and larynx because in our country to establish a more accurate epidemiological data in the Philippines.

Specimens from the tongue and larynx turned out to be p16 negative. This result is in agreement with data from other studies showing that high-risk HPV infection is present in low percentage (about 5%) in the oral cavity, larynx and hypopharynx.[25,26] In terms of keratinization, the p16 positive cases tended to have non-keratinizing histology while those that are p16 negative are likely to have keratinizing features. This is consistent with the data seen in some studies.[21,27] In terms of mitosis, results show that the slight difference in the mean ranks between p16 positive and p16 negative is not significant at α-0.05. This result may indicate that HPV-related SCCs in the p16 positive cases does not necessarily imply higher mitotic activity. This also means that lower mitotic activity does not necessarily confer the absence of HPV-association. This is in contrary to the claim in one study by Stevens and Bishop (2017), that HPV-related SCCs have high mitotic rates.[23]

CONCLUSION

There is a low prevalence of HPV-related SCC of the oral cavity, oropharynx, and larynx among patients in Rizal Medical Center. This study also highlights the need to address issues on the lack of available local data and clear-cut diagnostic protocols or consensus given that there are prognostic and treatment implications. The findings in this research confirm that tumor histomorphology, specifically the keratinization, significantly predicts HPV status in oropharyngeal SCC. Mitotic activity may not be a reliable marker in predicting the HPV status or p16 IHC reactivity of a case. Keratinization in oropharyngeal SCCs may provide valuable information in certain instances, particularly when HPV testing is not immediately available, although the combined tumor morphology and p16 IHC is more ideal.

RECOMMENDATIONS

Further studies with larger sample size may strengthen the prevalence of p16-positive oropharyngeal SCCs in Rizal Medical Center. This may also help in better assessing histomorphologic characteristics like mitotic activity. Larger sample sizes may determine whether mitotic activity could predict p16 IHC result or HPV status of oropharyngeal SCCs. Studies of more histomorphologic features like desmoplasia, nuclear-cytoplasmic ratio, tumor-infiltrating lymphocytes and tumor necrosis are recommended to better compare p16 positive and p16 negative oropharyngeal SCCs in H&E-stained slides. Given its low occurrence in the oral cavity and larynx, the researcher recommends focusing more on p16/HPV studies of tissues coming from the oropharynx and tonsils. Additional pathologists are also recommended in assessing histomorphologic features for a higher and reliable measurement of agreement and lower inter-observer subjectivity and variability. Further diagnostic studies involving PCR-based techniques and DNA testing are also recommended for an optimum diagnosis and to give clinicians substantial evidence for the optimal approach to these kinds of tumors.

ETHICAL CONSIDERATIONS

This paper has been reviewed and granted approval by the Rizal Medical Center Institutional Review Board. Consent forms from patients were not needed for this study because no personal data of the patients were disclosed in any way. The slides for the collected specimens were covered and labelled with assigned numbers to assure anonymity. All physical documents, if any, that may have contained confidential information were disposed using a paper shredder immediately after the study results were obtained.

STATEMENT OF AUTHORSHIP

Both authors certified fulfillment of ICMJE authorship criteria.

AUTHOR DISCLOSURE

Both authors declared no conflict of interest.

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