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ORIGINAL RESEARCH

# PD-L1 Expression and Its Association With p16 in Head and Neck Squamous Cell Carcinoma in Southwestern Uganda

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**Purpose:** Head and Neck squamous cell carcinoma (HNSCC) is the seventh most common cancer in the world. The prognosis of patients with HNSCC remains unsatisfactory, with a 5-years survival rate of only approximately 50%. In western Uganda, the 1-year survival rate is only 1%. Programmed cell death ligand 1 (PD-L1) and p16 have been shown to predict the progression of HNSCC lesions, prognosticate survival, reveal new therapeutic targets, and predict response to therapeutic agents. HNSCC patients with positive PD-L1 expression have been reported to benefit from immunotherapy; however, data on PD-L1 expression in HNSCC in Uganda and Africa are still lacking. This study aimed to determine the expression of PD-L1 in HNSCC and its association with p16 expression in Southwestern Uganda.

**Patients and Methods:** We retrospectively studied 165 formalin-fixed paraffin-embedded specimens from the head and neck region with a previous histological diagnosis of squamous cell carcinoma (SCC). The specimens were retrieved from June 2012 to June 2022. Hematoxylin and eosin (H&E) staining was performed to confirm the diagnosis, followed by immunohistochemical (IHC) staining for PD-L1 and p16 using the laboratory developed technique.

**Results:** Of the 165 specimens included in the study, 80% of source patients were male and the majority (51.5%) were older than 60 years. The oral cavity was the predominant site (51.52%). PD-L1 was expressed in 32.1% (95% C.I: 25.4–39.68) of the specimens, whereas p16 was expressed in 16.36% (95% C.I: 11.4–22.9). There was a statistically significant association between PD-L1 and p16 expression.

**Conclusion:** The prevalence of PD-L1 expression in HNSCC in southwestern Uganda is low, implying that a number of patients with HNSCC can benefit from immunotherapy. PD-L1 expression may predict p16 expression and therefore HPV infection in HNSCC.

**Keywords:** head and neck squamous cell carcinoma, PD-L1, p16, Uganda

## Introduction

Head and Neck squamous cell carcinoma (HNSCC) is the 7th most common cancer in the world.[1] In 2018, there were over 800,000 new HNSCC cases and 450,000 fatalities globally.[2] By 2030, it is predicted that there will be approximately one million new cases every year.[3] According to the Global Cancer Statistics report Globocan 2020, HNSCC accounts for approximately 3.9% of new cases of cancer and 3.7% of all cancer deaths, whereas in Uganda, it accounts for 2.24% of new cases and 2.29% of deaths due to cancer.[4] Over the past 20 years, the incidence of general cancers in Uganda has increased. According to a Kampala survey conducted from 2004 to 2009, 219 patients had HNSCC, of which approximately 56% had lymph node metastases and 6.8% had distant metastases at diagnosis.[5] In western Uganda, patients presented with advanced disease and had a 1-year survival rate of 1%, most of whom would not afford radiotherapy.[6]

Programmed cell death ligand 1 (PD-L1) is a transmembrane protein expressed on tumor infiltrating immune cells or tumor cells. Its interaction with programmed cell death 1 protein causes inactivation of T cell immune response against

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tumor cells leading to immune tolerance and tumor survival.[7] This promotes the escape of these tumor cells from the immune system by protecting themselves from being destroyed by the immune cells and also being eliminated by PD-L1.

Cyclin-dependent kinase inhibitor 2A (p16) is a tumor suppressor gene activator that plays a key role in HNSCC tumorigenesis. At least 30% of HNSCC biopsies carry human papillomavirus (HPV) genomic DNA, mainly type 16 and infrequently type 58.[8] Immunohistochemical expression of p16 is regarded as a marker representative of carcinogenic Human Papilloma Virus infection, as viral oncoproteins E7 and E6 inactivate Retinoblastoma gene and lead to p16 overexpression.[9] With sensitivity and specificity of 94% and 90%, respectively,[10] many significant international guidelines propose that p16 immunohistochemistry (IHC) be used to subgroup cases as HPV positive or HPV negative. HPV-positive and HPV-negative HNSCC differ in histological features, differentiation, risk factors, and prognosis. HPV-positive HNSCC has a better prognosis and survival rate because of its favorable response to radiation.[11]

Identifying molecular biomarkers such as PD-L1 and p16 is now useful for predicting the progress of precursor HNSCC lesions, overall survival, unveiling new treatment modality targets, and response to therapeutic agents. PD-L1 is among the most widely used biomarkers and is linked to a better response and lengthening of the survival period with immune checkpoint inhibitors in cases of recurrent and metastatic advanced tumor to reduce on drug toxicity with chemoradiotherapy.[12] Highly expressed PD-L1 has been revealed to be the most powerful marker of poor prognosis, even when additional prognostic indicators such as tumor extent, lymph node involvement, and metastatic disease are present.[13] In contrast, Karpathiou et al reported that high PD-L1 expression is associated with better prognosis because advanced HNSCC has favorable response to immunotherapy[14] hence, the prognostic role of PD-L1 expression in HNSCC remains controversial.

Studies have already recommended that PD-L1 and p16 be included in the standard diagnostic IHC in the workup of HNSCC,[10,15] but there is a dearth of information about PD-L1 expression and its association with p16 in Ugandan and Africa. Hence, we aimed to determine PD-L1 expression and its association with p16 in HNSCC in Southwestern Uganda.

## Materials and Methods

This retrospective study was conducted at the Mbarara University of Science and Technology (MUST) Pathology Laboratory which is a teaching laboratory and serves Mbarara Regional Referral Hospital. We reviewed the laboratory records for histological diagnosis of squamous cell carcinoma in the head and neck region (oral cavity, oropharynx, hypopharynx, and larynx). Tissue blocks (165) that had been archived from June 2012 to June 2022 were retrieved and repeated Hematoxylin and Eosin (H&E) staining was performed to confirm the diagnosis. Nonprobability convenient sampling was employed. All tissue blocks with corresponding biodata (age and sex) were included, and those lacking biodata were excluded. This study was approved by the MUST Research Ethical Committee and a waiver of consent was obtained from the hospital.

### Laboratory Methods

#### Immunohistochemistry

The manual laboratory developed test was used. Selected tissue blocks were sectioned at a thickness of 3 µm, placed on charged microscope slides, and dried in a tissue-drying oven for 45 min at 60°C. The sections were deparaffinized in 3 changes of xylene for 5 min, rehydrated, and gently rinsed with distilled water for 5 min. Antigen retrieval was performed by steaming slides in 0.01 M sodium citrate buffer at pH 6.1 and temperature for 20 min. The slides were then cooled and rinsed in tris-buffered solution (TBS) for 1 min. Peroxidase blocks were then added to all the slides for 10 min. The slides were then rinsed carefully with tris buffer for 3 min. The primary antibody Anti-PD-L1 rabbit monoclonal antibody EPR19759 (diluted 1:250, Abcam, Berlin, Germany) or p16INK4a (JC2) mouse monoclonal antibody (diluted 1:150, Zeta corporation) was added to the tissue sections, incubated for 1 h at room temperature, and then carefully rinsed in Tris buffer three times as described above. 1–2 drops of horseradish peroxidase anti-mouse and anti-rabbit secondary antibodies were added to tissue sections stained for p16 and PD-L1, respectively, and incubated for 30 min at room temperature. The slides were rinsed again in Tris buffer as described above. The 3,3′-diaminobenzidine tetrahydrochloride chromogen was added to the tissue and incubated for 5–10 min. Counterstaining was performed with hematoxylin, mounted, and interpreted under a light microscope.

### Evaluation of Immunoreactivity

For PD-L1 immunostaining, the Combined Positive Score (CPS) was used to interpret IHC status as either positive or negative. It was calculated as the sum of lymphocytes, tumor cells, macrophages positive for PD-L1 divided by the sum of viable tumor cells x100. A minimum of 100 viable tumor cells in PD-L1 stained slide were manually counted for the specimen to be considered adequate for PD-L1 evaluation. Positivity was confirmed by partial or complete membranous staining(brown) of the tumor cells of any intensity. Membranous and cytoplasmic staining of lymphocytes and macrophages within the tumor nests and surrounding stroma were considered positive. PD-L1 expression was divided into two classes: negative when CPS was less than one and positive when CPS was at least one.[16]

For p16 immunostaining, positivity was reported when at least 70% of the tumor cells showed brown nuclear and cytoplasmic staining.[17] Positive controls were human tonsils for PD-L1 and uterine cervical cancer for p16 expression. For the negative control, the antibody was omitted during staining. Manual counting method of the cells was used.

### Data Analysis

The data collected were double-entered into a Microsoft Excel worksheet and exported to Stata version 17, cleaned, and analyzed. Continuous variables were expressed as means with respective standard deviations, while categorical variables were expressed as frequencies with their respective proportions. Age was grouped into <60 or ≥60 years based on the median age. The prevalence of PD-L1 and p16 expression was expressed as frequencies and proportions. The association between PD-L1 and p16 in HNSCC was estimated using the chi-square test and adjusted using Fisher's exact method in univariate analysis. A $p$-value $\leq 0.05$ was considered statistically significant.

## Results

### Baseline Source Patients' Demographics

Table 1 summarizes the baseline characteristics of the patients. The majority of the patients were male (132 of 165, 80%). The mean ages of the males and females were 58.88(SD ± 15.25) and 58.06 (SD ± 12.76), respectively. The majority of the patients were between 60 and 69 years with 18 years as the youngest patient and 90 years as oldest.

**Table 1** Baseline Characteristics of Sample Source Patients

| Characteristic | Frequency | Proportion (%) |
|---|---|---|
| **Age** | | |
| 10–19 | 1 | 0.61 |
| 20–29 | 2 | 1.21 |
| 30–39 | 13 | 7.88 |
| 40–49 | 28 | 16.97 |
| 50–59 | 36 | 21.82 |
| 60–69 | 40 | 24.24 |
| 70–79 | 31 | 18.79 |
| 80–89 | 13 | 7.88 |
| 90–99 | 1 | 0.61 |
| **Mean** | 58.72 | |
| **Sex** | | |
| Male | 132 | 80 |
| Female | 33 | 20 |

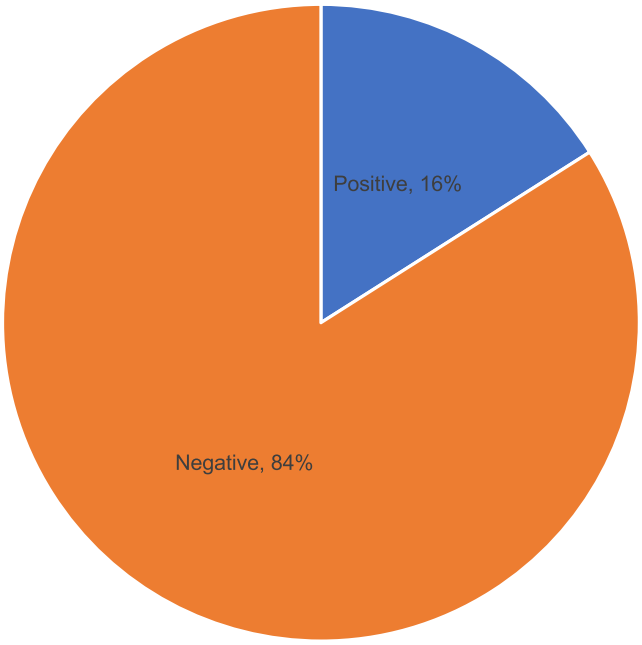


**Figure 1** Prevalence of p16 expression in HNSCC.

## Tumor Characteristics

The most commonly affected site was the oral cavity (51.52%), followed by the larynx (24.24%), oropharynx (22.42%) and hypopharynx (1.82%). The majority (59% (97/165) (95% C.I:51.1–66.1) of HNSCC tumors were well differentiated. Moderately differentiated tumors constituted 32% (53/165) (95% C.I:25.4–39.7) while poorly differentiated tumors accounted for 9% (15/165) (95% C.I:5.5–14.6) of HNSCC cases.

## p16 Expression (HPV) Status in Relation to Source Patients' Demographics and Tumor Characteristics

p16 expression was positive in 27 of 165, (16.36%: 95% C.l: 11.4–22.9). The oral cavity had the highest number of positive p16 cases (21.2%). Females had a higher prevalence of p16 (27.3%) than males (13.6%). Among all age groups, those ≥ aged 60 years had the highest prevalence of p16 positivity (91.76%). The results are summarized in Figure 1 and Table 2.

**Table 2** p16 Expression (Denoting HPV) in Relation to Source Patients' Demographics and Tumor Site

| Variables | Total Number | p16 Positive N (%) | p16 Negative N (%) |
|---|---|---|---|
| **All HNSCC cases** | 165 | 27(16.36) | 138(83.64) |
| **AGE:** <60 | 80 | 20(25) | 60(75) |
| ≥60 | 85 | 7(8.24) | 78(91.76) |
| **SEX:** Male | 132 | 18(13.6) | 114(86.4) |
| Female | 33 | 9(27.3) | 24(72.7) |

(*Continued*)

**Table 2** (Continued).

| Variables | Total Number | p16 Positive N (%) | p16 Negative N (%) |
|---|---|---|---|
| **TUMOR SITE** | | | |
| Oropharynx | 37 | 5(13.5) | 32(86.5) |
| Larynx | 40 | 4(10) | 36(90) |
| Oral cavity | 85 | 18(21.2) | 67(78.8) |
| Hypopharynx | 3 | 0(0) | 3(100) |

## PD-L1 Expression in Relation to Source Patients' Demographics and Tumor Characteristics

PD-L1 expression was positive in 53 of 165 patients (32.12%: 95% C.l: 25.4–39.68). The mean CPS score was 12.06 (SD] = ±24.95). The oral cavity had the highest number of positive PD-L1 cases (38.82%); however, there was no significant difference in PD-L1 expression between the oral cavity and other tumor sites (p = 0.265). PD-L1 positivity was the highest in those aged <60 years, and there was a significant difference between those aged 60 years and above (p = 0.045). The females showed more PD-L1 positivity compared to the males but there was no significant difference in PD-L1 expression between the two sexes (p = 0.094). The results are summarized in Figure 2 and Table 3.

Figure 3 shows pictographs of PD-L1 and p16 immunoreactivity in HNSCC.

# Discussion

PD-L1 expression in tumor cells is associated with the inactivation of macrophages, lymphocytes, and antigen-presenting cells. This promotes the escape of these tumor cells from the immune system by protecting themselves from destruction by immune cells and elimination by PD-L1. When expressed at high levels, this may imply that the body's immunity is inactivated, resulting in unfavorable overall survival compared to those whose tumors are non-expressive.[18] And when there is high expression of PD-L1 in the HNSCC tumor, the efficacy of immune checkpoint inhibitors is also better.[1]

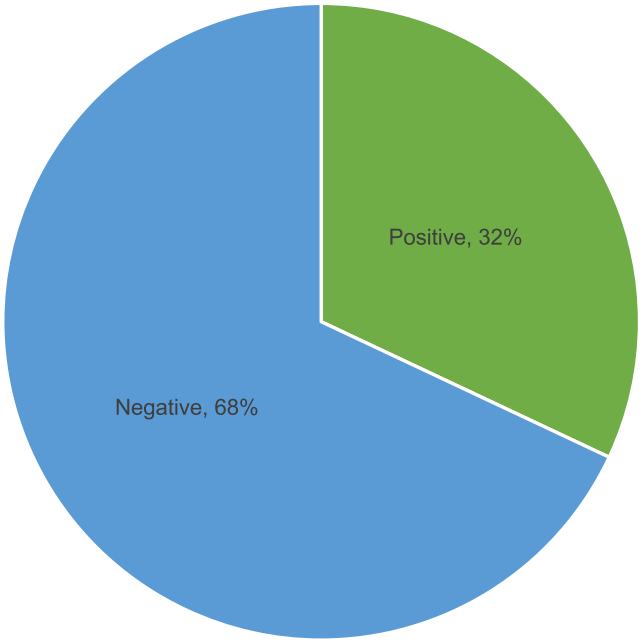


**Figure 2** Prevalence of PD-L1 expression in HNSCC.

**Table 3** Association Between PD-L1 Expression and Clinico-Pathologic Characteristics of HNSCC

| Parameter | Total Cases | PD-L1 Positive (N=53) | PD-L1 Negative (N=112) | Fischer's Exact |
|---|---|---|---|---|
| **All HNSCC cases** | 165 | | | |
| **AGE:** <60 | 80 | 32(40) | 48(60) | 0.045 |
| ≥ 60 | 85 | 21(24.71) | 64(74.3) | |
| **SEX:** Male | 132 | 38(28.79) | 94(71.21) | 0.094 |
| Female | 33 | 15(45.45) | 18(54.55) | |
| **TUMOR SITE** | | | | |
| Oral cavity | 85 | 33(38.82) | 52(61.18) | 0.265 |
| Oropharynx | 37 | 9(24.32) | 28(75.68) | |
| Larynx | 40 | 10(25) | 30(75) | |
| Hypopharynx | 3 | 1(33.33) | 2(66.67) | |
| **Histologic grade** | | | | |
| Well | 97 | 32(32.99) | 65(67.01) | 0.968 |
| Moderate | 53 | 16(30.19) | 37(69.81) | |
| Poor | 15 | 5(33.33) | 10(66.67) | |
| **p16** | | | | |
| Positive | 27 | 19(70.37) | 8(29.63) | <0.001* |
| Negative | 138 | 34(24.64) | 104(75.36) | |

**Note**: *Statistically significant.

In our study, we used CPS ≥ 1 to characterize PD-L1 expression as either positive or negative, as recommended by the Food and Drug Administration after the KEYNOTE 048 study.[2] Positive PD-L1 expression was observed in 32.1% of the patients when a CPS of ≥1 was used which was lower compared to many other studies. We analyzed the present literature and found that PD-L1 expression varied over a wide range; Mishra et al reported a PD-L1 expression positivity of 63.4% at CPS ≥ 1[3] while Downes et al reported 67–78% when CPS ≥ 1 and 19–22% when CPS ≥ 25 was used.[4] Other studies which reported varied PD-L1 expression include:.[5–7] The main setback in quantifying PD-L1 expression is that there is neither a clear optimal cut-off nor a specific scoring system that identifies patients best for immunotherapy.[18] Some studies have used the tumor proportion score (TPS), where PD-L1 expression on tumor cells was assessed, while others used CPS, which evaluates PD-L1 expression on both tumor cells and immune cells. Kulangara et al recommended the use of CPS because PD-L1 expression on immune cells in the tumor microenvironment may predict a patient's response to immunotherapy.[8] The lower PD-L1 expression in the current study compared to other studies may also be explained by differences in the type of specimens. PD-L1 expression is underestimated in tissue biopsies compared to resection specimens and it is high in lymph node metastasis compared to primitive tumor.[9] Majority of our tissue specimens (at least 95%) were biopsies.

Other possible explanations for variations in PD-L1 expression include the use of different clones of antibodies with different binding affinities and different IHC staining protocols. Food and Drug administration recommends the use of clone 22C3 anti-PD-L1 antibody for immunohistochemical assays assessing HNSCC patients for treatment with pembrolizumab. However, this clone requires use of the Dako auto staining platform which is not available in our local setting. In the current study, we used clone EPR19759 anti PD-L1 antibody[10] which is not ready to use, is for research only and a manual laboratory

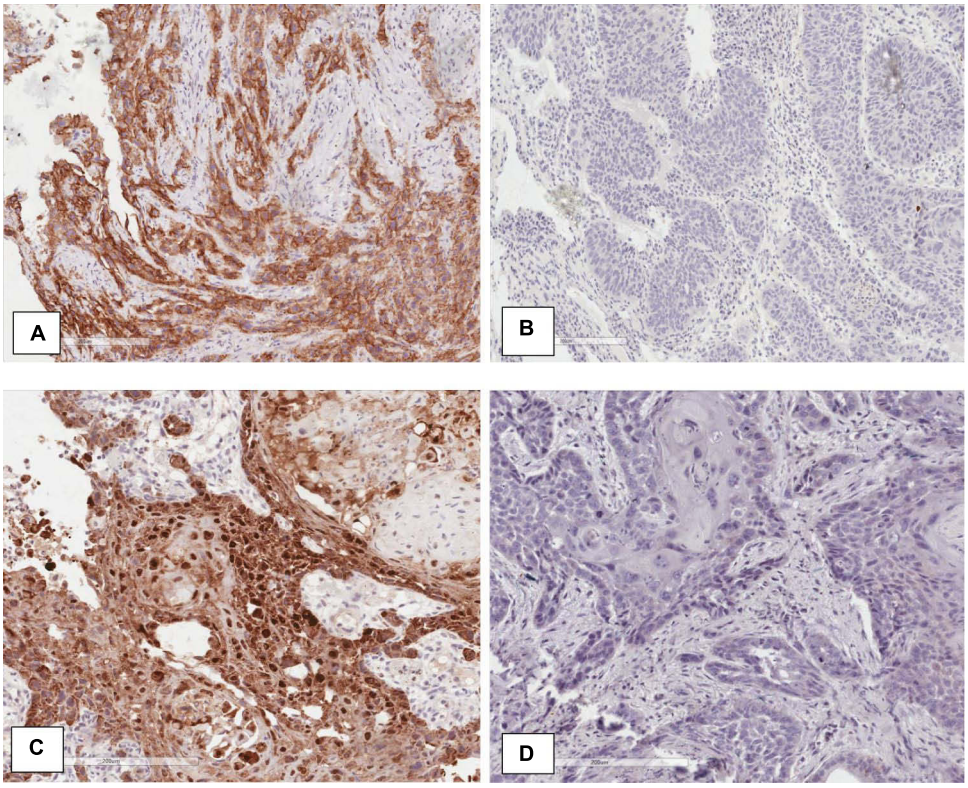


**Figure 3** Pictographs showing PD-L1 and p16 immunoreactivity.
**Notes**: (**A**) PD-L1 positive, (**B**) PD-L1 negative, (**C**) p16 positive, (**D**) p16 negative, x200.

developed technique. Hence, we recommend other diagnostic studies using FDA approved 22C3 anti-PD-L1 antibody and other antibodies in this setting. In addition, the differences in processing tissues, embedding, dilution of reagents, incubation times, temperatures, intra-tumor diversity and observer variations in the interpretation of the results may cause inconsistencies.[18]

Various studies have been conducted to determine the disparity between the immune microenvironment of HPV-positive tumors and that of HPV-negative tumors, because HPV infection is now a known association of a subclass of HNSCC, the occurrence of which is increasing year after year. These studies have reported differences in PD-L1 expression. Although a small number of studies have revealed that PD-L1 expression is not associated with HPV positivity,[11,12] the majority have shown that PD-L1 expression levels are favorably associated with HPV infection.[13,14]

In the present study, the expression of p16 (denoting HPV infection) was observed in 27 of the 165, (16.36%: 95% C.I: 11.4–22.9) patients with HNSCC. Our findings are in agreement with those of previous studies in Uganda by Nabukenya et al, who reported an HPV prevalence of 11.8%[15] while Kabagenyi et al reported a prevalence of 20%.[16] The prevalence of p16 in HNSCC in Uganda is still low because majority of patients are of low socioeconomic status and have no idea about risky sexual behaviors.[15]

The current study showed an association between PD-L1 and p16 expression in archived HNSCC ($p < 0.001$). No association was found between PD-L1 expression and sex, tumor site and histologic grade. No local studies or studies from other African countries have investigated this association before a comparison with studies conducted in a similar geographical setting was not possible. Further studies in this field are required.

Our findings are in accordance with reports from China,[14,17,19] United States,[20] Italy.[21] In the present study, the positivity rate of PD-L1 positive HNSCC (70.37%) was significantly higher than that of their HPV-negative counterparts (24.64%). We postulate that in addition to tumor cells activating immune cells, HPV infection escalates immune system activation, which increases the expression of PD-L1 on cell surfaces. This led to high PD-L1 expression in the HPV-positive group. Co-expression of PD-L1 and p16 may attenuate tumor growth and convert tumor cells into senescent cells, offsetting tumor aggression.[22]

In contrast, Blatt et al reported no association between PD-L1 expression and p16 expression.[23] This discordance may be explained by differences in the methodology; Blatt et al used image analysis software to interpret IHC results. Several recent studies have demonstrated that artificial intelligence and digital pathology provide accurate and consistent results comparable to manual scoring, which is affected by inter- and intra-observer variations.[24] Therefore, pathologists may find that image analysis score is a valuable tool when performing PD-L1 diagnostic testing.

Despite these insightful results, our study has some limitations. As this was a retrospective study, we could not obtain some important variables, such as risk factors such as immune status and history of tobacco use, which are potential confounders. Second, because of the small number of available specimens, probability sampling could not be performed, which might have caused selection bias.

## Conclusion

Positive PD-L1 expression among patients with HNSCC in southwestern Uganda was low, implying that some patients with HNSCC at MRRH can benefit from immunotherapy. PD-L1 expression is associated with p16 expression in HNSCC; however, further studies are required to confirm this association with prospective study design, larger sample size and using better sampling methods including probability sampling. We recommend multicenter based laboratory studies of PD-L1 expression in HNSCC to validate the findings of the current study.

## Ethical Approval

Permission to do the study was approved by the Mbarara University of Science and Technology Research Ethics Committee for Scientific and Ethical Approval under reference number MUST-2022-737. Consent was waived off by the same ethics committee as the study was using tissue blocks.

Site clearance was also obtained from the Hospital Director of Mbarara Regional Referral Hospital. The study was done in compliance with the Declaration of Helsinki and source patient identity remained anonymous throughout the study as a way of maintaining confidentiality.

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## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; agreed on the journal to which the article has been submitted; and agreed to be accountable for all aspects of the work.

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## Disclosure

The authors report no conflicts of interest in this work.

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