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A Descriptive Study of Human Papilloma Virus in Esophageal Squamous Cell Carcinoma in Uganda Assessed by p16 Immunohistochemistry

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Abstract

Background: Esophageal cancer is the eighth most common cancer and the sixth common cause of cancer deaths worldwide. p16 expression is associated with a better prognosis in esophageal squamous cell carcinoma (ESCC) in high risk regions. **Methods**: Formalin fixed paraffin embedded tissue blocks from 110 patients with a diagnosis of ESCC were tested for p16 immunohistochemistry. The analysis of the data was done using Stata version 17.0. The Chi square test and corresponding p-values were used to compare the positivity of p16 with the independent variables of the study. The level of significance was assessed at a 95% confidence interval with a p-value of less than or equal to 0.05. **Results**: 110 cases diagnosed with ESCC were analyzed. The mean age of patients from whom specimens were collected was 59 years (SD ± 11). Most of the samples were from males, 73 (66.4%), while females were 37 (33.6%). The majority of the specimens were of conventional histological type, 108 (98.2%). Moderately differentiated squamous cell carcinoma was the most common grade (48.2%), followed by poorly differentiated squamous cfoma (19.1%). p16 expression was higher in ≥55 year olds compared to 45 - 54 and 35 - 44 age groups (25%, 13%, and 4%) respectively. Moderately differentiated squamous carcinoma constituted the majority of the samples that stained positive for p16 (54.8%), followed by poorly differentiated squamous cell carcinoma (31%), and well differentiated squamous cell carcinoma (14.2%). **Conclusion:** The prevalence of p16 expression in esophageal squamous cell carcinoma in our study was 38.2%. This study may help to bridge the gap in knowledge of p16 expression in ESCC in Uganda.

Subject Areas

Pathology

Keywords

Esophageal Squamous Cell Carcinoma, Human Papilloma Virus, P16

1. Introduction

Esophageal cancer is the eighth most common cancer and the sixth most common cause of cancer deaths worldwide. Once it develops, the disease rapidly metastasizes to lymph nodes and surrounding tissues due to the lack of early symptoms, late presentation, and invasiveness of the cancer [1]. It is estimated that there were 572,000 new cases and 509,000 deaths worldwide in 2018 [2].

In Africa, about 27,900 new cancer cases and 26,600 deaths from esophageal cancer (predominantly squamous cell carcinoma) were estimated to have occurred in 2008 [3]. In Uganda, from 1991 to 2015, the incidence of esophageal cancer has been increasing 2.7% annually [4].

There are significant geographic variations in esophageal squamous cell carcinoma (ESCC) incidence rates, with differences between regions that are more than 10-fold. The highest incidence rates stretch from eastern to central Asia, with another band running along the Indian Ocean coast of Africa along the Great Rift Valley countries [5]. Human Papilloma Virus subtypes 16 and 18 have been linked to the carcinogenesis of ESCC, and it is detected in a varied range of resected samples of esophageal squamous cell carcinoma worldwide [6]. p16 is widely considered a surrogate marker for HPV in squamous cell carcinoma. It is a cost-effective method, easy and has high sensitivity for detecting HPV infection [7]. p16 expression in ESCC was associated with better survival compared to negative p16 ESCC [8]. These findings are consistent with reports from the literature that HPV positive tumors have improved outcomes in head and neck squamous cell carcinoma p16 expression can be used as a predictive marker for response in patients undergoing radiotherapy. Furthermore, most of the responders to neo-adjuvant chemotherapy were p16 positive cases, which indicates that expression of p16 in ESCC is associated with favorable outcomes for patients in terms of reaching a complete pathological response [9]. The aim of this cross sectional study was to describe the expression of p16 in ESCC with associated demographic and pathological features seen in the Department of pathology, Makerere University.

2. Materials and Methods

2.1. Patients and Tissue Samples

A total of 110 archived tissue blocks with a histological diagnosis of ESCC were

analyzed. This was a cross-sectional, laboratory-based study. The study was conducted in the Department of Pathology Laboratory at Makerere University, located in Kampala, Uganda.

The Department of Pathology is hosted within the School of Biomedical Sciences at Makerere University College of Health Sciences. The department receives about 5000 samples across Uganda per year. Tissue blocks from January 1st, 2009 to December 31, 2021, were considered in this study. The convenience sampling method was used until the required sample size was reached. The list of tissue blocks with the diagnosis of ESCC was made using the information from the histology request forms and/or sample reports by the principal investigators with the help of a qualified laboratory technician. The sample size was determined using Kish Leslie—1965 formula.

$$n = \frac{z^2 \times p(1-p)}{e^2}$$

Since the sample size according to the formula was not achievable, finite population correction formula was used. The minimum sample size required for our study were 110 tissue blocks.

2.2. Histology and p16 Immunohistochemistry Procedure

Sections were subjected to H&E staining according to standard operating procedures. Sections were then prepared for immunohistochemistry. Sections were dewaxed in 3 changes of xylene, hydrated in alcohol, placed in the epitope retrieval buffer solution (Leica Novocastra) at pH 6, incubated with peroxidase, washed with Tris buffer saline, incubated with mouse monoclonal primary antibody p16 (JC2-CELL MARQUE) diluted 1:100 for 30 minutes, washed with Tris buffer saline solution, incubated with Novolink post primary antibody, stained with Mayers hematoxylin, cleared in 3 changes of xylene, mounted with DPx and then cover slipped. Cervical squamous cell carcinoma was used as a positive control. An omission of the primary antibody was used as a negative control.

2.3. Immunohistochemistry Scoring Method

Positive staining for p16 was considered when the nucleus and/or cytoplasm stain brown for more than 70% of the tumor cells in moderate and strong intensity. Less than 70% of p16 staining of the cells and weak intensity for p16 were considered negative.

2.4. Statistical Method

Data was collected from the paper-based data extraction forms, which were cross-checked, and then entered into an Excel sheet. Data cleaning was done and then transferred to Stata version 17.0 (Stata corp) for analysis and checked for normality before analysis. The Chi square test and corresponding p-values were used to compare the positivity of p16 with the independent variables of the

study.

Bivariate analysis was done using logistic regression, and this was fitted to get crude odds ratios (OR). Interaction and confounding were assessed before fitting the final model. Confounding was determined if there was a >10% change in the adjusted and crude ORs for each variable. The level of significance was assessed at a 95% confidence interval and p-value of less than or equal to 0.05.

2.5. Ethical Consideration

Approval to conduct this study was sought and obtained (SBS-2022-21) from the School of Biomedical Sciences Research and Ethics Committee (SBS-REC) in the Department of Pathology, Makerere University, College of Health Sciences. A waiver of consent was obtained to use the archived biopsy materials from the Department of Pathology and SBS-REC. Confidentiality was ensured by using unique study numbers to link tissue blocks and the data abstraction forms. The information in the request forms, including names and biopsy numbers, was not included in the data abstraction forms or in the data analysis.

3. Results

3.1. Patient Characteristics

One hundred and ten tissue blocks of esophageal squamous cell carcinoma were analyzed. The mean age of the patients from whom specimens were collected was 59 years (SD ± 11).

The male gender constituted the majority of the samples 73/110 (66.4%) while females constituted 37/110 (33.6%) Almost all specimens were of conventional histological subtype 108/110 (98.2%), basaloid squamous cell carcinoma 1/110 (0.9%) and adenosquamous carcinoma 1/110 (0.9%) had one case each (see Table 1).

Table 1. Characteristics of esophageal squamous cell carcinoma specimens.

Characteristics	Frequency	Proportion (%)
Age		
35 - 44 yrs	9	8.2
45 - 54 yrs	32	29.1
≥55 yrs	69	62.7
Sex		
Male	73	66.4
Female	37	33.6
Histological type		
Conventional	108	98.2

Continued

Basaloid	1	0.9
Adenosquamous carcinoma	1	0.9
Histological grade		
Well differentiated	21	19.1
Moderately differentiated	53	48.2
Poorly differentiated	36	32.7
Intensity staining		
Strong	19	17.3
Moderate	39	35.5
Weak	25	22.7
No staining	27	24.6
P16 expression status		
Positive	42	38.2
Negative	68	61.8

The majority of the specimens were of moderately differentiated histological grade 53/110 (48.2%) followed by poorly differentiated squamous cell carcinoma 36/110 (32.7%) and well differentiated squamous carcinoma 21/110 (19.1%) respectively (Figure 1). Moderate intensity of p16 immunohistochemistry was the most frequent finding at 39/110 (35.2%), while strong intensity had the least frequent finding at 19/110 (17.3%) (Figure 2).

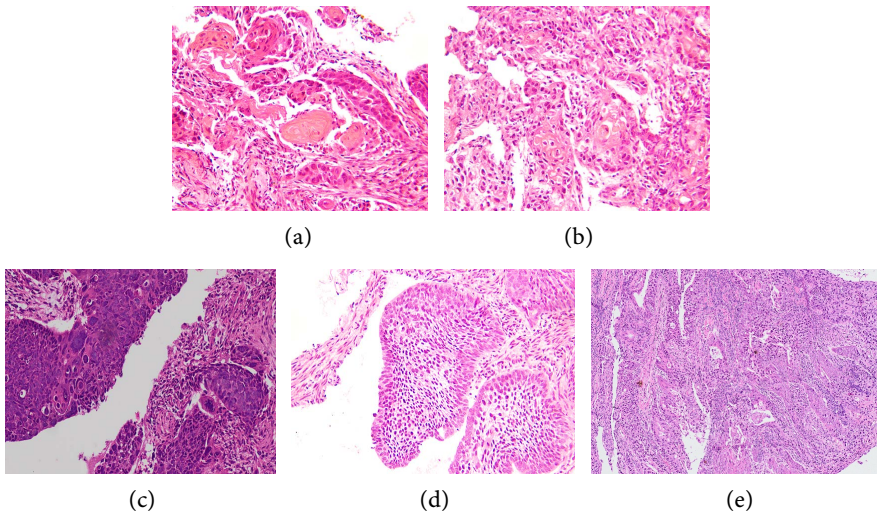
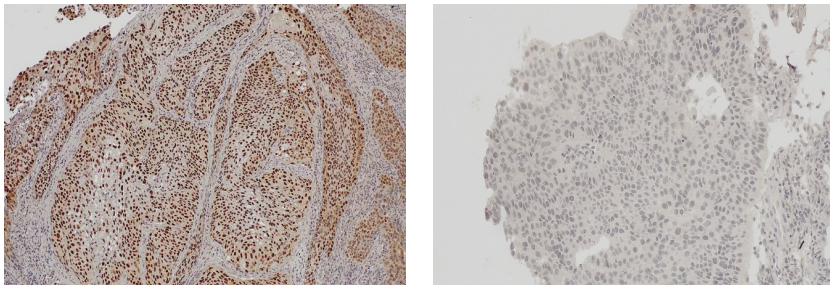


Figure 1. Photomicrograph showing: (a). Well differentiated squamous cell carcinoma. (b). Moderately differentiated squamous cell carcinoma. (c). Poorly differentiated squamous cell carcinoma. (d). Basaloid squamous cell carcinoma, E. adenosquamous carcinoma, E (H & E 100×)



(a) (b)

Figure 2. Photomicrograph showing positive p16 expression in ESCC: (a): numerous tumor cells are showing brown p16 IHC staining (100×). (b): photomicrograph showing negative expression of p16 in ESCC. The tumor cells are negative for p16 immunohistochemistry (100×).

Out of 108 cases of conventional squamous cell carcinoma, 41 cases were positive for p16 (38.0%). The case of basaloid squamous cell carcinoma was positive for p16, while the adenosquamous carcinoma case was negative for p16, as shown in Table 2.

Table 2. Association between p16 expression and study variables.

Characteristics	p16 reactivity		p-value
	Positive	Negative	
Age			
35 - 44 yrs	4 (44.4)	4 (55.6)	
45 - 54 yrs	13 (40.6)	19 (59.4)	
≥55 yrs	25 (36.2)	44 (63.8)	0.843
Sex			
Male	25 (34.3)	48 (65.8)	
Female	17 (46.0)	20 (54.0)	0.722
Histological type			
Conventional	41 (38.0)	67 (62.0)	
Basaloid	1 (100)	0 (0)	
Adenosquamous carcinoma	0 (0)	1 (0)	0.326
Histological grade			
Well differentiated	6 (28.6)	15 (71.4)	
Moderately differentiated	23 (43.4)	30 (56.6)	
Poorly differentiated	13 (36.1)	23 (63.9)	0.473

3.2. Association between p16 Expression and with Patient's Age and Sex

The results of our study showed no statistically significant association with the

expression of p16, age, or sex.

Although patients who were older than 55 years had higher p16 expression compared to those who were younger than 55 years, this was not statistically significant (p = 0.843). Although, the males in our study were more than females (73 males and 37 females), females had a higher expression of p16 (46%) compared to the males (34%), however that, was not statistically significant (p = 0.722).

3.3. Association between p16 Expression with Histological Subtypes and Grade of ESC

In our study, no statistically significant association was found between p16 expression, histologic subtypes and grades of ESCC. Out of 108 cases of conventional squamous cell carcinoma, 41 cases were positive for p16 (38.0%). The case of basaloid squamous cell carcinoma was positive for p16 while the adenosquamous carcinoma case was negative for p16. Moderately differentiated squamous cell carcinoma stained p16 mostly 23/53 (43.4%) followed by poorly differentiated 13/36 (36.1%) and well differentiated 6/28 (28.6%) respectively, but this was not statistically significant (p = 0.473) as shown in **Table 2**.

4. Discussion

In our study, the expression of p16 in ESCC was 38.2%. These results are comparable to the findings of studies done in China [8] and Iran [10] which reported prevalence of 37.1% and 42.9% of p16 expression in ESCC respectively. In contrast, a case-control study done in Mexico reported that p16 was positive in 11.6% of the ESCC samples analyzed [11]. On the other hand, another study done in Mexico for p16 expression in ESCC reported that, out of 121 samples analyzed, 13 cases (10.7%) of ESCC samples were positive for p16 [12]. This variation in the prevalence of p16 in ESCC could be due to the different methodologies used. It has been observed that the prevalence of p16 in ESCC is widely variable in the literature [13]. The difference in prevalence can be attributed to several factors. First and foremost, HPV-high-risk geographic regions, or so called high risk regions, have a higher expression of p16 in ESCC compared to low risk geographic areas. Geographic differences among the populations studied were the most important determinant factor for the assessment of HPV infection rates and p16 expression in ESCC [8] [14] [15]. Secondly, the methodologies for p16 expression in the different studies, including sample size, scoring criteria, and clones used in the different studies, vary significantly.

The cutoff of p16 expression varies in different studies, while some studies used a cutoff of ≥1% of the tumor cells by analyzing them through semi-automated image analysis software [16], others considered ≥50% of the tumor cells [9] and finally, there were some studies that considered ≥70 positive tumor cells [11]. The variation in the methodologies used resulted in differences in the results in the literature. In the present study, p16INK4A (JC2) clone was used to analyze the p16 expression, same clone was used in a study done in China, where 177

ESCC samples were analyzed. The results were grouped according to the p16 expression into negative, focal positive, and diffuse positive. Only diffusely positive tumor cells were considered positive, and the prevalence of p16 in ESCC was 2.82% [17]. Different scoring methods used in our study and the study in China could have contributed to this difference.

In our study, a total of 110 samples were analyzed, and the mean age was 59 years. These findings are comparable to those reported from Mexico, where the median age was 60 years [8]. The male-to-female ratio in our study was 2.08, and these results are comparable to the findings in a study done in India [9]. Although the male gender was predominant in our study, the expression of the p16 protein was more common in females.

Similar findings were reported in a study done for the determination of p16 expression of ESCC in patients who underwent neo-adjuvant chemotherapy [9].

The findings of our study showed no association of p16 expression with age or sex. Similar findings were reported by other studies [1] [10], where gender and age were not statistically significant. In contrast to the findings of our study, a study done to determine p16 expression in malignant ESCC histologic types, reported an association between p16 expression and gender ($p = 0.047$), but reported no association with age [16]. The disagreement in these findings may be attributed to the method used in that study, in which 82 samples of highly malignant esophageal squamous cell carcinoma were analyzed and considered ≥1% for the cutoff of p16 positivity. In our study, p16 expression was not associated with the histologic subtypes of ESCC. There is scanty literature on p16 expression in different histologic subtypes of ESCC. The most commonly reported histologic subtype is conventional squamous cell carcinoma, since it is the most common subtype.

The conventional squamous cell carcinoma constituted 98.2% of the samples analyzed. This is in agreement with the literature, where the conventional subtype is the most common subtype of ESCC [18].

In our study, there was one case of basaloid squamous cell carcinoma, which was positive for p16. In disagreement with the findings of our study, a study done to determine p16 expression in basaloid squamous cell carcinoma reported that none of the samples were positive for p16 [19]. This supports the other findings in literature which have established that basaloid squamous cell carcinoma of the esophagus has a distinct molecular pathway and that the expression of p16 is due to dysregulation of the retinoblastoma 1 signaling pathway but not due to HPV [16]. The other histological subtype was adenosquamous carcinoma, which was negative for p16 protein expression. The negative expression can be explained by the findings in the literature, which showed that loss of p16 expression was more common in poorly differentiated squamous cell carcinoma than in well- and moderately differentiated squamous cell carcinoma due to promoter methylation [20]. The most common grade that stained positive for p16 in our study was moderately differentiated ESCC, followed by poorly differentiated and well differentiated ESCC by 48.2%, 32.7%, and 19.1%, respectively. In disagree-

ment with our findings, a study reported that p16 stained mostly with moderately differentiated ESCC, followed by well differentiated and poorly differentiated ESCC, respectively [9]. The difference may be attributed to the differences in the methodologies used in that study and our study. Furthermore, a study done in China reported that well differentiated squamous cell carcinoma was the most common histologic grade, stained for p16, followed by moderately differentiated squamous cell carcinoma and poorly differentiated squamous cell carcinoma, respectively [1]. This variation in the expression of p16 in different grades of ESCC could be due to the different sample sizes of the studies and the different methodologies used. Regarding the association of p16 in ESCC grades, our study found no association between histological grade and p16 IHC staining. In agreement with our study, other studies reported no association between histological grades of ESCC and p16 immunohistochemistry [10] [12]. In disagreement with our study, another study reported that histological grade was associated with p16 expression in ESCC [8]. This difference may be due to different methodologies in the studies.

5. Conclusion

The prevalence of p16 expression in esophageal squamous cell carcinoma in our study was 38.2%. P16 expression was not associated with age, sex, histological subtypes, or the histological grade of ESCC in the samples analyzed in the Department of Pathology at Makerere University. These findings can be used as baseline data on the p16 expression of esophageal squamous cell carcinoma in Uganda. Studies with a larger sample size, patient survival data, and a history of contributing risk factors for ESCC development, such as smoking and alcohol consumption, may be needed, as studies from the literature have reported that alcohol, smoking, and HPV have a synergistic effect on the development of ESCC.

6. Limitation

A difference in the fixation of tissues could have reduced the immunoreactivity of p16 antibody.

As p16 is a direct method for the detection of HPV, combined tests along with p16 IHC, such as PCR and in-situ hybridization techniques, are preferred to confirm the presence of HPV DNA and HPV subtypes in ESCC patients in Uganda.

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Data Availability

The data of this research article is available on request from the corresponding author.

Conflicts of Interest

The authors declare no conflicts of interest.

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