Original Article

Immunohistochemical Study of the Role of Human Papillomavirus, Epstein–Barr Virus, and P16^{INK4a} Expression in Head-and-Neck Squamous Cell Carcinomas

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Abstract

Background: Studies over the years have established that human papillomavirus (HPV) and Epstein–Barr virus (EBV) are major etiological agents in subsets of head-and-neck squamous cell carcinomas (HNSCCs). This study further explores the concurrence of HPV and EBV together with P16^{INK4a} expression in HNSCCs, providing additional insights into their unique role in establishing a virus-induced carcinogenesis. **Materials and Methods:** A retrospective cross-sectional study utilizing immunohistochemistry was employed to establish the presence of HPV, EBV, and P16^{INK4a} expression in HNSCC archived tissue samples. **Results:** A total of 121 selected HNSCC cases were included in the study, with male preponderance (n = 86) and majority of the cases occurring in patients ≤54 (n = 62). The most common site of occurrence was the oral cavity (n = 29), followed by larynx (n = 27) and nasal cavity and paranasal sinuses (n = 24), respectively. The study recorded 18 (14.9%) HPV-positive tumors, 7 (5.8%) EBV-positive tumors, and 2 (1.7%) tumors coinfected with HPV and EBV. P16^{INK4a} expression was recorded in 42.1% (n = 51) of the tumors. Although P16^{INK4a} expression correlated weakly with both HPV (r = 0.116) and EBV (r = 0.205) positivity, it showed a statistically significant expression with EBV positivity (P = 0.024). **Conclusion:** The observed pattern of HPV association with P16^{INK4a} overexpression was consistent with earlier reported studies, and as such, the study reinforces the assertion that P16^{INK4a} can be used as a surrogate marker for HPV-positive tumors. However, additional studies are required to validate its suitability in tumor sites other than oropharyngeal squamous cell carcinoma.

Keywords: Epstein–Barr virus, head-and-neck squamous cell carcinoma, human papillomavirus, p16

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Introduction

Head-and-neck squamous cell carcinoma (HNSCC) encompasses malignancies arising from the upper aerodigestive tract (oral cavity, pharynx, larynx, nasal cavity, and paranasal sinuses) and it constitutes about 95% of head-and-neck cancers.[1-3] Globally, HNSCC is the sixth most common tumor with the main risk factors being alcohol and tobacco abuse.[2] However, recent studies have reported a surge in virus-induced HNSCC, implicating human papillomavirus (HPV) and Epstein–Barr virus (EBV) in unique subsets of the carcinoma.[4-7]

Studies have shown that HPV is particularly associated with oropharyngeal squamous cell carcinoma (OPSCC) recording a decline or stability in tobacco-and alcohol-induced HNSCC but a rise in HPV-induced HNSCC.[8-10] Smith *et al*.[11] report that 20%–30% of subjects with OPSCC do not have the traditional risk factors of tobacco and alcohol use and HPV appears to be the major driver of this new trend. This observed trend has been related to increase in oral sexual behaviors exacerbated by earlier age of sexual debut, multiple sexual partners, and genital warts history.[12-15] The implication of HPV in HNSCC has thus led to the creation of a new patient profile in the

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clinic, as observed by Goon *et al*.[16] that patients now present at a much younger age with weak or no history of alcohol or tobacco. High oncogenic HPV types in HNSCC particularly HPV-16 have been known to mediate their carcinogenic effect through the viral oncoproteins E6 and E7 which inactivate tumor suppressor proteins P53 and PRb, respectively. HPV positivity in HNSCC patients has, however, been linked to favorable prognostic outcomes, with reports indicating that HPV-positive tumors respond better to chemotherapy and radiation than HPV-negative tumors.[17-19]

EBV as a human herpesvirus has been identified to persist for life in a quiescent state in resting memory B lymphocytes in almost all humans.[20] However, there is occasional reactivation of EBV to infect new B cells and has been associated with nasopharyngeal carcinoma, with a recent meta-analysis also suggesting a significant association with oral squamous cell carcinoma (OSCC).[7,21] Persistent infection with EBV has also been deemed as a precursor for the development of other malignancies including HPV-positive OSCC, but the role it plays is poorly understood.[7] $P16^{INK4a}$ on the other hand is a tumor suppressor protein that is downregulated in many cancers but overexpressed in HPV-related tumors due to inactivation PRb.[22,23] Overexpression of $P16^{INK4a}$ has thus been harnessed as a surrogate marker of HPV infection in OPSCCs while its use in cancers of the other parts of the upper aerodigestive tract is not well established.[24] This study, therefore, sets out to further investigate the co-carcinogenic role of HPV and EBV in HNSCC while examining the suitability of $P16^{INK4a}$ as a surrogate marker for HPV-positive tumors.

Materials and Methods

Design

A retrospective cross-sectional study to investigate the presence of HPV and EBV and the expression of $P16^{INK4a}$ in archived tissue samples of HNSCC retrieved from the Pathology department. Approval for the study was obtained from the Committee on Human Research, Publications, and Ethics of our study site (CHRPE/AP/438/17).

Tissue samples

In consecutive order (from 2007 to 2016), archived, formalin-fixed paraffin-embedded HNSCC tissue block and slides were retrieved. HNSCC cases in which the archived tissue blocks could not be retrieved or have inadequate information were excluded from the study. The hematoxylin and eosin-stained slides of the selected cases were then reviewed independently by two pathologists to confirm the diagnosis. A total of 121 HNSCC cases were considered suitable for further analysis using immunohistochemistry. From these suitable tissue blocks, tissue microarray (TMA) was prepared using Micarray by Micatu Inc., USA.

Immunohistochemistry

Immunohistochemical staining was performed according to standard procedures. Antibodies for HPV, EBV, and $P16^{INK4a}$ were used. 3 μm-thick sections were made from each TMA block onto SuperFrosted Plus slides and were deparaffinized using xylene. The sections were then rehydrated using a series of ethanol solutions of decreasing grades (absolute – 95%–70%), diluted with tris-buffered saline, and washed with distilled water. Antigen retrieval was performed by incubating the TMA sections in citrate buffer (pH 6) in a pressure cooker (10 min). Background staining and nonspecific antibody binding were prevented using hydrogen peroxide (3%) in methanol for 10 min and casein solutions. The sections were incubated with the primary antibodies according to the manufacturer specifications. Secondary antibody conjugated with peroxidase and antiperoxidase (DAKO) was added. Sections were later developed with diaminobenzidine tetrahydrochloride (DAB). The sections were counterstained in hematoxylin, dehydrated in increasing grades of ethanol (70%–95% – absolute), and mounted using DPX mountant.

Statistical analysis

Statistical analysis was performed for clinical and demographic data, virus positivity, and $P16^{INK4a}$ positivity using IBM SPSS statistical software (version 23) (Armonk, NY, USA). Statistical significance was defined as $P < 0.05$.

Results

A total of 121 HNSCC cases were included in the study. Mean age was 52.22 years (standard deviation, ± 18.7 years; median, 54 years). Majority of the patients were ≤54 years (51.2%) with male preponderance (71.1%). The most common site of infection was the oral cavity (24%), followed by the larynx (22.3%) and nasal cavity (19.8%), respectively. Majority of the cases were high-grade tumors (76%). The tumor sites were classified according to guidelines of the American Academy of Otolaryngology–Head and Neck Surgery Foundation.[25] The distribution is shown in Table 1.

Out of the 121 cases analyzed, the study recorded 18 (14.9%) HPV-positive tumors, 7 (5.8%) EBV-positive tumors, and 2 (1.7%) tumors coinfected with HPV and EBV. In both HPV and EBV-positive cases, males were more likely to be infected compared to females. When patients were stratified into young (≤54 years) and old (>54 years) based upon the median of the study population, older patients in the HPV-positive group had a higher susceptibility, while younger patients in the EBV-positive group were more susceptible. However, this observation was not statistically significant. The distribution of the HPV-positive cases according to tumor site showed that HPV was prevalent in 35.7% of oropharyngeal cancers, 33.3% of salivary gland cancers, 20.8% of nasal cavity cancers, and 17.2% of oral cavity cancers. Similarly, EBV was prevalent in 33.3% of salivary gland cancers, 28.6% of nasopharyngeal cancers, 8.3% of nasal cavity cancers, and 6.9% of oral cavity cancers. The two co-infected cases were recorded in oropharyngeal and nasal cavity cancers. Majority of both HPV and EBV-positive cases occurred in high-grade tumors. The distribution is shown in Table 1.

P16^{INK4a} expression was recorded in 42.1% of the tumors. The most common site of P16^{INK4a} expression the nasal cavity (17/24;(70.8%), followed by oropharynx (7/14;50%) and oral cavity (13/29;44.8%), respectively [Table 1]. All the HPV-positive and EBV-positive cases were found to be positive for P16^{INK4a}. Although P16^{INK4a} expression correlated weakly with both HPV (r = 0.116) and EBV (r = 0.205) positivity, it showed a statistically significant expression with EBV positivity (P = 0.024) [Table 2]. The immunohistochemical staining pattern is shown in Figure 1.

Discussion

The study employed immunohistochemistry in detecting the presence of HPV, EBV, and P16^{INK4a} expression in archived tissue samples of HNSCC. Immunohistochemistry represents a valuable screening tool with comparable sensitivity for the detection of high-grade tumors.[26] This study to the best of our knowledge is one of the first few in Africa to explore P16^{INK4a} expression in addition to the detection of both HPV and EBV in HNSCC. The results showed a similar clinical profile to earlier reported studies in HNSCC, recording a higher association with males and majority of the tumors occurring in oral cavity followed by larynx.[2,4,27]

In the 121 HNSCC cases analyzed, the study recorded 14.9% HPV-positive tumors with a higher percentage in OPSCC. This was in line with several studies that have reported a rise in HPV-induced HNSCC and a higher association with OPSCC.[8-11] However, the percentage of HPV-positive tumors recorded was lower than that reported by Aboagye *et al*.[4] (18%), Asante *et al*.[28] (19.4%), and Kaba *et al*.[29] (19.2%) within the same geographical region. This may probably be due to the difference in detection methods, as PCR utilized in the earlier works has been reported to have better sensitivity and successful detection of HPV.[30] The detection of HPV in HNSCCs is, however not enough proof of viral causation, as it might just reflect a transient infection unrelated to the carcinogenic process.[23] Thus, it is necessary to explore other biomarkers such as P16^{INK4a} which have been

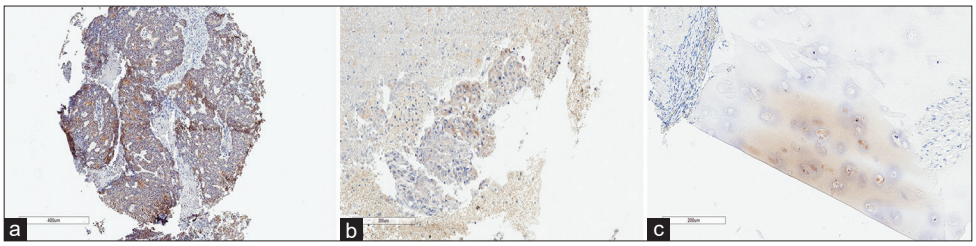

Figure 1: Immunohistochemical staining pattern of p16 (a); human papillomavirus (b); and Epstein–Barr virus (c). The golden brown appearance shows positive expression

Table 1: Characteristics of the study population, human papillomavirus, and Epstein-Barr virus status and the expression of P16^{INK4a}

Parameters	Study population (n=121)	HPV$^+$ (n=18)	EBV$^+$ (n=7)	Co-infection HBV-EBV (n=2)	P16^{INK4a+} (n=51)
Gender					
Male	86	15	5	1	37
Female	35	3	2	1	14
Age (years)					
≤54	62	8	5	1	27
>54	59	10	2	1	24
Tumor site					
Oral cavity	29	5	2	0	13
Oropharynx	14	5	0	1	7
Hypopharynx	1	0	0	0	0
Larynx	27	2	0	0	6
Nasopharynx	7	0	2	0	3
Nasal cavity and paranasal sinuses	24	5	2	1	17
Salivary gland	3	1	1	0	2
Undetermined	16	0	0	0	3
Tumor grade					
Well differentiated	29	4	2	0	15
Moderately differentiated	52	7	3	1	22
Poorly differentiated	40	7	2	1	14

Undetermined*- written as head and neck without specification. HPV: Human papillomavirus, EBV: Epstein-Barr virus

Table 2: Correlation of $P16^{INK4a}$ with human papillomavirus and Epstein-Barr virus

	HPV status	$P16^{INK4a}$ expression	EBV status
HPV status			
Pearson correlation	1	0.116	0.043
Significance (two-tailed)		0.206	0.636
n	121	121	121
p16 expression			
Pearson correlation	0.116	1	0.205*
Significance (two-tailed)	0.206		0.024
n	121	121	121
EBV status			
Pearson correlation	0.043	0.205*	1
Significance (two-tailed)	0.636	0.024	
n	121	121	121

*Correlation is significant at the 0.05 level (two-tailed). HPV: Human papillomavirus, EBV: Epstein-Barr virus

clinically validated in addition to HPV detection to detect oncogenically active HPV infection, especially in OPSCCs.[31] In the present study, all (100%) the HPV-positive tumors showed positive for $P16^{INK4a}$. This percentage was higher than that reported by Ndiaye *et al*.[23] (45%) in their systematic review and meta-analysis of 5 studies (all from Europe) that reported on HPV-positive/$P16^{INK4a}$ positive cases. Furthermore, the observed percentage by cancer site in the HPV-positive tumors differs with that reported in literature, which records a higher percentage of $P16^{INK4a}$ positive cases in OPSCC relative to other sites of HNSCC.[31-33] However, the correlation between $P16^{INK4a}$ expression with HPV positivity was weak and nonsignificant. This was expected considering the higher percentage (42.1%) of $P16^{INK4a}$ positive tumors relative to the lower percentage of HPV-positive tumors (14.9%), as opposed to the expected commensurate increase in HPV-positive samples as reported in other studies.[31-33] This observation could be attributed to factors including sensitivity of the test, the stage of the tumors, and the tissue preservation method used.[23] Notwithstanding, this study reinforces the assertion that $P16^{INK4a}$ expression in addition to HPV detection could be used to characterize oncogenically active HPV infection in HNSCC and that $P16^{INK4a}$ can serve as a suitable biomarker for HPV-positive tumors. However, additional studies are required to validate its suitability in tumor sites other than OPSCC as observed in the present study.

The study also recorded 5.8% EBV-positive tumors. This was not surprising considering the number of nasopharyngeal carcinomas ($n = 7$) involved in the study. The association of EBV with nasopharyngeal carcinomas is well established, but scanty information exists on the association with other sites of HNSCC.[7,28] The present study recorded EBV association with other sites including salivary gland, nasal cavity, and oral cavity. A recent meta-analysis has recorded a significant association of EBV with oral cavity cancers, but additional studies with large sample sizes are required to confirm the relationship of EBV with other nonnasopharyngeal sites like those found in this study.[21] Furthermore, the study recorded 2 tumors that were coinfected with HPV and EBV. This may point to the role of EBV as a precursor for the development of other malignancies, however, additional studies are needed to elucidate the relative roles played by both HPV and EBV in coinfected tumors.[7] Furthermore, all the EBV-positive tumors were positive for $P16^{INK4a}$. This was illustrated by a weak but significant correlation of $P16^{INK4a}$ with EBV. The observation was in line with several studies that have reported $P16^{INK4a}$ expression in EBV-positive tumors.[34,35]

Both HPV and EBV association with HNSCC have been identified with better prognosis in patients.[32,34] This observation is mostly linked to $P16^{INK4a}$ overexpression resulting from the direct or indirect inactivation of retinoblastoma protein (Rb) which releases $P16^{INK4a}$ from its negative feedback control, thereby causing paradoxical increase in levels of the protein.[36] $P16^{INK4a}$ thus acts to inhibit uncontrolled cellular proliferation and its overexpression in virus-induced cancers points to the unsuccessful attempt.[37] Studies have thus shown that virus-related cancers presenting $P16^{INK4a}$ overexpression are very sensitive to treatment and have better prognosis than those unrelated to virus.[32,34,38,39] Therefore, there is the possibility of reducing the amount of therapy required by both HPV-and EBV-positive HNSCC patients in the near future and thereby reducing long-term side effects.

Conclusions

The study revealed a similar clinical profile to earlier reported studies in HNSCC. $P16^{INK4a}$ overexpression was recorded in 42.1% of the study population, out of which 14.9% were HPV positive, 5.8% EBV positive, and 1.7% coinfected with HPV and EBV. The observed pattern of HPV association with $P16^{INK4a}$ overexpression was consistent with earlier reported studies, and as such, the study reinforces the assertion that $P16^{INK4a}$ can be used as a surrogate marker for HPV-positive tumors. However, additional studies are required to validate its suitability in tumor sites other than OPSCC.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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