HEAD AND NECK CANCER

original reports abstract

Epstein-Barr Virus Latent Membrane Protein-1 Expression in Nasopharyngeal Carcinoma

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PURPOSE Nasopharyngeal carcinoma (NPC), a malignant neoplasm of the epithelium covering the nasopharynx, is a rare disease in most parts of the world. Epstein-Barr virus (EBV), the most potent oncogenic virus, coupled with environmental and genetic factors has been identified to play a role in the development of NPC. An array of methods for detecting the virus do exist, from serologic detection of antibodies to DNA amplification. There is paucity of local data on the status of EBV infection in relation to NPC within the region, and this study attempts to shed more light on the subject.

METHODS This was a retrospective cross-sectional laboratory-based study on histologically confirmed, archived tissues from July 2015 to June 2019. Immunohistochemistry expression of latent membrane protein-1 (LMP-1) was used to detect EBV infection in the tissues.

RESULTS A total of 71 cases were enrolled in this study. The mean age was 47.87 years ± 16.84 years with a male-to-female ratio of 1.5:1. There was a unimodal distribution of EBV detection, with the peak (26.8%) at 36-45 years. About 45.1% of the 71 samples tested positive for LMP-1, all of which were nonkeratinizing carcinoma. Nonkeratinizing carcinoma was the most common histopathologic subtype (n = 67; 94.4%), with the majority (38 of 67; 56.7%) being undifferentiated and 29 of 67 (43.3%) differentiated. Keratinizing and basaloid subtypes had two cases each, representing 2.8%.

CONCLUSION A significant proportion of NPC, particularly nonkeratinizing histologic subtype, seems to show LMP-1 positivity by immunohistochemistry, which may be adopted in resource-constrained settings to detect EBV infection in these tissue biopsies.

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INTRODUCTION

Nasopharyngeal carcinoma (NPC) is a malignant neoplasm of the epithelium covering the nasopharynx and is the most common tumor affecting this anatomic area. In early stages, it has a tendency to localize to the lateral wall of the nasopharynx, particularly the fossa of Rosenmuller.[1-3]

A rare disease in most parts of the world, it has a high incidence in regions such as Southeast Asia (Thailand and Philippines) and North Africa (Algeria and Morocco) with the highest incidence in Hong Kong.[1,4] In Tanzania, it accounts for 14.2% of head and neck cancers at Muhimbili National Hospital.[5]

WHO has classified NPC into three distinct entities on the basis of the histologic findings. They are nonkeratinizing carcinoma, keratinizing squamous cell carcinoma, and basaloid squamous cell carcinoma. This is a modification from the traditional numerical classification of WHO I, II, and III, which has been replaced.[1,2,6] Of the three variants, keratinizing carcinoma is of a greater proportion in the low-incidence areas in comparison with the higher-incidence areas.[1,7,8]

Genetic and environmental factors together with Epstein-Barr virus (EBV) infection play the greatest role in the development of NPC.[9,10] EBV is detected in 100% of nonkeratinizing NPC[1] regardless of geographical region, with low levels detected in keratinizing and basaloid in high endemic areas in comparison with higher levels in low-incidence areas for NPC. The association of EBV with NPC was postulated because of the presence of elevated IgG and IgA antibody titers to the viral capsid antigen, which correlated with the tumor burden, remission, and recurrence.[11]

EBV is a DNA virus of the *Herpesviridae* family and *Gammaherpesviridae* subfamily. It exhibits tropism for epithelial and lymphoid tissues because of the cellular

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CONTEXT

Key Objective

To determine a difference in expression of Epstein-Barr virus (EBV) infection among the different histologic types of nasopharyngeal carcinoma (NPC).

Knowledge Generated

There is a unimodal peak in age distribution among patients with NPC and EBV, latent membrane protein-1 detection, and only nondifferentiated keratinizing carcinoma, the most prevalent histologic type, tested positive for EBV.

Relevance

With the prospects of immunotherapy for NPC, adoption of routine EBV testing, even with immunohistochemistry in resource-constrained settings, would be an added advantage in identifying those who would benefit from this modality of treatment.

expression of its receptor, which is also C3d receptor for the complement system. EBV has been linked to conditions such as infectious mononucleosis, oral hairy leucoplakia, and malignancies such as Burkitt's lymphoma, B-cell lymphomas, and gastric carcinomas.[12] Virtually all human beings become infected with EBV at some point in their lifetime. For those in developing countries and lower socioeconomic status, infection is acquired in early childhood and remains subclinical but for those in the higher socioeconomic status, it is acquired in adolescence, mostly presenting with infectious mononucleosis.[11] Transmission is primarily through saliva.[11,13]

Three types of latency infections have been identified with EBV depending on the viral products expressed. For NPC, it is type 2 latency infection with the expression of EBV nuclear antigen-1, latent membrane protein-1 (LMP-1), latent membrane protein-2, and EBV early RNA.[14] These products are what are assayed to detect presence or absence of EBV infection. Detection methods include in situ hybridization (ISH), polymerase chain reaction, and immunohistochemistry (IHC). Serologic assays are becoming a commonplace in testing for EBV but they have their drawbacks such as a wide variability and lack of specificity with false positives seen in autoimmune disorders unrelated to EBV.[15]

LMP-1 regulates a number of signaling pathways in the pathogenesis of NPC, mainly through the three functional domains of its C terminal activating regions 1, 2, and 3.[16,17] Through the NF-κB signaling pathway, LMP-1 regulates cell proliferation, apoptosis, transformation, metastasis, and invasion ultimately causing immortalization through the p65 subunit. LMP-1 plays a crucial role in the chemokine ligand 5–mediated cancer angiogenesis by activating the phosphoinositide 3-kinase protein kinase B (PI3K AKT) and hypoxia-inducible factor 1α signaling pathways. The Janus tyrosine kinase and signal transducer and transcription activator pathway activated by LMP-1 mediates expression of programmed cell death protein ligand 1 aiding the cancer cells escape the body's immune surveillance.[17] LMP-1 makes more cells susceptible to the virus by secretion of matrix metalloproteases, which facilitate the degradation of the extracellular matrix. The stability of p53, a tumor suppressor gene, is interfered with by LMP-1, such that there is a lack of induced cell apoptosis in the cell cycle. LMP-1 also mimics the CD40 causing an overexpression of cancer stem cell markers leading to high metastatic features in NPC.[18]

METHODS

This was a retrospective, cross-sectional, laboratory-based study conducted at Muhimbili National Hospital, Tanzania. The study population was nasopharyngeal biopsies submitted to the histopathology unit to confirm diagnosis of NPC.

Inclusion Criteria

All histologically confirmed NPC biopsies submitted to the histopathology department from July 2015 to June 2019 were included.

Exclusion Criteria

Crushed tissues, unavailable tissue blocks, and cases lacking patient information were excluded.

Histopathologic Evaluation and Microscopy

This was done as previously described.[19] Briefly, review of the diagnoses was done by the first author (V.E.S.) and a qualified, senior anatomical pathologist (A.R.M.) on hematoxylin and eosin sections and classification using the WHO system. EBV status was determined using immunohistochemical expression of LMP-1. Photomicrography was performed by the first author (V.E.S.) and the senior histopathologist (A.R.M.) using an Olympus (CX31RBSF Model) light microscope equipped with a digital camera (Olympus Corporation, Tokyo, Japan) as previously described.[19,20]

IHC Staining

This was done according to methods previously described.[20] The paraffin-embedded tissue blocks were cut into 3 μm–thick sequential sections; the slides were dried, deparaffinized in xylene, and rehydrated through graded

alcohol; tissue sections were circled with a hydrophobic pen (Dako pen); and endogenous peroxide activity was blocked for 15 minutes using peroxidase blocking solution (Dako ready-to-use reagent) and antigen retrieved by pressure cooking for 10 minutes in citrate buffer (pH = 6). Slides were allowed to cool using tap water for another 10 minutes and then rinsed with wash buffer (phosphate-buffered saline [PBS]) for 5 minutes. Sections were incubated with mouse antibody anti-EBV-LMP (CS1-4) prediluted by Medaysis for 30 minutes, washed with wash buffer for 5 minutes and thereafter incubated with a universal Horseradish peroxidase for 30 minutes and washed with PBS twice each for 3 minutes. Sections were then incubated with 3,3′diaminobenzidine (Dako DAB) for 10 minutes followed by rinsing in water for 2 minutes then counterstaining with hematoxylin for 17 dips and bluing for 5 minutes. Sections were dehydrated in the ascending grades of alcohol (ethanol 70%, ethanol 80%, ethanol 95%, and ethanol 100%) and then cleared in two changes of xylene for 5 minutes in each and covered using mounting medium by using Sakura Tissue Tek coverslipper. All these procedures were performed in the humidity chamber to make sure slides are not drying in between the steps. Both negative and positive controls were run alongside the tests.

IHC Evaluation

The slides were then reviewed under a light microscope. Brown granular cytoplasmic and membrane staining was interpreted as positive for EBV LMP-1, whereas bluish staining of the cytoplasm and membrane was interpreted as negative for EBV LMP-1. A positive control included a tissue known to have EBV infection, whereas for negative controls the test antibody was omitted and replaced by PBS.[19]

Furthermore, an internal negative control included parts of the section (stroma) that did not stain to the antibody.

Data Analysis

Data analysis was performed using Statistical Package for Social Sciences SPSS version 26. Results were presented in cross tabulations and figures. Association of EBV with age and sex as well as histologic classification of NPC was analyzed using Fisher's exact *t* test and a *P* value of $< .05$ was considered significant.

Ethical Approval

Ethical clearance was sought from the Research and Publication Committee of the School of Medicine and from the Senate Research and Publications Committee of the Muhimbili University of Health and Allied Sciences. Administrative permission to conduct the study and waiver of consent, to be allowed access to the tissue bank, were granted from Muhimbili National Hospital as this was a retrospective study.

RESULTS

General Findings

A total of 71 cases were enrolled to this study. The patients' age ranged from 16 to 82 years with a mean age of 47.87 years ± 16.84 years. The most frequent (26.8%) age group was 36-45 years, reflecting a unimodal peak. There were 43 males enrolled, with a male-to-female ratio of 1.5:1. Thirty-two (45.1%) of the 71 samples tested positive for LMP-1. There is a unimodal peak in EBV tissue immunoexpression across the age groups, with the highest positivity rate seen among the 36-45 years group (26.8%) and the lowest being among the 26- to 35-year-olds (8.5%) although this was not statistically significant (*P* value of .111; Table 1).

Histopathologic and Immunohistologic Results

Nonkeratinizing carcinoma (Fig 1) was the most common (67 of 71; 94.4%) histopathologic subtype, majority (38 of 67; 56.7%) of which were undifferentiated and 29 of 67 (43.3%) differentiated, although this difference was not statistically significant (*P* value = .277). Furthermore, keratinizing squamous cell carcinoma and basaloid type each had two cases, representing 2.8%, respectively.

A significant number (32 of 71 [45.1%]) of the tissues tested positive for LMP-1 antibody by IHC and all these were nonkeratinizing carcinoma (Figs 2 and 3). On the contrary, keratinizing and basaloid carcinomas all tested negative for LMP-1, although this difference in EBV immunoreactivity across the three histologic types of NPC was not statistically significant, with a *P* value of .248 (Table 2). Moreover, majority (41 of 71 [58.1%]) of the males tested negative for LMP-1, whereas the females showed equal distribution of the same, although this difference was also not statistically significant, with a *P* value of .333 (Table 1).

TABLE 1. LMP-1 Immunoexpression by Age and Sex

	LMP-1 Immunoexpression, No. (%)			
Characteristic	**Positive**	**Negative**	**Total**	***P***
Age, years				.111
16-25	7 (77.8)	2 (22.2)	9 (12.7)	
26-35	1 (16.7)	5 (83.3)	6 (8.5)	
36-45	11 (57.9)	8 (42.1)	19 (26.8)	
46-55	4 (30.8)	9 (69.2)	13 (18.3)	
56-65	4 (30.8)	9 (69.2)	13 (18.3)	
> 65	5 (45.5)	6 (54.5)	11 (15.5)	
Total	32 (45.1)	39 (54.9)	71 (100)	
Sex				.333
Male	18 (41.9)	25 (58.1)	43 (60.6)	
Female	14 (50)	14 (50)	28 (39.4)	
Total	32 (45.1)	39 (54.9)	71 (100)	

Abbreviation: LMP-1, latent membrane protein-1.

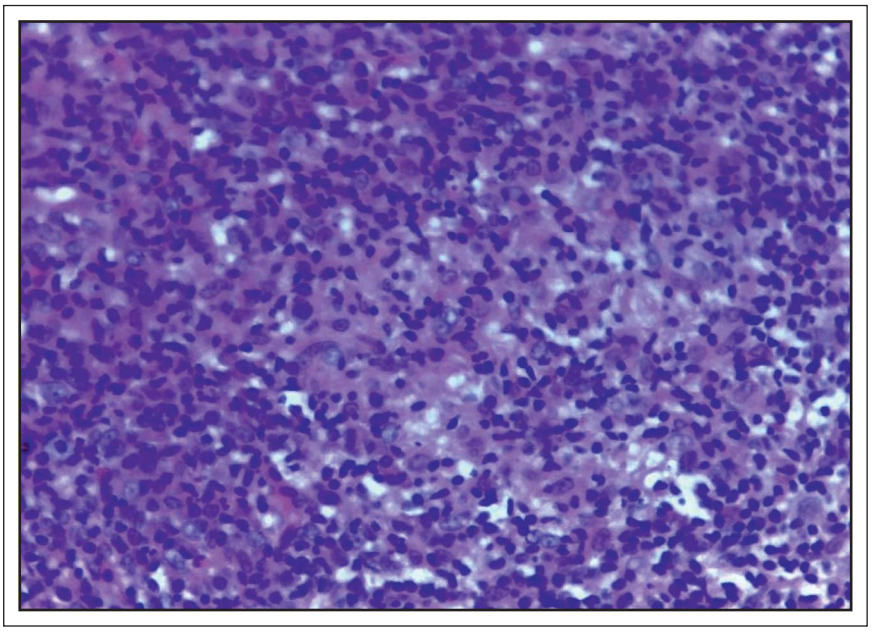

FIG 1. Hematoxylin and eosin–stained photomicrograph showing nonkeratinizing undifferentiated carcinoma, ×40 magnification.

DISCUSSION

Sub-Saharan Africa is considered to be a low endemic group for this particular malignancy with expected bimodal peak in age incidence and a male predominance.[1,2,7,21] In this study, there was a unimodal peak at the 36-45 years age group similar to other studies within the continent. Edris et al[22] in Sudan found the same unimodal peak at 41-60 years, so did other studies in Nigeria and Kenya with a peak at 40-49 and 31-40 years, respectively.[8,23] Mremi et al[24] in Tanzania found a peak at the 31-50 years age group. This similarity might be suggestive of comparable pathogenetic mechanisms and risk factors. However, a more extensive study on NPC in the region should be carried out to establish whether there is a change in age distribution pattern. In this study, there were more males who tested positive for EBV, a finding that is replicated in a study from Finland with 74% positive cases being male. Edris et al in Sudan found 60% of men tested positive for EBV, whereas Abdalazez et al found an equal number of positive and negative cases among men. EBV detection rates were high amongst females with NPC, with positivity rates of 82.7%, 66.6%, 77% in Finland and the last two in Sudan respectively.[22,25,26]

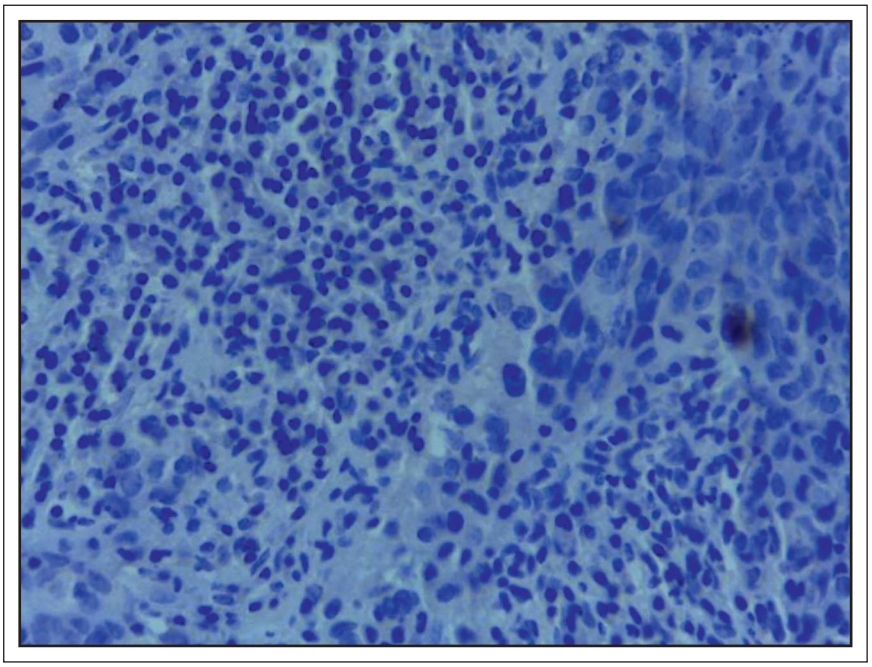

FIG 2. LMP-1 immunohistochemistry photomicrograph showing absence of staining of the nuclei and cytoplasm, which is defined as negative for LMP-1 immunoexpression, ×40 magnification. LMP-1, latent membrane protein-1.

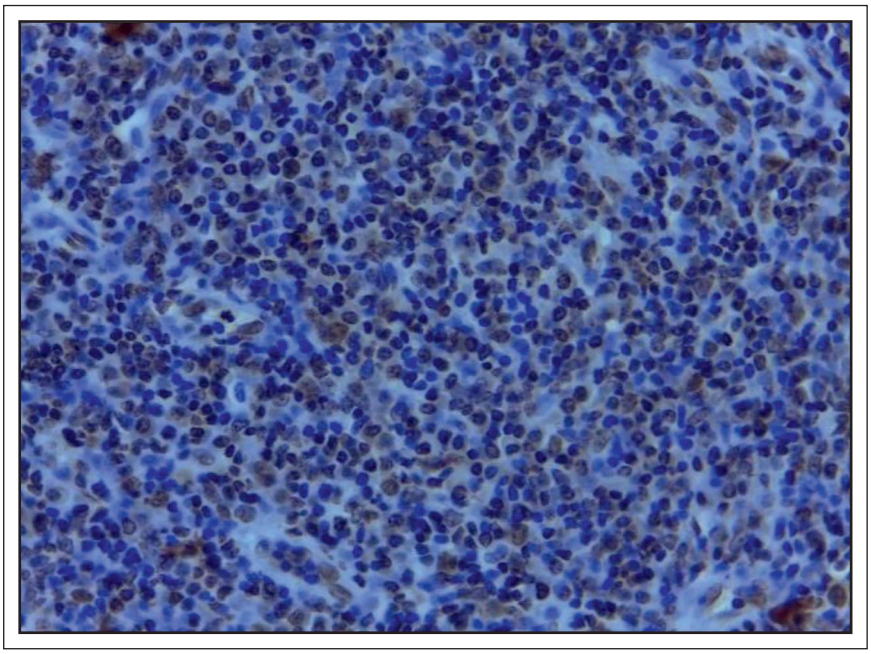

FIG 3. LMP-1 immunohistochemistry photomicrograph showing diffuse staining of the nuclei and cytoplasm, which is defined as positive for LMP-1 immunoexpression, ×40 magnification. LMP-1, latent membrane protein-1.

In our current study, the prevalence of EBV infection in NPC tissue sections was found to be 45% using LMP-1 IHC, although this was not statistically significant, which could partly be because of the small sample size. This compares well with a previous study from Sudan that showed almost similar findings at 41%.[25] However, among studies that used a similar EBV detection method, a previous report from Finland showed a prevalence of 62%,[26] whereas two in Nigeria showed 77.3% and 86%,[27,28] respectively. These studies were in low endemic regions for NPC but the type of antibody used and the detection system applied might partly account for the differences seen in the rates. On the contrary, a previous report from Ghana showed a low 25% detection rate despite using EBV DNA polymerase chain reaction, a method considered to be superior to IHC.[12] LMP-1 is expressed in all NPCs but the cells express variable levels of the protein within the tumor foci and this might explain the low detection rate of EBV observed in this study.[29] Despite EBV early RNA ISH being considered the gold standard[1] for EBV detection, other methods such as IHC are now being adapted in low-resource settings.[25,27,28] This is especially true considering that a number of studies have noted that there is no significant difference among the various tests in detection rates.[4,30-32] However, Fanaian et al[33] compared the ISH and IHC methods of EBV detection and found that automated ISH had a sensitivity and specificity of 94% and 69%, respectively, whereas IHC had a sensitivity of 44% and specificity of 93%.

TABLE 2. Histologic Type of Nasopharyngeal Carcinoma by EBV Status

Histology	EBV Status, No. (%)		Total, No. (%)	*P*
	Positive	Negative		.248
Keratinizing	0 (0)	2 (100)	2 (2.8)	
Nonkeratinizing	32 (47.8)	35 (52.2)	67 (94.4)	
Basaloid	0 (0)	2 (100)	2 (2.8)	
Total	32 (45.1)	39 (54.9)	71 (100)	

With advancements in genomic research, there are biomarkers such as p53R2, fibronectin, Mac-2 binding protein, plasminogen activator inhibitor-1, ceruloplasmin, and serum amyloid A that are being identified to be key in early diagnosis, response to treatment, and prognosis of NPC.[34,35] It has been shown that expression of LMP-1 and Cripto-1, a member of the epidermal growth factor and a modulator in embryogenesis and oncogenesis, is positively related. LMP-1 can therefore be used as biomarker in tumor progression and metastasis.[36] There is also promising use of immunotherapy in NPC with LMP-1–specific autologous cytotoxic lymphocytes–targeted therapy for recurrent disease. A vaccine based on LMP-1 has also shown tumor growth and metastasis suppression in mouse models; however, human trials are yet to be done.[37]

The histopathologic classification of NPC has undergone several changes, with the current classification by WHO identifying three distinct types: nonkeratinizing, keratinizing, and basaloid carcinoma.[1,2] Nonkeratinizing carcinoma was the most prevalent type of NPC in this study, at 94.4%, which seems to be similar to the global picture across all risk strata.[1,2,6,8,23,24,38] This histologic type is further classified into undifferentiated and differentiated with the former accounting for majority of the cases at 36%-95%, but this classification has no clinical significance and there might be cases wherein the two coexist in one tumor.[1,2,6,23,26] In our current study of the various histologic types, EBV was apparently only detected in nonkeratinizing

carcinoma tissues, a finding that seems comparable with reports from elsewhere.

In conclusion, this current study determined the association of EBV infection in NPCs at Muhimbili National Hospital, Tanzania, using the LMP-1 IHC and found that apparently about half (45%) of the cases tested positive, reflecting almost one in every two patients, although this appears to be lower than in majority of previous reports from elsewhere.

The age and sex distribution of NPC appeared similar to other studies from Africa showing a unimodal peak, but this is contrary to previous studies that indicate a bimodal peak with a majority of those tested positive for EBV being males and younger than 45 years. The most common histologic type of NPC appeared to be nonkeratinizing carcinoma, and only this type seemed to be associated with LMP-1 tissue positivity.

With the prospects of immunotherapy for NPC, adoption of routine EBV testing would be an added advantage in identifying those who would benefit from this modality of treatment.

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Final approval of manuscript: All authors
Accountable for all aspects of the work: All authors

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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