Original Article

Immunohistochemical Expression of Epstein–Barr virus Latent Membrane Protein-1 in Nasopharyngeal Carcinoma

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Abstract

Background: Epstein–Barr virus (EBV) has a consistent global association with nasopharyngeal carcinoma (NPC). The detection of EBV in NPC has been shown to have diagnostic and prognostic importance. Latent membrane protein-1 (LMP-1) immunochemical staining is a well-recognized, rapid, and effective method of EBV detection. This study aims to determine the EBV status of NPC seen over 22 years, from 1992 to 2013 in Ahmadu Bello University Teaching Hospital (ABUTH). **Materials and Methods:** It was a retrospective study of all NPCs diagnosed at the Department of Pathology, ABUTH from January 1, 1992 to December 31, 2013. The paraffin-embedded tissue blocks of all histologically diagnosed NPCs within the study period were retrieved and examined. Cases in which the tissue blocks were missing, badly damaged, or had insufficient tissue were excluded from the study. Histopathological diagnosis was in accordance to the 2005 World Health Organization classification. EBV detection was done using immunohistochemistry (IHC) for EBV LMP-1. Data were analyzed using SPSS 24. Fisher's exact test statistic value is 0.545, which is not significant at $P < 0.05$. **Results:** A total of 112 NPC cases were histologically diagnosed, 66 (58.9%) of these were available for EBV LMP-1 IHC and 46 (41.1%) were excluded. EBV LMP-I IHC was positive in 51 samples (77.3%), while 15 (22.7%) were negative. There were 63 (95.5%) cases of nonkeratinizing carcinoma (NKC) seen, of which 49 (77.8%) were EBV positive and 14 (22.2%) were negative. Keratinizing squamous cell carcinoma (KSCC) constituted 3 (4.5%) cases, 2 of which were EBV positive (66.7%) and 1 was negative (33.3%). No basaloid squamous cell carcinoma case was available for the study. **Conclusions:** There is a high prevalence of EBV in NPC. However, there is no statistical difference in the prevalence of EBV in NKC and KSCC. NPC showed an association with EBV irrespective of histological type. LMP-1 IHC has proved useful in detecting EBV in NPC in this study.

Keywords: Epstein-Barr virus, immunohistochemistry, latent membrane protein-1, nasopharyngeal carcinoma

Introduction

Epstein–Barr virus (EBV) (human herpesvirus 4) is a ubiquitous virus that latently infects over 90% of the world population and was first identified by Epstein *et al.* in Burkitt Lymphoma, making it the first isolated human viral carcinogen.[1,2] The relationship between EBV and nasopharyngeal carcinoma (NPC) was first suggested in 1966 by Old *et al*.[3] based on the presence of high titers of EBV antibodies in diseased patients, but the initial *in situ* demonstration of EBV DNA in biopsies of NPC was by Zur Hausen *et al*. in 1970.[4] Since then, EBV has been consistently detected in NPC, particularly nonkeratinizing carcinoma (NKC), and the virus is detected in virtually every primary and metastatic tumor, irrespective of the degree of tumor differentiation or the geographic location.[5-7] EBV has been shown to have a stronger association with NKC than with keratinizing squamous cell carcinoma (KSCC); consequently, it is almost always positive in high-risk populations where NKC predominates and positive in only a proportion of cases in low incidence areas where KSCC predominates.

EBV DNA within the tumor cells are monoclonal, suggesting that NPC occurs from the clonal proliferation of a single EBV-infected cell, and the consistent expression of the same EBV latency program in precursor lesions further support this.[8] The EBV infection in NPC exhibits Type II latency pattern with the expression of EBV nuclear antigen-1 (EBNA-1), latent

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membrane proteins (LMPs-1, 2A and 2B), EBV-encoded early RNAs (EBERs), and micro-RNAs.[1] All three of the LMPs are detected in NPC and are highly multifunctional.

LMP-1 exerts an antiapoptotic function by upregulating Bcl-2 and altering the ratio of initiator caspase-8 and its inhibitors.[1] It also constitutively activates signaling systems (nuclear factor- kappa B, JNK-kinase, and Janus kinase/signal transducers and activators of transcription-pathways), upregulates intercellular cell adhesion molecule-1, lymphocyte function antigen, and MHC Class I and II molecules, induces expression of epidermal growth factor receptor, and induces telomerase activity.[1,5] By and large, the LMP-1 has a significant effect on cellular gene expression and cellular growth which is consequential in the highly invasive malignant nature of NPCs.[8] A study by Hu *et al*. supports this and shows that LMP-1-positive NPC grows more rapidly and extensively than LMP-1-negative tumors.[9] However, only about two-thirds of NPCs express LMP-1.[1]

EBV can be demonstrated in NPC by the detection of its viral products. The detection of EBER transcripts by *in situ* hybridization (ISH) is the popular and most reliable method for the molecular diagnosis of EBV in NPC and considered the gold standard. LMP-1 immunochemistry is not as effective as EBER-ISH in detecting EBV in NPC as LMP-1 is often not detectable even in obviously EBER-positive cells.[10,11] Yet, LMP-1 immunohistochemical staining is a rapid, cheaper, and effective method of EBV detection, thus, retains a role in the clinical evaluation, especially in resource-poor locations.

Other useful methods of detecting EBV include viral nucleic acid amplification, that is, polymerase chain reaction and nucleic acid sequence-based amplification, serology (ELISA and immunofluorescent assays), Southern blot analysis of EBV DNA, gene expression profiling, culture of EBV or EBV-infected lymphocytes, and electron microscopy to examine the detailed morphologic changes associated with EBV infection.[11]

Significantly elevated IgG and IgA antibody titers against multiple viral antigens are consistently observed in NPC; these include antibodies against EBV viral capsid antigen, EBNA-1, EBNA-2, lytic antigens, and neutralizing antibodies against EBV-specific DNAse. These antibody titers are observed to precede tumor development by several years and are elevated in advanced disease correlating significantly with tumor burden, remission, recurrence, and survival.[12] Hence, EBV-specific serology is now employed in screening for the early detection of NPC as well as for differential diagnosis in fine-needle aspiration biopsies of metastatic cervical nodes and for monitoring of response to therapy.[13,14] However, false-positive results of 9%–30% may pose a limitation for its use.[15]

This study was conducted to determine the EBV LMP-1 expression of NPC seen over 22 years, from 1992 to 2013 in Ahmadu Bello University Teaching Hospital (ABUTH).

Materials and Methods

This was a 22-year retrospective study of all NPCs diagnosed at the Department of Pathology, ABUTH from January 1, 1992 to December 31, 2013. The paraffin-embedded tissue blocks (PETB) of all histologically diagnosed NPCs within the study period were retrieved and examined. Cases in which the tissue blocks were missing, badly damaged, or had insufficient tissue were excluded from the study. The histopathological patterns were analyzed using the hematoxylin and eosin-stained slides, and the histopathological diagnosis was in accordance to the 2005 World Health Organization classification.[5] Indirect IHC for EBV LMP-1 was done on each sample to determine the expression of EBV in the tumor cells.

Sections were made at 2–3 μ from the PETB and placed on negatively-charged slides. The sections were then deparaffinized in xylene, rehydrated in decreasing concentrations of alcohol, and then sequentially incubated with hydrogen peroxide block, protein block, anti-EBV LMP-1 antibody, complement, horseradish peroxidase conjugate, and diaminobenzidine chromogen/substrate mixture for about 10 min each and rinsed each time in a buffer. The slides were counterstained with hematoxylin, then dehydrated and coverslips applied. All steps were performed at room temperature. Both negative and positive controls were run alongside the tests.

The slides were viewed under the light microscope and brown granular cytoplasmic and membrane staining were interpreted as positive for EBV LMP-1, while bluish staining of the cytoplasm and membrane was interpreted as negative for EBV LMP-1. Furthermore, nuclear and paranuclear brown dot staining were interpreted as false positive. The data obtained were statistically analyzed using SPSS version 24 (IBM SPSS statistics for Windows, version 24).

Results

A total of 112 NPC cases were histologically diagnosed, 66 (58.9%) met the inclusion criteria of this study and 46 (41.1%) were excluded. There were 46 male (69.7%) and 20 female (30.3%) with a male-to-female ratio of 2.3:1. The ages ranged from 6 to 91 years with an overall mean age of 42.8 years and modal age of 40 years. The age group with the highest incidence is 40–49 years age group with 18 cases (27.27%) [Table 1].

Overall, 51 (77.3%) samples were positive for EBV LMP-I IHC, while 15 (22.7%) were negative [Figure 1]. A total of 63 (95.5%) of the tested samples were NKC, of which 49 (77.8%) were EBV positive and 14 (22.2%) were negative. KSCC constituted 3 (4.5%) cases, 2 of which were EBV positive (66.7%) and 1 was negative (33.3%) [Figures 2 and 3]. Fisher's exact test statistic value is 0.545, which is not significant at $P < 0.05$. No basaloid squamous cell carcinoma case was available for the study.

The nasopharynx was the most common site of surgical biopsy with 43 cases (65.2%), whereas laryngeal masses were the

least common with a single specimen (1.5%). Specimens from the nasopharynx and cervical lymph node show 81.4% and 83.3% EBV positivity, respectively, while those from the nasal cavity and oropharynx show 66.7% positivity each. The single laryngeal biopsy was EBV positive [Figure 4].

Figures 5-7 show representative photomicrographs of the EBV LMP-I IHC.

Table 1: The association of Epstein-Barr virus with age and sex

Age group (years)	Male		Female	
	EBV positive	EBV negative	EBV positive	EBV negative
0-9	0	1	0	0
10-19	4	0	0	1
20-29	3	2	1	1
30-39	7	1	2	2
40-49	10	2	5	1
50-59	7	2	1	1
60-69	4	1	4	0
Above 70	2	0	1	0
Total	37	9	14	6

EBV: Epstein-Barr virus

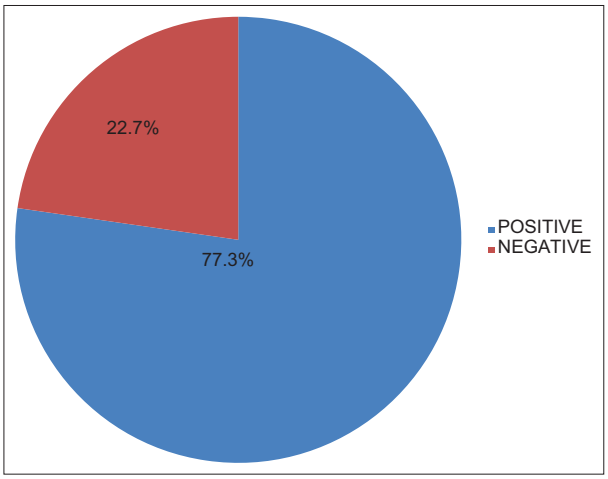

Figure 1: Pie chart showing the Epstein–Barr virus latent membrane protein-1 status of nasopharyngeal carcinoma

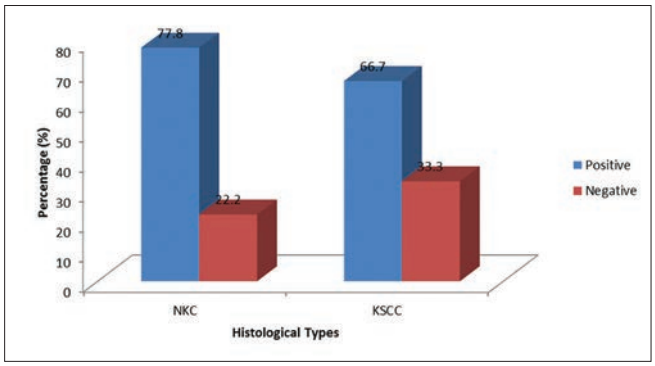

Figure 3: Bar chart showing the Epstein-Barr virus status of different histological types of nasopharyngeal carcinoma

Discussion

Immunohistochemical study of all available specimens showed 77.3% EBV LMP-1 positivity which attests to the strong association of EBV with NPC already documented in the literature.[5,8,12,16] This observation is similar to that made by Vera-Sempere *et al*. in Spain where 78.4% EBV LMP-1 positivity was observed.[17] On the contrary, a much lower incidence has been reported in a study in Israel with 13.3% positivity,[18] while a study in Morocco failed to detect EBV LMP-1.[19] The reason for this disparity is uncertain although the lack of constant expression of LMP-1, differences in the sensitivity of assays used and geographical variations may all be contributory to varying degrees.[1,18-22]

NKC showed 77.8% EBV LMP-1 positivity which is similar to observations by Pathmanathan *et al*. who detected 73% positivity.[23] Although several studies have shown that NKC is invariably EBV-positive, irrespective of geographical origin,[5-8,12,24] these studies were done using various methods of EBV detection and may therefore not be the best comparison. KSCC showed 66.7% EBV LMP-1 positivity. Existing literature have presented conflicting data concerning the association of EBV with KSCC: some studies have been unable to demonstrate the presence of EBV in KSCC [8,24,25] while others have.[22,23,26] This variation may be attributed to technical factors

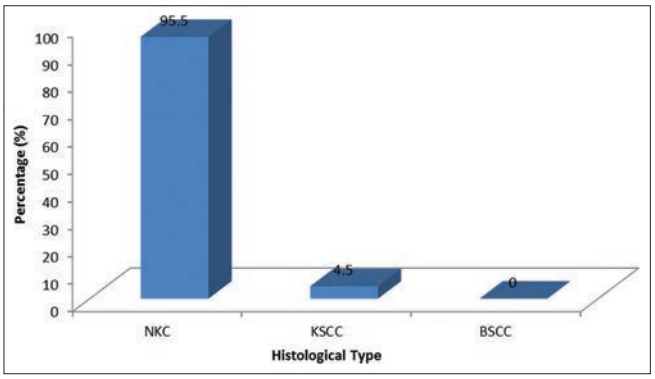

Figure 2: Bar chart showing the histopathological pattern distribution of the study samples

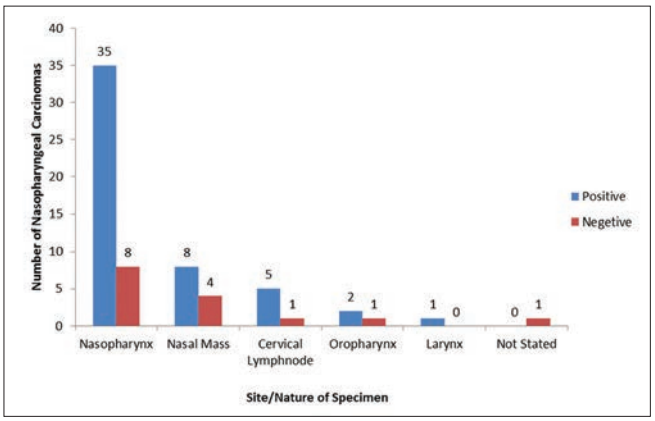

Figure 4: Bar chart showing the Epstein–Barr virus status and the site/nature of specimens received

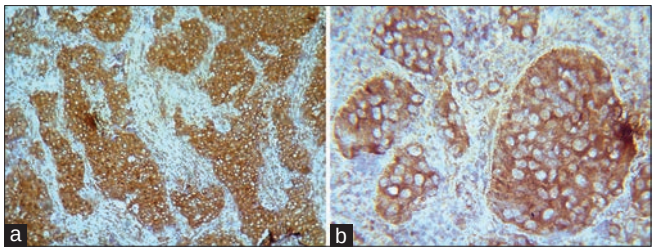

Figure 5: (a) Photomicrograph showing Epstein–Barr virus latent membrane protein-1 positive IHC staining in nonkeratinizing carcinoma (A. at ×100 and B. at ×400) (b) Photomicrograph showing Epstein–Barr virus latent membrane protein-1 positive IHC staining in nonkeratinizing carcinoma (A. at ×100 and B. at ×400)

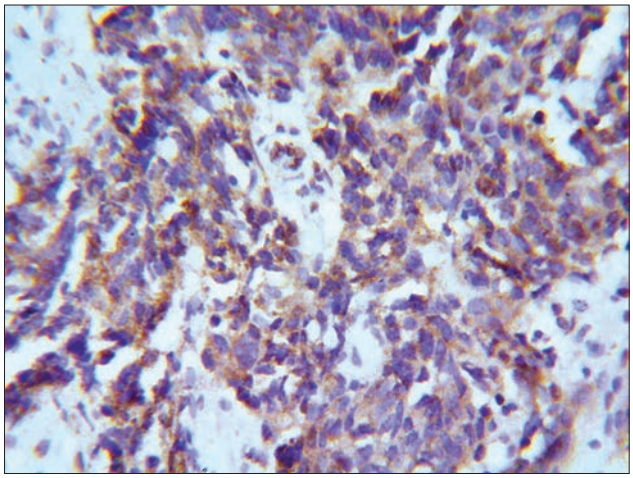

Figure 6: Photomicrograph showing Epstein–Barr virus latent membrane protein-1 positive IHC staining in nonkeratinizing carcinoma (×400)

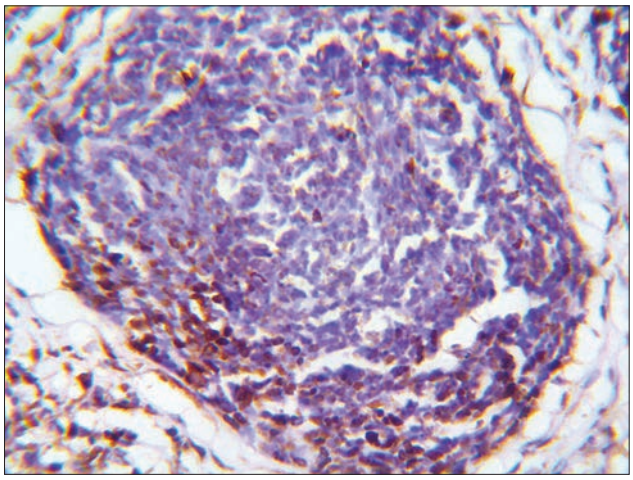

Figure 7: Photomicrograph showing Epstein–Barr virus latent membrane protein-1 negative IHC staining in nonkeratinizing carcinoma (×400)

and geographical variations. With regard to the conflicting reports of EBV association with the histopathological types of NPC, Pathmanathan *et al*. have convincingly argued that the existence of mixed histological types, the phenomenon of dedifferentiation seen in the clinical course and biological evolution of NPC strongly suggest that all types of NPC are variants of an EBV-associated malignancy.[23]

It is noteworthy that although EBER-ISH is more sensitive in detecting EBV in cancer cells,[10,11] LMP-1 IHC has proved useful in detecting EBV in this study and in other similar studies earlier mentioned. In addition, it is rapid, more economical, and has prognostic significance as studies have shown that LMP1-positive NPCs are more aggressive than LMP1-negative tumors.[9,27,28]

Although most specimens in this study were from the nasopharynx, presumably suggesting that they may be limited to the nasopharynx, the rest of the specimens are outside the nasopharynx, thus, suggesting advanced disease. Some of these head-and-neck mucosal sites occasionally develop primary lymphoepithelial carcinomas; however, in contrast to NPC, they do not show a strong association with EBV except in Asians.[5] The strong association with EBV seen in this study is therefore highly suggestive of NPC rather than primary lymphoepithelial carcinomas.

Conclusions

There is a high prevalence of EBV LMP-1 in NPC. However, there is no statistical difference in the prevalence of EBV in NKC and KSCC. NPC has shown an association with EBV irrespective of histological type. LMP-1 IHC has proved useful in detecting EBV in NPC in this study. The findings in this study show several similarities and some peculiarities in comparison with those of other studies done in different parts of the country, Africa and globally. The superiority of EBER ISH over EBV LMP-1 IHC has been documented, however, more local studies are recommended to compare the sensitivity of various methods of EBV detection so as to choose the most appropriate method for our environment, one which is both sensitive and affordable.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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