Original Article

Molecular Detection of Epstein-Barr Virus in Nasopharyngeal Carcinoma

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Abstract

Background: Nasopharyngeal carcinoma (NPC) has been closely associated with the oncogenic virus, Epstein–Barr virus (EBV). Its latent membrane protein-1 (LMP-1) has been particularly implicated in the molecular induction of latency in premalignant epithelial cells, initiation and development of NPC through various signaling pathways. The main aim of the study is the detection of LMP-1 gene in formalin fixed paraffin embedded tissue blocks (PETB) of histologically diagnosed NPC at Ahmadu Bello University Teaching Hospital (ABUTH), Zaria from January 1992 to December 2013. **Materials and Methods:** The PETB of 43 previously diagnosed NPC were retrieved and real-time Polymerase Chain Reaction (PCR) was used for EBV LMP-1 detection. Tissue sections were deparaffinized with xylene and ethanol, DNA extracted using *QIAamp*® DNA FFPE Tissue kit, Qiagen (Hamburg, Germany) and was quantified using NanoDrop. The amplicons were subjected to gel electrophoresis to detect the specific amplified product for LMP-1 gene. Data were analyzed using SPSS 26. **Results:** The result showed a male predominance with ratio 2.9:1, a wide age range of 6 years to 83 years, a mean age of 42.8 years, median age of 45 years and a unimodal age group of 40-49 years. EBV LMP-1 was positive in 16 (37.2%) of cases, 14 (32.6%) of these were of the Non-keratinizing Carcinoma (NKC) subtype while 2 (4.7%) were Keratinizing Squamous Cell Carcinoma (KSCC). **Conclusion:** The study provides evidence of the association of EBV LMP-1 with NPC and might prove useful as a diagnostic, therapeutic and prognostic tool in the management of NPC.

Keywords: Nasopharyngeal Carcinoma, Epstein-Barr Virus, Latent Membrane Protein-1, Polymerase Chain Reaction

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is defined as "a carcinoma arising in the nasopharyngeal mucosa that shows light microscopic or ultrastructural evidence of squamous differentiation."[1] Globally, in 2012, it accounted for 0.6% of all cancers, ranking 24th most common cancer with an estimate of 86,700 new cases of NPC and 50,800 deaths from NPC.[2] NPC incidence is 2 to 3-fold higher in males than in females irrespective of the incidence rate or geographical location, an observation thought to be due to habits such as smoking and alcohol consumption.[3] Certain salted fish and

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environmental and genetic factors have also been associated with NPC.[4]

The strongest causal association with NPC, however, is with Epstein-Barr virus (EBV).[1,5] EBV DNA found inside the tumoural cells are monoclonal, this suggests that NPC occurs from the clonal proliferation of a single EBV-infected cell.[6] Furthermore, the EBV infection in NPC displays a type II latency pattern with the expression of EBV nuclear antigen-1 (EBNA-1) and latent membrane proteins (LMP-1, 2A and 2B).[7]

LMP-1exerts an anti-apoptotic function by upregulating Bcl-2 and altering the ratio of initiator caspase-8 and its inhibitors.[7] It also constitutively activates signaling systems (NF-κB, JNK-kinase, and JAK/STAT-pathways), upregulates ICAM-1, LFA and MHC Class I and II molecules, induces expression of epidermal growth factor receptor (EGFR) and induces telomerase activity.[8,9] It could also interact with DNA methyltransferases (DNMTs) or demethylases to modulate epigenetic and metabolic modifications.[10,11]

LMP-1 positive tumours grow more rapidly, are found to be more aggressive than LMP-1 negative tumours and prone to invade lymph nodes.[4,12] The expression level of EBV-encoded LMP-1 is variable in NPC, ranging from 20-60%,[8] specifically, only about two-thirds of NPCs expressing LMP-1 and only 50% express LMP-2A protein.[7,13]

LMP-1 expression is also significantly correlated with the expression of proteins that mediate invasion, angiogenesis and metastasis, such as Twist, MMP9, c-Met, Ets-1 in NPC tumors.[8,14] In addition, LMP1-positive NPC tissues are significantly associated with lymph node metastasis and poor overall survival, consequently, it is considered a strong risk factor for poor prognosis in NPC.[15] Furthermore, LMP-1 impairs Chk1 activation which induces G2 checkpoint defect allowing unrepaired chromatid breaks to undergo mitosis eventually leading to the propagation of chromosomal instability.[16] The genetic polymorphisms of the human leukocyte antigen (HLA) complex located at the major histocompatibility complex (MHC) region on chromosome 6p21 might confer susceptibility towards NPC due to impaired EBV antigen presentation to cytotoxic cells, hence contributing to ethnicity predisposition of EBV-associated NPC.[17]

This study's primary objective was the molecular detection of EBV LMP-1 using conventional PCR in histologically diagnosed NPC seen at ABUTH Zaria.

MATERIALS AND METHODS

The study was a retrospective study of all histologically diagnosed NPC seen at the Department of Pathology, ABUTH Shika-Zaria from January 1, 1992 to December 31, 2013. Formalin-fixed, paraffin-embedded tissue blocks (PETB) of all previously diagnosed NPCs within the study period were retrieved and examined. Excluded from this study were all cases in which the PETB were missing, badly damaged, or had insufficient tissue.

Deparaffinization:

About 5-8 freshly cut sections were made from the PETB, each with a thickness of up to 10 µm and a surface area of up to 250 mm^2. The tumoural tissues were retrieved by dewaxing the thin tissue sections in 1mL of xylene and vortexed for 10 seconds. Tubes were then centrifuged at 14,000 rpm for 2 minutes and the supernatant (xylene) was removed carefully by pipetting. To the pellets at the bottom of the tube, 1ml of 96% ethanol was added and vortexed vigorously for 10 seconds before centrifugation at 14,000rpm for 2 minutes. The supernatant (ethanol) was removed by pipetting and the tubes were opened and incubated at room temperature until the ethanol evaporated.

DNA Extraction:

EBV DNA was extracted by means of an enzymatic digestion. Genomic DNA extraction was carried using *QIAamp*® DNA FFPE Tissue kit and performed precisely according to the manufacturer's instruction except for *Proteinase K* digestion which was allowed to stay overnight for complete digestion of the tissues. Pellets were re-suspended in 180 µL ATL buffer and 20 µL of proteinase K was added and allowed to digest overnight at 56^0C. After overnight incubation, the tubes were allowed to cool then incubated at 96^0C for 1hr. Tubes were cooled again and 400 µL of Buffer AL-alcohol mixture (1:1) was added and vortexed vigorously. The entire mixture was carefully transferred to the *QIAamp MinElute* column (in a 2 ml collection tube) and centrifuged at 8000 *rpm* for 1 minute. Changing the collection tubes each time, AW1 and AW2 solutions were used to wash, by centrifuging for 1 minute at 8000 *rpm*. The spin column was dried to remove all traces of alcohol by centrifuging the spin columns at 14,000 *rpm* for 3 minutes. To the dried spin column, 100 µL of TE buffer was added directly to the spin column and allowed to equilibrate for 2 minutes before centrifugation at 8000 rpm for 1 minute. The eluted DNA was stored at -20^0C.

RESULTS

A total of 43 cases were included in the study, 32 (74.42%) of which were male and 11 (25.58%) were female with a male to female ratio of 2.9:1. The ages ranged from 6 to 83 years with an overall mean age of 42.80 years, median age of 45 years and modal age of 40 years. The females had a mean age of 44.73 years (age range 16 to 68 years) while the mean age for males was 42.16 years (age range 6 to 83 years). The peak age of incidence was 40-49 years age group with 11 cases (25.58%) closely followed by 50-59 years age group with 10 cases (23.26%) then 30-39- and 60-69-years age groups which had 6 cases each (13.95%) and then 20-29 years age group with 5 cases (11.63%). The remaining age groups recorded a much lower incidence with 3 cases seen in the 30-39 age group, one case each seen in 0-9 years and 80-89 years age groups (2.33%), and none seen in the 70-79 years age group. (Table 1)

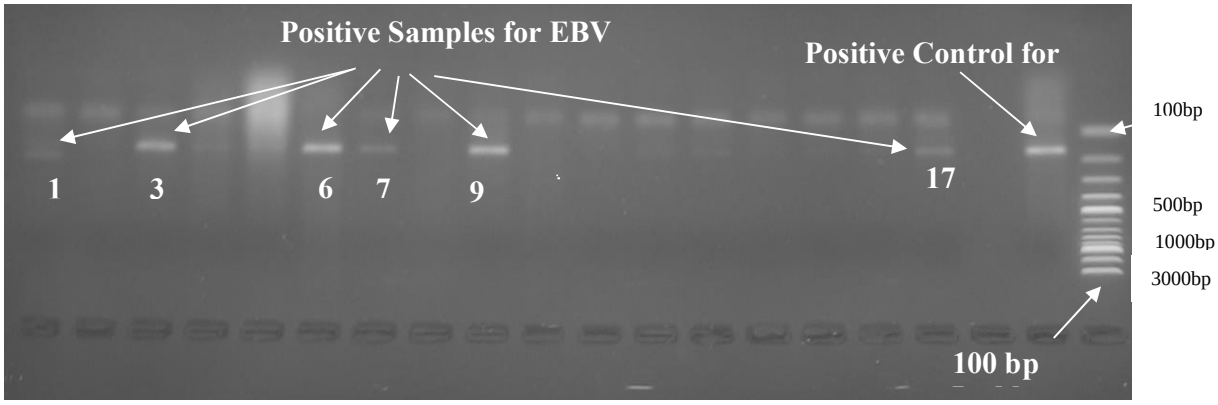
Real-time PCR detection for EBV LMP-1 done for 43 cases (Figures 1-3), showed that 16 (37.2%) were EBV-positive and 27(62.8%) were EBV-negative. NKC was the predominant subtype with 39 (90.7%) of cases, out of which 14 (32.6%) were EBV-positive and 25 (58.1%) were EBV-negative. There were 4 (9.3%) cases of KSCC, and half of these were EBV-positive 2 (4.7%). (Table. 2)

Table 1: Age and Sex Distribution of Study Population

Age Group	Male		Female		Total	
(Years)	Frequency	Percentage (%)	Frequency	Percentage (%)	Frequency	Percentage (%)
0-9	1	3.12	0	0	1	2.33
10-19	2	6.25	1	9.09	3	6.98
20-29	4	12.50	1	9.09	5	11.63
30-39	5	15.62	1	9.09	6	13.95
40-49	7	21.88	4	36.36	11	25.58
50-59	9	28.13	1	9.09	10	23.26
60-69	3	9.38	3	27.27	6	13.95
70-79	0	0.00	0	0.00	0	0.00
80-89	1	3.12	0	0.00	1	2.33
Total	32	100	11	100	43	100

Table 2: Frequency of EBV LMP-1 in Nasopharyngeal Carcinoma Histological Subtypes

Histopathological Type	Number	Positive	Negative
NKC	39 (90.7%)	14 (32.6%)	25 (58.1%)
KSCC	4 (9.3%)	2 (4.7%)	2 (4.7%)
Total	43 (100%)	16 (37.2%)	27 (62.8%)



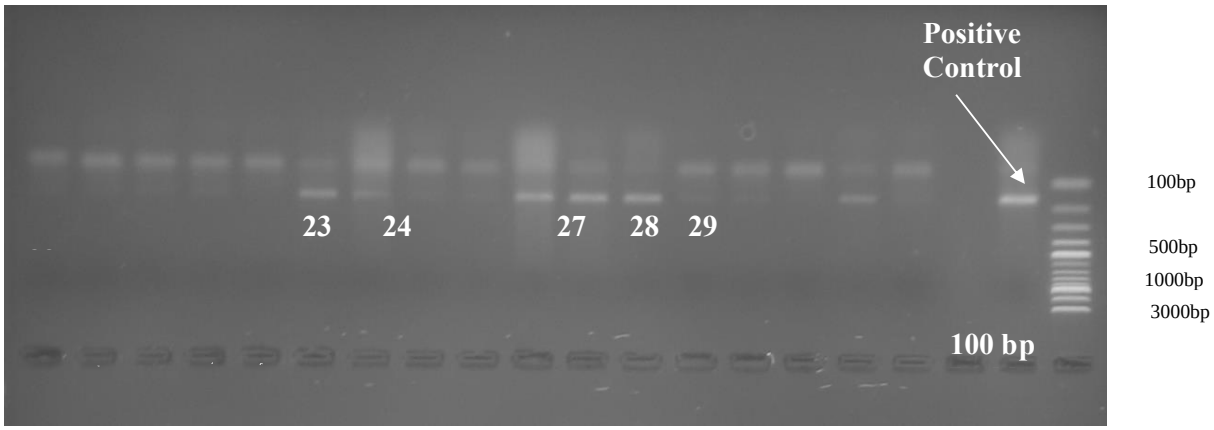


Figure 2: Continuation of gel electrophoresis of EBV detection showing positive bands in samples 23, 24, 27, 28, 29 and 33. Only wells with positive bands were labeled, wells not showing any band are negative.

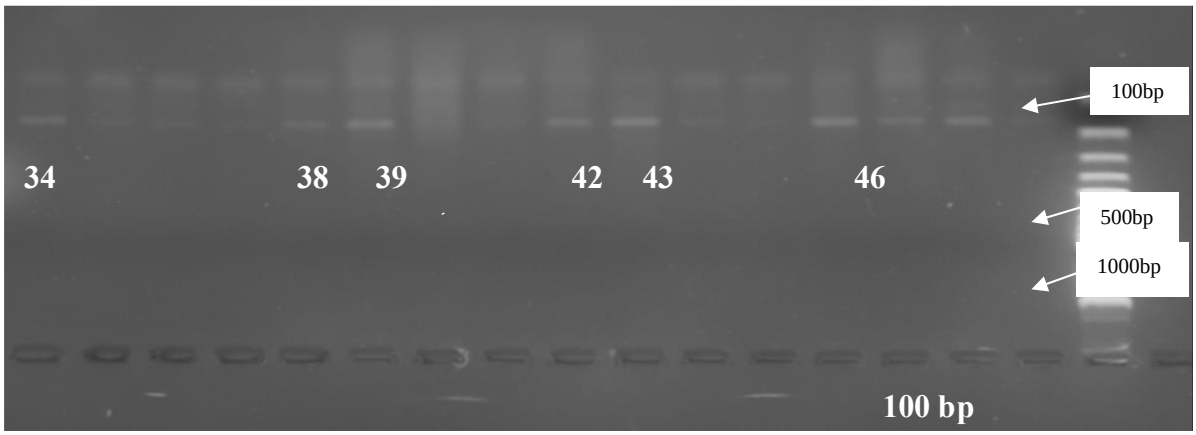


Figure 3: Continuation of gel electrophoresis of EBV detection showing positive bands in samples 34, 38, 39, 42, 43,46, 47 and 48. Only wells with positive bands were labeled, wells not showing any band are negative.

DISCUSSION

A male preponderance is observed in this study, 32 (74.42%) were male and 11 (25.58%) were female representing 2.9:1 male to female ratio. This sex distribution is consistent with local and international reports which have documented ratios of 2-3:1, regardless of the incidence rate or the geographical location.[19–23]

The mean age in this study is 42.80 years with an age range of 6 to 83 years. This is consistent with other reports from various parts of Nigeria that have reported mean age ranges from 41.10 to 48.70 years.[24–27] Conversely, the mean age of incidence from North America, Western Europe and China is about a decade higher.[23] Though the reason for this difference is unclear, it is thought to be due to the lower life expectancy reported in Nigeria compared to these countries.[28] The mean age for females is 44.73 years (age range 16 – 68 years) and 42.16 years (age range 6 – 83 years) for males. As observed in the literature, the age distribution is similar in males and females.[1]

The peak age of incidence in this study is 40-49 years (5th decade) and the modal age is 40 years. Comparable findings have been reported in other parts of Nigeria.[22,24,29] This unimodal age pattern is in contrast to the consistently bimodal age distribution reported in the low risk populations, irrespective of sex and geographical location.[30] In Nigeria, such bimodalities have been observed in studies done in Ibadan and Lagos.[25] Remarkably, comprehensive reviews have reported that NPC incidence increases monotonically with age in most of low-risk populations.[31] This is consistent with the findings of this study and most other local studies that do not demonstrate bimodality.

Numerous studies have since established the strong association of EBV with NPC, and the benefits of its analysis in the accurate diagnosis, appropriate treatment and prognostication of EBV-associated diseases.[1,8,9] The real-time PCR gene detection for EBV LMP-1 in this study demonstrated 37.2% positivity, attesting to the well-recognized association of EBV with NPC and further confirming the reported albeit widely variable reports of 20-60% expression of EBV LMP-1 in NPC.[8]

Interestingly, a previous immunohistochemical study of the same population reported a much higher prevalence of 77.3% EBV LMP-1 positivity.[32] This difference may be ascribed to the difference in the viral detection methods. Indeed the wide variability in the expression of EBV LMP-1 is demonstrated by the low incidence report from Israel with 13.3% positivity[33] and even the non-expression reported from Morocco[34] which is sharply contrasting with the 72% reported from another study in the same location.[35] This disparity could be attributed in varying degrees to the lack of constant expression of LMP-1, differences in the sensitivity of assays used and geographical variations. Moreover, PCR detection has been found to be affected by the type and duration of fixation, prolonged archiving of PETB, and the RNA/DNA extraction methods.[36–38] Nevertheless, higher values were observed with PCR detection in Maiduguri (63.4%)[39] and Sudan (61.3-62.2%).[18,40]

The vast majority of NPC seen were Non-keratinizing Carcinoma (NKC) accounting for 90.7% of the samples with Keratinizing Squamous Cell Carcinoma (KSCC) making up the remaining 9.3% of the samples; no Basaloid Squamous Cell Carcinoma (BSCC) is seen. NKC showed 32.6% EBV-positivity while KSCC accounted for 4.7% EBV-positivity. Notably, a number of studies have shown that NKC is invariably EBV-positive, irrespective of geographical origin.[4,41] On the other hand, there are conflicting data with regards to the association of EBV with KSCC.[42,43]

CONCLUSION

The prevalence of EBV LMP-1 is significant even in our environment which is geographically considered to be a low-risk location. This EBV-NPC causal relationship appears to persist irrespective of age, sex, geographical location, or histologic subtype. A comparative study of EBV detection methods is recommended to compare their sensitivity and specificity under similar conditions.

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