

RESEARCH ARTICLE

Expression of Epstein Barr Virus Encoded EBNA1 and LMP1 Oncoproteins in Nasopharyngeal Carcinomas from Northeast India

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Abstract

Background: Nasopharyngeal carcinoma (NPC), a malignancy arising from the epithelial lining of the nasopharynx, is distinct from others cancers in terms of its epidemiologic features. It is rare in most parts of the world except for a few regions with populations of Mongoloid origin. **Objectives:** To study the expression pattern of Epstein Barr virus (EBV) encoded oncoproteins EBNA1 and LMP1 in different histological types of NPC and to correlate expression patterns with sex, age and histological types. **Materials and Methods:** A total of 40 formalin-fixed, paraffin-embedded NPC biopsy samples and tissues from 20 healthy controls were collected to study the expression level of EBNA1 and LMP1 using immunohistochemistry. **Results:** EBNA1 and LMP1 expression was found in 92.5% and 90% respectively, of the cases and none of the control specimens. The expression patterns of EBNA1 and LMP1 were determined to be statistically significant ($p < 0.05$) when correlated with sex, age and histological distributions. Also immunohistochemistry was found to be a sensitive technique in the detection of EBV. **Conclusions:** The study reveals that the potent oncoproteins EBNA1 and LMP1 were over expressed in our population cohort. Our findings are to some extent inconsistent with earlier reports as our population showed a higher expression of both EBNA1 and LMP1 compared to other studies.

Keywords: Nasopharyngeal carcinoma - Epstein Barr virus - LMP1 - EBNA1 - North East India.

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Introduction

Nasopharyngeal carcinoma (NPC) is a rare malignant carcinoma that arises in the epithelial lining of the nasopharyngeal mucosa. NPC is distinct from others cancers in terms of its epidemiologic features. It is a rare cancer in most part of the world except for a few regions with mongoloid origin. The age adjusted rate-(AAR) of NPC in the north eastern region of India particularly Kohima of Nagaland ranks highest in India and third highest in the world (Kataki et al., 2011).

Reports show that the survival rate is approximately 90% for patient with an early diagnosis, however, most patients are diagnosed with an advanced-stage of the disease, and the survival rate decreases less than 50%. The diagnosis of NPC is often delayed due to the inaccessible location of the tumour leading to its detection in the advanced stage of the disease which results in high mortality of the patients (Razak et al., 2010). NPC is one of the most confusing malignant carcinoma. The major risk factors known to be associated with NPC include genetic susceptibility, (Feng et al., 2002; Xiong et al.,

2004), Epstein-Bar virus (EBV) infection (Niedobitek et al., 1991; Vasef et al., 1997), environmental risk factors (Vaughan et al., 2000; Mirabelli et al., 2000) and certain dietary factors (Armstrong et al., 1998; Edefonti et al., 2015).

The histological classification guidelines proposed by World Health Organization (WHO) in 1978 categorized NPC into 3 groups: Type I (keratinizing squamous cell carcinoma), Type II (Non-keratinizing carcinoma) and Type III (undifferentiated carcinoma). In 1991, WHO revised the classification of NPC and categorised them into two groups: Squamous cell carcinoma (Type I of former classification) and Non-Keratinizing carcinoma (former Type II and III) is further divided into differentiated and undifferentiated carcinoma (Robinson et al., 2013). Type II and type III has been found to be more common and related to EBV infection compared to Type I group. The WHO 2005 classification still maintains the separation between the keratinizing squamous cell carcinoma and non- keratinizing carcinoma, but, they are more flexible in their sub-classification (Barnes et al., 2005; Maumad et al., 2013).

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The high incidence of NPC is found in Southern China, particularly Guangdong and Guangxi provinces, North-East India, Arctic region, Southern Asia and Northern Africa (Simons, 2011). It has been observed that people from high risk areas if migrate to low risk areas, they continue to remain susceptible to NPC, however, it has been found that successive generations show a decreased risk to NPC, suggesting the vital role played by the environmental and genetic factors in NPC development (Muir, 1971). In almost all studies, the incidence of NPC is 2 to 3 fold higher in males compared to females (Parkin et al., 2002). NPC is a rare cancer in most regions of India except for the North-eastern regions bordering China. The National Cancer Registry Programme (NCRP), a three-year report on Population Based Cancer Registries (PBCRs), 2009-2011 has reported that most of the North-Eastern cancer registries had higher AARs (age adjusted incidence rates) of NPC than the rest of India. The AARs of NPC of males in Nagaland is 21.0, which is highest in India and third highest in the world (in comparison with AARs of international PBCRs), behind Zhongshan, China and Guangzhou City, China (Kataki et al., 2011).

The association of Epstein Barr virus was first demonstrated when the majority of NPC patients were tested to have high level of antibodies against EBV antigens (Henle et al., 1970). The direct proof of the association of EBV in NPC was demonstrated by the presence of EBV DNA in the epithelial cells of NPC patients with the use of radioactive EBV probe (Wolf et al., 1973). The correlation between consumption of salted and preserved foods with expression of EBV early antigen (EA) was demonstrated which suggested that development of EBV positive NPC could be related to dietary habits, which provides another dimension to the epidemiological studies of NPC (Shao et al., 1998). Patients with undifferentiated nasopharyngeal carcinomas are invariably EBV positive, irrespective of geographical origin (Khan et al., 1996; Khaled, 2014).

EBV is a prototype of human herpes virus family with a large 172kbs double stranded linear DNA genome encoding nearly 100 genes during lytic phase that is associated with several types of malignancies. Epithelial cell infection is primarily lytic while B-cell infection is usually latent thereby enabling the virus to establish a persistent infection (Babcock et al., 1998). In healthy individuals, EBV is restricted by the immune system to a small number of memory B-cells, approximately 50 cells per million of peripheral B-lymphocytes. However, in immune deficient patients EBV may escape from immune surveillance and may express up to 10 latent genes most of which has been found to be involved in the process of tumorigenesis (Korcum et al., 2006).

EBV infection is classified as type I, type II and type III latencies. EBV infects NPC by type II latent infection in which only EBV nuclear antigen-1(EBNA-1), latent membrane protein- 1(LMP1), LMP2, and EBV early RNA (EBER) expressions are associated (Young et al., 2004). Studies conducted on normal nasopharyngeal tissue and premalignant biopsies shows that genetic events like loss of heterozygosity at chromosomal regions 3p and 9p, occur early in the pathogenesis of NPC, probably

due to exposure of environmental factors such as dietary components (salted fish), which might predispose to subsequent EBV infection. Once the virus has set up, the latent genes may provide growth and survival signals, leading to the development of NPC (Tabyaoui et al., 2013). Among the molecules of EBV latency, EBNA1 and LMP1 are the main oncogenes of EBV because of their abilities to recruit an array of cellular genes and interaction with a number of critical cellular signalling pathways causing apoptotic inhibition, cell proliferation, metastasis and thereby leading to severity and pathogenesis of the disease (Yoshizaki et al., 2013; Tsao et al., 2015).

The present study was aimed to investigate the expression of the two viral oncoproteins EBNA1, LMP1 in the NPC patients cohort of Northeast India particularly Nagaland and to determine any significant correlation between expression of the two viral oncoproteins with histological types, age and sex. The study may provide an insight in the high prevalence of the disease among our study population considered. It was also aimed to investigate if immunohistochemistry can be used as a regular test to determine any association of EBV with NPC.

Materials and Methods

Study subject

This study included 60 paraffin embedded tissue samples which include 40 paraffin tissue blocks of patients diagnosed positive for NPC and 20 normal paraffin tissue blocks which were diagnosed as negative for NPC. Blocks were collected from Naga Hospital authority, Kohima. Of the 40 NPC sections, the histotypes were 12 non-keratinizing carcinoma, 18 undifferentiated carcinoma, 10 squamous cell carcinoma. The histological classification of the tissues along with age, sex and tumor grade is depicted in Table 1.

The work conducted in this study has been approved by the Institutional Ethics committee with ethical clearance application number GUEC-08/2015. The members of the ethics committee were as follows: Dr. Dhaneswar Kalita, Ex-Principal, Govt. Ayurvedic College, Guwahati; Dr. N.N Talukdar, CMO, Gauhati University Hospital; Prof. R.C Borpatra Gohain, Dept. of LLM, G.U; Prof. Anuradha Dutta, Nabagraha Road, Guwahati; Dr. Diganta Narzary, Dept. of Botany, G.U.

Immunohistochemical staining

Four micron sections of the targeted paraffin embedded tissues were taken in Poly L Lysin (Sigma) treated glass slides (Bluestar) using a semi automated microtome (Leica) and baked overnight in a hot air oven (BioCraft). Hi Def Polymer Secondary Detection kit (Cell Marque, USA) was used for the immunohistochemical study.

The steps of the immunohistochemical protocol are as follows. 1. Deparaffinization in 2 wash of xylene. 2. Hydration in alcohol gradient, starting from 100% alcohol to 50% alcohol for 2mins each. 3. Antigen retrieval in Tris EDTA buffer at higher to lower power level in a microwave oven. 4. Treatment with blocking solution for 15mins. 5. Treatment with primary respective antibodies

overnight. 6. Washing three times in Tris buffer for 5mins each. 7. Treated with amplifier for 30mins. 8. Washing three times in Tris buffer for 5mins each. 9. Treated with secondary antibody for 30mins. 10. Washing three times in Tris buffer for 5mins each. 11. Treatment with 3,3-diaminobenzidine tetrahydrochloride (DAB) solution with hydrogen peroxidise for 5 minutes. 12. Washed with clean running water. 13. Treated with Hematoxylin for 2mins. 13. Washed with clean distilled water. 14. Dehydrated in two xylene treatment for 5mins each. 15. Cleaned and mount with DPX (Distrene, Plasticiser, Xylene). A fluorescence microscope (Olympus) was used to analyse the IHC slides. The antibodies used were anti-EBV Nuclear Antigen Antibody (Abcam), anti EBV LMP1 Antibody (Abcam).

Analysis of EBNA1 and LMP1 expression

EBNA1 expression was considered to be positive for those samples where nuclear immunostaining was more than 10% and LMP1 expression was considered positive for samples when membrane pattern of staining was observed in more than 10% of cancerous cells. Expression analysis for EBNA1 and LMP1 was based on scoring system (percentage of stained cells and intensity of staining) performed by pathologist. The scoring pattern has been shown in Table 2. The observed data was also correlated using Immunoratio online application.

Statistical analysis

Evaluation among different categorical data was done using Fisher's exact probability test and the level of significance was estimated. Probability scores less than 0.05 were considered to be significant. LMP1 and EBNA1 expressions were analyzed based on various clinicopathological parameters. Epi-Info 3.5.4 software was used for statistical analysis of the data.

Results

Clinical data

A total of 40 NPC positive paraffin embedded tissue sections and 20 paraffin embedded healthy control tissue sections are included in the study. A male preponderance of the disease is observed with a male: female ratio to be 3.44:1 with 31 males and 9 females. The age groups of the patients ranged between 21 and 70 years with mean

age ranging 50 ± 12.78

The samples were segregated based on WHO classification. 40 primary NPC cases are classified as 18 (45%) undifferentiated carcinoma (UC, WHO type III), 12 (30%) of non-keratinizing carcinoma (NKC, WHO type II) and 10 cases (25%) of squamous cell carcinoma (SCC, WHO type I).

EBNA1 expression

EBNA1 expression was detected in 40 NPC tissue sections and 37 (92.5%) samples revealed positive staining. The negative control did not show any immunohistochemical staining. The positive control was prepared for a NPC positive sample showing strong immunostaining for both EBNA1 and LMP1. Serum sample was collected for that sample and viral DNA was isolated using standard phenol chloroform method. The sample was found positive for EBV by PCR (using detection primer specific for EBNA1) and ELISA.

LMP1 expression

Immunohistochemical staining was used for detection of LMP1 proteins. Of the 40 NPC tissue sections 36 (90%)

Table 1. Histological Classification of the NPC Tissue Sections in Terms of Age, Sex and Tumor Grade

Sl no	Histological Type	Cases	Age	Sex	Tumor grade
1	Non Keratinizing Carcinoma (NKC)	12	45±14.96	9:03	I 4
					IIA 2
					IIIB 3
					IIIA 1
					IIIB 0
					IIIC 0
2	Undifferentiated Carcinoma (UC)	18	51±10.05	13:05	IV 2
					I 2
					IIA 4
					IIIB 3
					IIIA 3
					IIIB 0
3	Squamous Cell Carcinoma (SCC)	10	54±13.34	9:01	IIIC 0
					IV 6
					I 3
					IIA 4
					IIIB 0
					IIIA 3
IIIB 0					
					IIIC 0
					IV 0

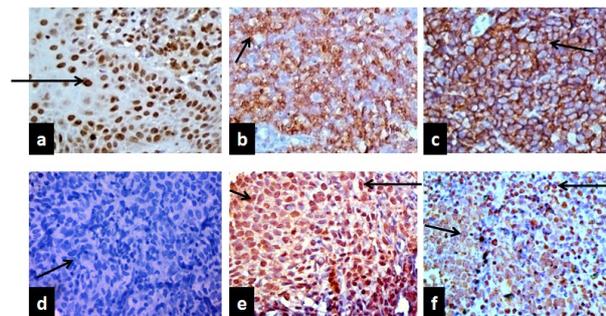


Figure 1. Immunohistochemical Findings. a) EBNA1 protein staining, b) LMP1 protein staining, c) positive control using CD45 staining, d) negative control, e) EBNA1 co-stain and f) LMP1 co-stain

Table 2. Allred Score = Proportion Score + Intensity Score

Percentage of stained cells	Score	Intensity of staining	Score	Total score
NIL	0	None	0	0
<1%	1	Weak	1	2
1%-10%	2	Moderate	2	3
11%-33%	3	Strong	3	4
34%-66%	4			5
67%-100%	5			6
				7
				8

0-2: Negative >3: Positive

Table 3. EBNA1 and LMP1 Protein Detection in NPC Specimens: Correlations with Clinical and Histopathological Data

Criteria	Co-relative Data	Specimen Nos.	EBNA1 expression		P value [chi]	LMP1 expression		P value [Chi]
			Positive	Negative		Positive	Negative	
SEX	Male	31(77.5)	28 (90.3)	3 (9.6)	0.0001 [10.5] Odd Ratio 0.0	27 (87.0)	4 (12.9)	0.003[12.7] Odd Ratio 0.0
	Female	9(22.5)	9 (100)	0 (0)		9 (100)	0 (0)	
AGE	<45	10(25)	10 (100)	0 (0)	0.001 [10.5] Odd Ratio 0.0	10 (100)	0 (0)	0.0001[4.9] Odd Ratio 0.0
	>45	30(75)	27 (90)	3 (10)		26 (86.6)	4 (13.3)	
HISTOLOGICAL TYPE	UC	18 (45)	18 (100)	0 (0)	0.001 [23.0]	17 (94.4)	1 (5.5)	0.005[10.5]
	NKC	12 (30)	11 (91.6)	1 (8.3)		11 (91.6)	1 (8.3)	
	SCC	10 (25)	8 (80)	2 (20)		8 (80)	2 (20)	

samples showed positive staining for LMP1. The negative control did not reveal any immunostaining.

EBNA1 and LMP1 protein expressions were co related with patient's age, sex and histological type statistically and the data obtained are summarized in Table 3. All 31 males were found positive for both EBNA1 and LMP1. Of the 9 females 3 were negative for EBNA1 and 4 were negative for LMP1. However, the data were found to be statistically significant for both EBNA1 and LMP1 when compared with sex, age and histological types of the patients. ($p < 0.05$). Undifferentiated carcinoma (UC) showed 100% (n=18) expression for EBNA1 and 94.4% (n=17) for LMP1, Non keratinizing carcinoma (NKC) showed 91.6% (n=11) expression for EBNA1 and 91.6% (n=11) for LMP1 and Squamous cell carcinoma (SCC) showed 80% (n=8) expression for EBNA1 and 80% (n=8) for LMP1.

Discussion

Our study reveals the association of EBV in Nasopharyngeal carcinoma and demonstrates that immunohistochemical staining detects the presence of latent EBV in NPC tumour biopsy tissues. The results of our study reflect the situation in our population as NPC is endemic in this segment of study. It has been observed that admixture of Chinese blood in an ethnic group, irrespective of their country of immigration, elevates the nasopharyngeal carcinoma incidence rates (Feng et al., 2002). The diagnosis of NPC is often delayed due to the hidden location of the tumour; as a result it is often detected in the advanced stage at the time of presentation, leading to the high mortality of the patients.

We noticed a predominance of undifferentiated carcinomas (UC, WHO type III) (45%) compared with non-keratinising carcinomas (NKC, WHO type II) (30%), whereas keratinising squamous-cell carcinomas (SCC, WHO type I) were 25%. This was quite logical as NPC is endemic in this region of India and the vast majority of NPC belongs to the undifferentiated forms, namely WHO types II and III (Busson et al., 2002).

EBV has been found to be constitutively expressed almost in all cases of NPC (Zhang et al., 2013). NPC is by far the third most common virus associated carcinoma in humans (Chang et al., 2006). In healthy individuals, EBV is restricted by the immune system to a small number of memory B-cells, approximately 50 cells per million of peripheral B-lymphocytes. However, in immune deficient patients EBV may escape from immune surveillance and

may express up to 10 latent genes most of which has been found to be involved in the process of tumourigenesis (Liu et al., 2000). Of all the viral encoded proteins EBNA1 and LMP1 have been found to be constitutively expressed and involved in pathogenesis of the disease.

Epstein-Barr Nuclear Antigen-1 (EBNA1) plays a crucial role in Epstein-Barr virus (EBV) infection and it is the only latent viral protein known to be consistently expressed in all EBV-associated malignancies, including nasopharyngeal carcinoma (NPC). A study conducted showed that EBNA1 was expressed in 79.5% of NPC, and 28% of non- NPC tissues (Xiao et al., 2014).

Over the last few years, LMP1 has attracted immense interest as it has been found to be associated with critical cellular signalling pathways. Latent membrane protein 1 (LMP1) is one of the major EBV encoded oncogenes associated with viral mediated transformation. It is known to encode an oncoprotein that functions as a constitutively active tumour necrosis factor receptor (TNFR) and acts as a key modulator in NPC pathogenesis and progression, particularly in invasion and metastasis. The clinical significance of LMP1 has not yet been fully determined, with some studies reporting that it is not a prognostic factor, rather an adverse prognostic factor or a good prognostic marker (Guo et al., 2012). The study we conducted shows a high expression of EBNA1 and LMP1 in our patient cohort. 37 of a total of 40 patients showed positivity for EBNA1 and 36 for LMP1.

The samples were also processed for PCR detection of EBNA1 and LMP1. Some studies have reported LMP1 expression in 50% to 65% of EBV-positive NPC cells (Chan et al., 2002). A study on 52 Spanish patients showed a 78.4% positivity of LMP1 by immunohistochemistry (Vera-Sempere et al 1996), what was inconsistent with another study where only one LMP1 positive case of the undifferentiated NPC (WHO type 3) have been reported on a total of 44 cases (Kouvidou et al., 1995). On another study, 23 Moroccan NPC patients were included in the study and were found negative for LMP1 expression. (Tabyaoui et al., 2013). A study performed on 87 NPC patients from China found 75 patients (86.2%) to be positive for NPC by PCR detection of LMP1 (Guo et al., 2012). One more study in Pakistani population reported a 75/92(81%) positive LMP1 cases and all cases of Keratinizing Squamous Cell Carcinoma were EBV-LMP1 negative (Umar and Ahmed 2014). In one recently reported study LMP1 was detected in 35 NPC positive cases according to the histological subtypes in 55% of undifferentiated squamous cell carcinoma, 28%

of keratinized squamous cell carcinoma and 21% in Non keratinized squamous cell carcinoma (Ibrahim et al., 201).

It is reported earlier that immunohistochemistry may be less sensitive than Western blotting (65% positive) or RT-PCR (nearly 100% of expression) for detecting LMP1. Some studies also take into consideration the quality of the anti-LMP1 clone used; it claims that S12 or anti-136 clones provide better results compared to the CS 1-4 clone. Some studies have reported 20-30% of LMP1 expression on NPC frozen tissues compared to 0% of expression in paraffin embedded tissues. It has also been stated that LMP1 expression may only rarely detectable using immunohistochemistry (Niedobitek et al., 2000).

The data we have reported is in concordance with the high risk of NPC in our study population. We observed that immunohistochemistry is a sensitive technique and showed better results in contrast to other studies. We included a total of 40 paraffin embedded NPC tissues and 20 healthy control tissues. All the samples were collected from the population of Nagaland. We could find a higher expression for both EBNA1 and LMP1, with 92.5% - EBNA1 positivity and 90% - LMP1 positivity. Of the 20 controls 2 were found positive for EBNA1 with weak immuno-stain but were negative for LMP1. We used Anti-EBV Latent Membrane Protein 1 antibody [CS 1-4] (Abcam) and Anti-EBV Nuclear Antigen antibody [E1-2.5] (Abcam) clones in our study and both the antibodies were found to be sensitive and showed good results. The study performed by us reports for the first time a high expression of both EBNA1 and LMP1 in North east India. We also reported discordance in the expression of LMP1 and EBNA1. In 15 of our study samples we found a co-expression of LMP1 and EBNA1 in the cytoplasm as well as in the nucleus. However, it is an established fact that EBNA1 is a nuclear protein while LMP1 is expressed in the cytoplasmic portion of the plasma membrane. To find the efficacy of our results we repeated our experiment on selected samples and our results came out to be same. We although could not come to any conclusion with this anomaly in our result. This result has been reported for the first time and it requires further elucidation which in future may bring new insights and help in better diagnostics and therapeutics of the disease.

The results we concluded may be further investigated using other molecular techniques which may help in better understanding of the carcinogenesis and progression of NPC. The study shall bring new insight in the pathology of the disease in North East India and may introduce new biological factors which can be used as better prognostic markers.

In conclusion, the study confirms that Epstein Barr virus (EBV) may play a crucial role in the pathogenesis and high prevalence of nasopharyngeal carcinoma in North Eastern segments of India, particularly Nagaland. The potent oncoproteins EBNA1 and LMP1 were found to be overexpressed in our population cohort. Our findings are inconsistent with earlier reports in so far as our population showed a higher expression of both EBNA1 and LMP1 compared to other studies.

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