

Epstein Barr Virus in Head and Neck Extranodal Non-hodgkin's Lymphoma in Egypt

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ABSTRACT

Background: Primary extranodal lymphoma of the head and neck (PELHN) represents 16.18% of all malignant lymphoma cases diagnosed at the NCI, Cairo University between 1985-1990. Epstein Barr virus (EBV) infection is highly associated with lymphoma in immunocompromised patients however, the situation in immunocompetent patients is still unclear.

Material and methods: We investigated 50 PELHN, 5 reactive lymph nodes, 15 normal nasopharyngeal tissue (NPTs) and 25 throat washes (TW) of healthy people from Egypt for EBV association, expression of *p53*, *Ki-67*, *bcl-2*, *Bax* and the clinical outcome.

Results: Phenotypic analysis showed that 31 cases were B cell and 19 were T cell lymphomas. Eleven cases were present in the nose and paranasal sinuses, 11 in the nasopharynx, 13 in the tonsils, 7 in the oropharynx and 8 in the oral cavity. EBV positivity was reported in 90% and 70% of the cases using EBER in situ hybridization and PCR respectively (concordance = 70%). All cases of nasal type lymphoma were EBV positive. Strain typing revealed the presence of type 1 in all positive cases. 30 base pair deletion of the LMP-1 (del-LMP1) gene was detected in 24/35 EBV-positive cases by PCR (68.6%) whereas 11 cases had wild type variant either alone or mixed with del-LMP1. There was a statistically significant difference in the frequency of del-LMP1 between lymphoma and normal tissues. Overexpression of *Ki-67*, *p53* and *bcl-2* was detected in 78.1%, 62.5% and 20% of cases whereas absence of *Bax* was reported in 18% of cases. Univariate analysis showed that *p53* overexpression, a high *Ki-67* index, advanced disease stage and T phenotype are poor prognostic factors whereas only *p53* overexpression and advanced disease stage are poor prognostic factors in multivariate analysis.

Conclusion: EBV infection is frequent in PELHN in Egypt. Possible pathogenetic mechanisms involve deregulation of *p53* and enhanced proliferation (as detected by high *Ki-67* index). The presence of del-LMP1 variants, *p53* overexpression and advanced disease stage are poor

prognostic factors associated with reduced survival and poor response to therapy.

Key Words: Head and neck lymphoma - EBV - *p53* - *Ki-67* - *bcl-2* and *Bax*.

INTRODUCTION

Primary extranodal lymphoma of the head and neck (PELHN) is frequently encountered in Egypt both in pediatric and adult age groups. It represents 16.18% of total lymphomas and includes lymphoma of the oral cavity (5.35%), hard palate (3.25%), tonsils (2.9%), nasopharynx (2.3%), oropharynx, nose and paranasal sinuses (1.34% each) [1].

EBV is a human herpes virus, which is associated with a variety of lymphoid and epithelial malignancies. Therefore, it has been classified in a recent IARC monograph as a group 1 carcinogen [2]. EBV has been identified with variable frequencies in Burkitt's lymphoma (BL), nasopharyngeal carcinoma (NPC), Hodgkins Disease (HD), sinonasal and peripheral T cell lymphomas, lymphoepithelioma, gastric carcinoma and leiomyosarcoma in immunosuppressed patients, some head and neck cancers and lymphoma and in almost all cases of NK/T cell nasal lymphomas in certain geographical areas [3-7]. In these tumors, it is present as a single episomal form localized to the neoplastic cell population. This is in keeping with latent infection of a single precursor cell prior to clonal expansion and suggests a possible pathogenetic role of the virus at least in some tumors [8].

A limited number of genes are expressed in EBV-associated tumors, including six nuclear antigens (EBNA-1,2,3-A,3B, 3C and -LP) and three latent membrane proteins (LMP-1, 2A, 2B) [2]. The LMP-1 is considered a viral oncogene because it is the only EBV-encoded protein that has a transforming ability in isolation and induces DNA synthesis in human B cells [9]. Also, expression of LMP-1 protects EBV-infected cells from apoptosis through regulation of the bcl-2 and related proteins (bcl-X_L, Bax, MCL1) which block or promote apoptosis by forming homo- or heterodimers with each other [10]. A partial deletion located within the carboxy terminal domain of the LMP-1 protein was detected in a proportion of EBV-associated lymphomas. The characterized 30 base pair (bp) deletion have involved an identical DNA segment corresponding to amino acids 346-355 [11]. Inactivation of the *p53* gene was mentioned as a possible pathway through which EBV can abrogate apoptosis and lead to sustained genetic damage [12]. Moreover, Calzolari et al. [13], Liu et al. [14] and Petit et al. [15] demonstrated that, overexpression of *p53* and Ki-67 strongly modulates tumor response to chemo- and radiotherapy and has a significant impact on the mean survival time in some EBV-associated neoplasms.

The present study was conducted to determine the frequency of EBV and to identify the possible pathogenetic mechanisms in PELHN. We also investigated the impact of EBV infection on the clinical outcome of PELHN patients using tumor response to therapy, disease free and overall survival rates as indicators for patients' outcome.

MATERIAL AND METHODS

Patients and tissues: Fifty cases of PELHN were selected from the files of the Pathology Department, NCI, Cairo, Egypt covering 6 years period from January, 1995 to December, 2001. Thirteen cases were located at the tonsils, 11 in the nose and nasal sinuses, 11 in the nasopharynx (NP), 8 in the oral cavity (OC) including the hard palate and 7 in the oropharynx (OP) (Table 1). Histological diagnosis and grading were done following "REAL classification of NHL". Subtyping of the lymphoma was done using a panel of B-, T- and NK-cell specific antibodies. Special attention was given to an angioinvasive/angiocentric growth pattern and

the presence of necrosis for the preliminary diagnosis of nasal/ nasal type lymphoma (Fig. 1-A). Five reactive lymph node biopsies, 25 throat washings (TW) from healthy individuals and 15 normal nasopharyngeal mucosal tissues (NPT) obtained at post-mortem examination of patients who died of causes unrelated to the respiratory tract and had no evidence of EBV-related diseases were included for comparison. Throat washings were collected from healthy individuals by asking them to gargle with phosphate buffered saline (PBS). The collected material was centrifuged at 3000 g for 20 min. Cells were then divided into two portions, one portion was kept at -80°C for detection and typing of EBV and the other was used to make a cell block for EBER-ISH and IHC. The cell lines Raji and Jijoye (EBV positive Burkitt's lymphoma cell lines) and MOLT (EBV negative) were obtained from the ATCC (Rockville, MD) and cultured in RPMI-1640 (GIBCO, Paisely, UK) supplemented with 10% fetal calf serum, L-glutamine and gentamycin (8µg/ml) according to the supplier's instructions. All cultures were grown at 37°C in 5% CO₂ and passaged weekly. Full clinical data were available for 39 patients. The overall survival was calculated from the date of initial diagnosis till the date of death. Complete remission was defined as total disappearance of all symptoms and clinically detectable disease. Partial remission was defined as a 50% or greater disappearance of all measurable tumors and no appearance of new lesions for at least one month.

1- Immunophenotyping of lymphoma: Was performed on paraffin-embedded material using the following antibodies (All from Dako Ltd., Cambridge, United Kingdom): CD45 (LCA, T29), CD20 (L-26), CD79acy (HM57), CD3 (PC3), CD45RO (UCHL1), CD5 (CD5/45/F6), CD4 (OPD4), CD8 (C8), CD10 (SS2/36) and CD56 (NK1). A reaction was considered positive when at least 50% of the tumor cells showed staining.

2- In situ hybridization for EBV-encoded RNA (EBER) transcripts (EBER ISH): EBER ISH was carried out using the EBV-ISH kit from Novo castra (NCL-EBV) according to manufacturer's instructions [16]. The Raji cell line embedded in paraffin was used as positive control. Hybridization buffer without probe and the MOLT cell line were used as negative control.

3- Molecular detection of viral sequences: Extraction of DNA from Raji, MOLT, Jijoye cell lines and paraffin embedded normal and tumor tissues was performed as described by Khanim et al. [17]. Two micrograms of genomic DNA were amplified in a DNA thermal cycle 480 (Perkin Elmer Corp., Foster City, CA) using primers sets covering three different regions of the EBV genome: EBER-2, BZLF1 (BamHI Z Leftward Frame 1) and BNL1 (BamHI N Leftward Frame 1) as previously described [3]. The expected products were 108 bp for EBER-2, 994 bp for BZLF1 and 337 or 307 bp according to BNL1 polymorphism. Samples were considered positive for EBV if amplification of all three genes occurred. DNA amplification for all cases was performed according to Bonnet et al. [3]. The standard protocol was 5 min denaturation at 95°C (1 cycle) followed by 40 cycles of 30 sec denaturation at 95°C, 1 min of annealing, and 2 min extension at 72°C. Two microliters of the BZLF1-PCR product were used in a second round of PCR using internal primers. Amplification of the GAPDH was used to confirm the adequacy of DNA and absence of inhibitors. PCR products were analyzed by electrophoresis through 1.5% agarose gel. Control template DNA [DNA extracted from Raji cell line (EBV+) and MOLT cell line (EBV negative)] accompanied each PCR run.

4- PCR Analysis for LMP-1 Deletion and EBNA-2 Strain Typing: Primers flanking the clustered LMP-1 deletion segment were used as previously described by Kingma et al. [11]. Control template DNA for LMP-1 PCR included DNA extracted from Raji cell line (EBV+, wt-LMP-1) and MOLT cell line (EBV negative). LMP-1 PCR products were analyzed on a 2% agarose gel and the product length for clinical samples was compared with that obtained from PCR amplification of Raji cell line. Cases exhibiting equivalent PCR product lengths when compared with Raji were considered wild type (wt-LMP1) whereas samples exhibiting shorter PCR product lengths were interpreted as containing deletion (del-LMP-1).

The primer sequences used for EBNA-2 strain typing were chosen from the highly sensitive and type-specific polymorphic region of the EBNA2 gene as previously described [18]. PCR conditions were identical to those described by Khanim et al. [17]. Briefly, 2 separate PCRs were carried out with a type-common 5' primer

(B95-8 coordinates 48810-48829) and a type 1-specific 3' primer (B95-8 coordinates 49058-49039) or a type 2-specific 3' primer, followed by hybridization with a type 1-specific (B95-8 coordinates 48997-49028) and a type 2-specific internal oligonucleotide probe, respectively. Control template DNA for EBNA-2 PCR included DNA extracted from Raji (type 1) and Jijoye (type 2) cell lines. EBNA-2 PCR products were analyzed on a 2% agarose gel and compared with PCR products of Raji and Jijoye. Cases exhibiting equivalent PCR product lengths when compared with Raji were interpreted as containing EBV strain 1. Cases with PCR product lengths equivalent to Jijoye were interpreted as containing EBV strain 2.

Specificity of the PCR reactions was confirmed by hybridization with oligonucleotide probes. Gels were denatured (0.2 mol/L NaOH, 0.5 mol/L NaCl), neutralized (4X TBE, 1X TBE) and DNA transferred onto nylon membranes (Hybond N+, Amersham, UK) by electroblotting at 200 V for 2 hr. Nonspecific binding was blocked by incubation of the membrane in prehyb solution (0.5% sodium dodecyl sulphate [SDS], 4XSSC, 5X Denhardt's and 200 µg/ml denatured salmon sperm DNA) for 60 min at 42°C. Following hybridization with 10 pmoles of ³²P-5' end labelled probe for a minimum of 4 hr at 42°C, membranes were washed at RT with 2XSSC, 0.1% SDS and 1XSSC, 0.1% SDS and exposed to Kodak autoradiographic film.

5- Immunohistochemistry: Five micron thick sections of the studied samples were cut onto sialinized slides, air-dried overnight at room temperature (RT), dewed in xylene and rehydrated through graded alcohol. Slides were incubated for 10 min in 3% H₂O₂ to abolish endogenous peroxidase activity and the antigen retrieval method was performed. The primary antibody was added at a working concentration and incubated for 2 hr at RT. After washing, all sections were immunostained with the universal labeled streptavidin-biotin method (Vector) according to the manufacturer's instructions. Positive staining was detected with 0.3% 3,3'-diaminobenzidine tetrahydrochloride in DW and nuclei were counterstained with hematoxylin.

The following antibodies were used: the antihuman Bcl-2 mouse monoclonal antibody (Dako Ltd., Cambridge, United Kingdom), the antihuman Bax polyclonal antibody (Biogenex,

Biogenex, San Romano, CA), the monoclonal antibody (DO7, Biogenex, San Romano, CA) against the wild/mutant type *p53* and the monoclonal Ki-67 (MIB1, Dako Ltd., Cambridge, United Kingdom). Sections obtained from a lymph node with follicular lymphoma, a normal tonsil and a case of invasive breast carcinoma were used as positive control for bcl-2, Bax as well as for *p53* and Ki-67/MIB1, respectively. Negative controls were obtained by substituting the primary antibody by bovine serum albumin. Bcl-2 and Bax positive cells were identified by the presence of brown cytoplasmic reactivity whereas *p53* and Ki-67 positive cells were identified by the presence of brown nuclear staining. Scoring of the bcl-2, Bax and *p53* immunoreactivity results was performed as follows: (-), <10% immunostaining; (+), 10-25% stained cells; (++) , 25-50% stained cells and (+++) , > 50% stained cells [5]. Scoring of the Ki-67 was done by determining the percentage of positive cells in 1000 cells (proliferation index, PI).

6- Follow-up: The clinical outcome and the survival data were obtained from the patients' records. Tumor response to therapy, disease-free (DF) and overall survival (OAS) rates were assessed. The follow-up period was calculated from March 1995 to December 2001.

7- Statistical Methods: Proportions were compared with Fisher's exact test for unordered or ordered categorical variables. The impact of different variables on survival was determined using the Cox proportional hazards model. *p*-values less than 0.05 were considered significant. The Kaplan-Meier method was used to create survival curves which was analyzed by the log-rank test.

RESULTS

Clinical and histological features of patients: Out of the 50 PELHN cases examined 22 were diagnosed as diffuse large (DL) B cell lymphoma, 5 Burkitt's lymphoma, 10 peripheral T cell lymphoma, 8 anaplastic large cell lymphoma, 2 cases of MALT, 2 small lymphocytic lymphoma and a case of lymphoblastic lymphoma (Table 1). Patients were categorized into pediatric (<18 years, 8 patients) and adult (>18 years, 42 patients) age groups. The age ranged from 4-79 years (mean = 47.8 and median = 45 years). In the pediatric age group, the majority

of cases were BL (5 cases), whereas in the adults, the majority of cases were DL (20 cases). In 30 patients, the lymphoma was confined to the site of origin whereas 20 cases showed regional lymph node metastasis, 12 of them revealed disseminated disease after the staging work-up mainly to the liver, blood, CNS and skin. Local destruction of the anatomical structures with extension into the surrounding tissues were evident in 5 cases of nasal and nasopharyngeal lymphoma only.

Immunophenotyping of lymphoma: Out of the 50 cases analyzed, 31 (62%) revealed the expression of B-cell surface markers and 19 (38%) revealed the expression of T-cell surface markers. Ten out of the 19 cases with T-cell surface markers (20%) were also positive for the NK-specific marker, CD56. These cases showed the characteristic morphological features of nasal/ nasal type lymphoma (Table 1 & Fig. 1-B,C,D).

EBER ISH revealed the presence of EBV in 90% of the tumor samples analyzed (45/50). All positive cases contained hybridization signal in more than 95% of tumor cells. Positive signals were localized to the nuclei of the neoplastic cells in a relatively even distribution throughout the section (Fig. 1-E). EBER positive cells ranged from small to large size with round or irregular nuclei. Non-neoplastic epithelia or glandular epithelia were seen in some biopsies and were uniformly negative for EBER except in some cases from the NP which showed focal positivity in some samples.

PCR amplification of viral sequences: Revealed the presence of EBV in 70% of the cases (35/50). All cases of nasal NK/T cell lymphoma (10 cases) were positive for EBV infection. The concordance between ISH and PCR was 70% (35 cases). Out of these cases, 10 were in the nose and the maxillary sinus, 9 in the tonsils, 9 in the NP, 4 in the oral cavity, and 3 in the OP. EBV was also detected in 18/45 (40%) of the normal samples examined; 10 NPT and 8 normal TWs.

Each isolate reported in this study was analyzed for LMP1 30 bp deletion and EBNA2 strain typing. All EBV positive cases harbored type 1 (Fig. 2-A). A single dominant EBV population with del-LMP1 gene product was found mainly in cases of lymphoma. Out of the 35 tumor samples positive for EBV-DNA, del-

LMP1 genotype was found in 24 cases (68.6%), the wt-LMP1 product predominated in 7 cases (20%) whereas mixed del- and wt-LMP1 variants were detected in 4 cases (11.4%) (Figure 2-b). All nasal NK/T cell lymphoma cases revealed del-LMP1 variant only. In normal tissues, a wt-LMP1 genotype predominated in 50% of the samples (9/18), 3 of them were NPTs and 6 were normal TWs. A single del-LMP1 band was detected in 2 cases (11.1%) of NPTs, whereas mixed infection with wt- and del-LMP1 variants was present in 6 cases (38.9%) (4 NPTs and 2 TWs). There was a significant difference in the frequency of del-LMP1 and wt-LMP1 between tumor and normal tissues (68.6% vs. 11.1% $p < 0.001$ and 20% vs 50%; $p = 0.03$ respectively). However, there was no significant relation between the presence of del-LMP1 and any of the clinicopathological parameters studied.

The results of IHC: Are illustrated in fig. (1-F,G,H,I) A high Ki-67 index was reported in 35/50 (70%) cases. All cases with a high index were EBV+. p53 overexpression was detected in 24/50 (48%) cases, 23 of them were EBV+ ($p < 0.001$). On the other hand, a high expression level of bcl-2 protein was reported in 10 cases (20%) and reduced expression of Bax protein was detected in 9 cases (18%) all were B-cell lymphomas (Table 2).

There was a significant association between p53 overexpression and T phenotype and between the expression level of bcl-2, Bax and B phenotype ($p < 0.001$ & 0.004 respectively). A significant relationship was also noticed between p53 overexpression and lymph node involvement, advanced disease stage, poor performance status and the incidence of locoregional recurrence ($p < 0.001$). Similarly, a high Ki-67 index was significantly associated with advanced

disease stage ($p < 0.001$) (Table 1).

The association between the presence of EBV infection, p53 overexpression and a high Ki-67 index was statistically significant ($p = 0.01$) as well as the association between EBV and the expression of bcl-2 and Bax in B-cell lymphoma cases ($p < 0.001$).

Estimates of survival calculated using Kaplan-Meier analysis showed that p53 overexpression, a high Ki-67 index, advanced disease stage, T phenotype and the presence of del-LMP1 were poor prognostic factors (Table 3, Fig. 4). In Multivariate analysis, only p53 overexpression, advanced disease stage and the presence of del-LMP1 were poor prognostic factors.

During a median follow up of 20 month, (range 1-72), 19/30 patients (63.3%) with available clinical, pathological and virology data responded to therapy. The initial treatment of all patients consisted of chemotherapy (a combination of CHOP, vincristine, prednisone, cyclophosphamide, doxorubicin), local radiotherapy or a combination of both. Whereas 11/30 patients (36.7%) had local, regional or systemic relapse. Locoregional relapses occurred in 7 cases whereas systemic failure occurred in 4 cases. Nineteen of the 30 patients (63.3%) responded to therapy, 10 (33.3 %) with complete remission (CR) and 9 (30%) with partial remission (PR) whereas 11 cases (36.7%) revealed disease progression (DP). Treatment with a combination of radio- and chemotherapy (11 cases) resulted in a CR in 8 cases, partial remission in 2 cases and disease progression in one case. In contrast, CR was seen in only 1/10 of the patients who were initially treated with radiotherapy alone and another case that was treated with chemotherapy alone.

Fig. (1-A)

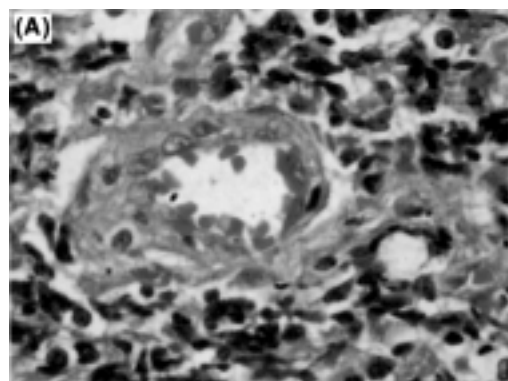


Fig. (1): Hematoxylin and eosin-stained section for a case of polymorphic, medium-sized T-cell lymphoma showing angiocentricity (a), positive immunostaining for CD20 in a case of B-cell lymphoma (b), positive immunostaining for CD45-RO in a case of T-cell lymphoma with angioinvasion (c) and a case of nasal NK/T cell lymphoma with CD56 expression on the atypical cells (d). EBER-in situ hybridization in a case of nasal NK/T cell lymphoma (e) with strong signals localized to the nuclei of virtually all tumor cells. A case of T cell lymphoma showing high Ki-67 proliferation index (f) and marked overexpression of p53 (g). Positive immunostaining for bcl-2 (h) and Bax (i) proteins in cases of B-cell lymphoma.

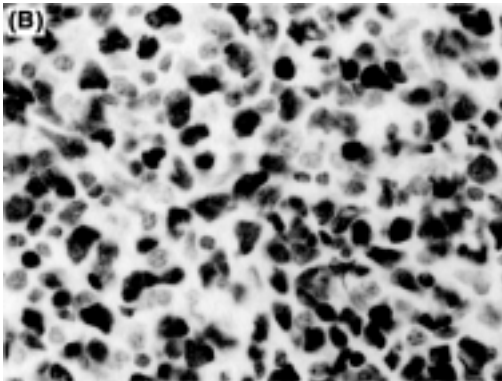


Fig. (1-B)

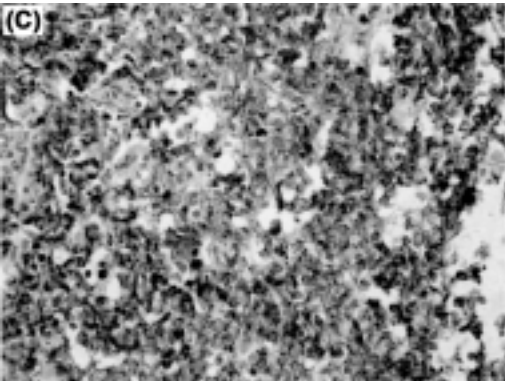


Fig. (1-C)

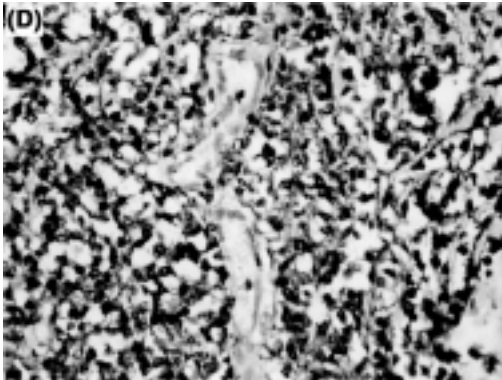


Fig. (1-D)

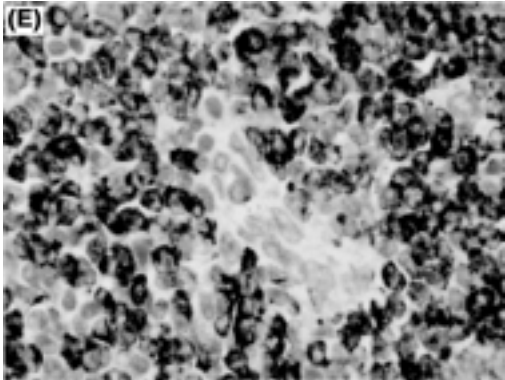


Fig. (1-E)

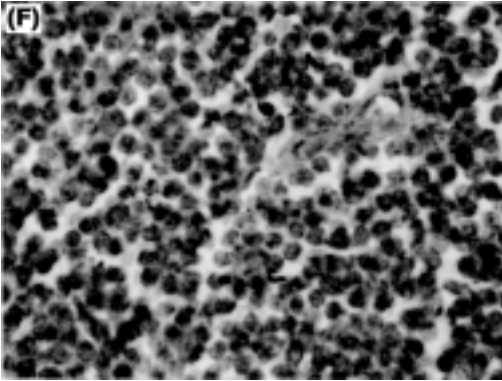


Fig. (1-F)

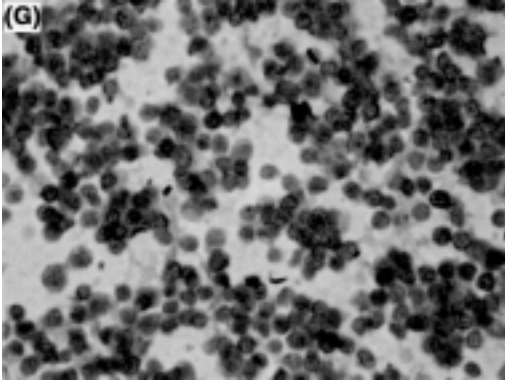


Fig. (1-G)

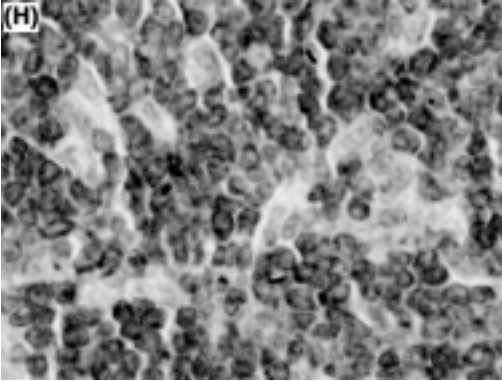


Fig. (1-H)

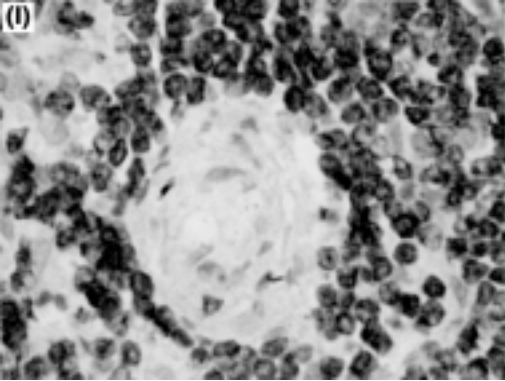


Fig. (1-I)

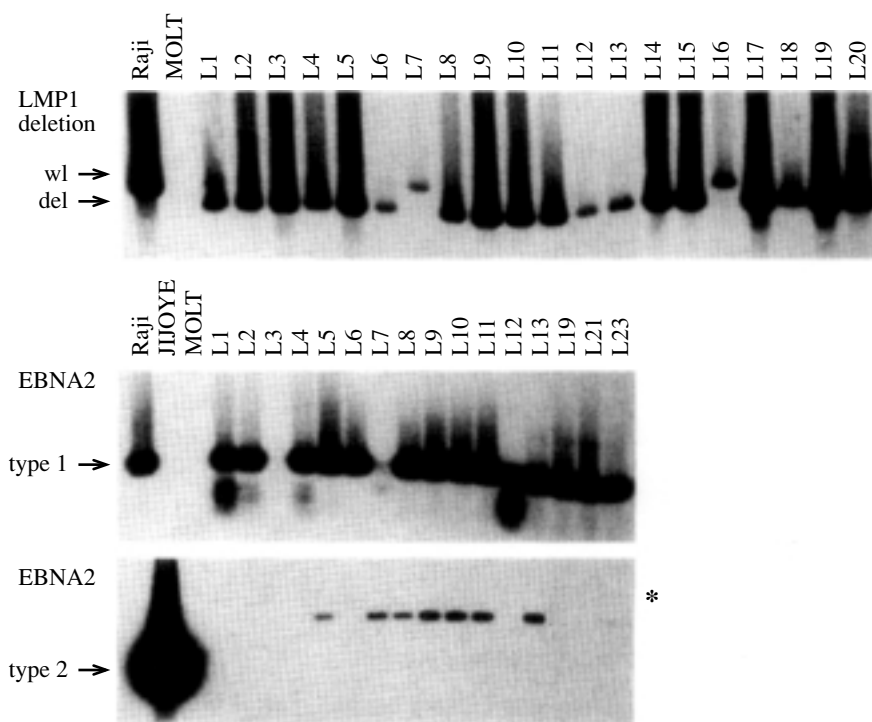


Fig. (2): EBV genome analysis in cases of PELHN. Raji and Jijoye were reference type 1 and 2 cell lines. The MOLT cell line served as negative control in all assays. Each case is associated with a single dominant virus strain which is consistent with the clonal nature of the tumor. (a) The majority of cases show a c-terminal 30 bp LMP1 deletion, case no. 7 and 16 show the wt-LMP1 and case no. 1 contains both the del- and wt-LMP1. (b,c) EBNA 2 strain typing shows that all lymphoma cases were of type 1.

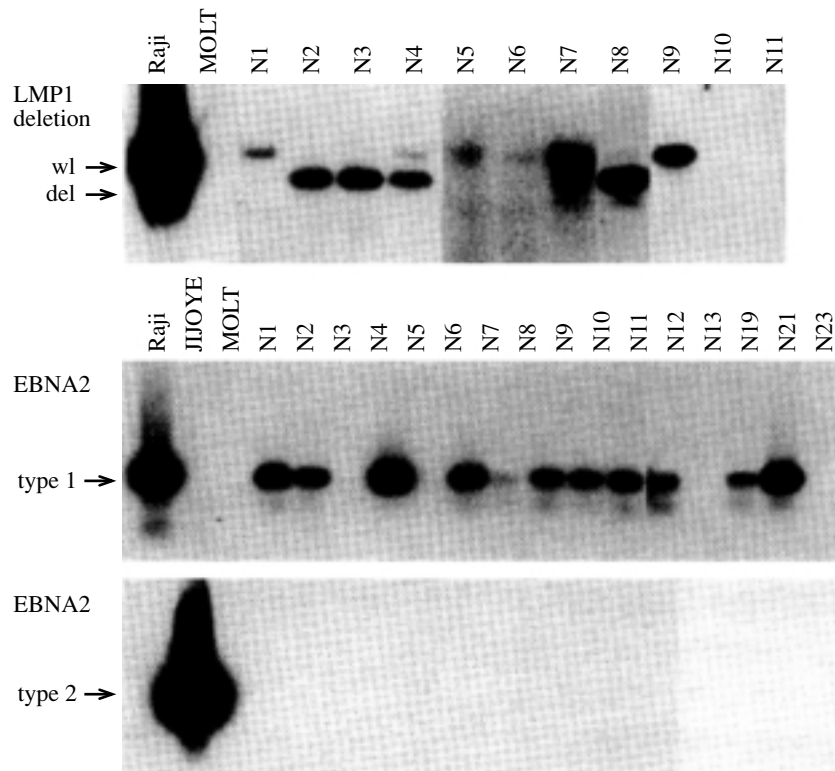


Fig. (3): EBV genome analysis in normal nasopharyngeal tissues and throat washings. EBNA 2 strain typing shows that all cases were of the type 1 (a,b). PCR at the LMP1 gene locus revealed an unexpected extent of diversity. The wt-LMP1 predominated in 4 cases (Lanes no. 3,7,8,11), the del-LMP1 variant predominated in 3 cases (lanes no. 4,5,10) whereas both the del- and wt-LMP1 were detected in 2 cases (Lanes no. 6,9) (c).

Fig. (4): Kaplan-Mayer curves showing reduced overall survival rates in cases with p53 overexpression (a), advanced disease stage (b) and del-LMP1 variants (c).

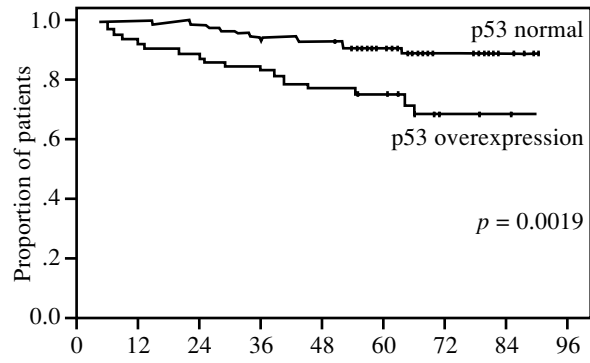


Fig. (4-A): Overall survival.

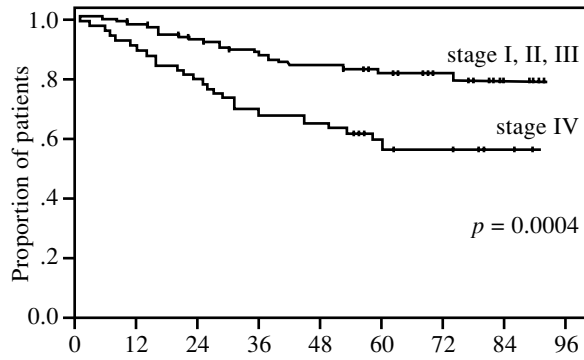


Fig. (4-B): Overall survival.

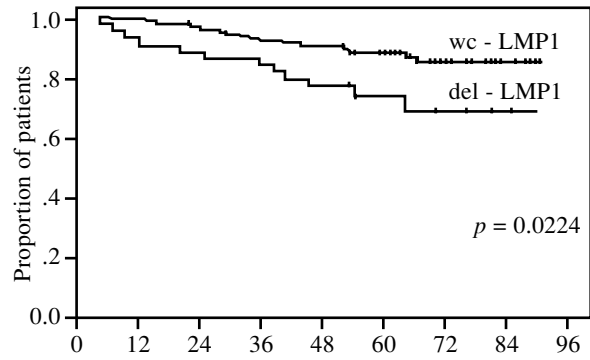


Fig. (4-C): Overall survival.

Table (1): Association between p53 overexpression and clinicopathological features of patients.

Clinicopathological features	No.	p53 overexpression	p
<i>Age:</i>			
Pediatric (<18 years)	8	2	0.063
Adults (>18 years)	42	22	
<i>Sex:</i>			
Male	35	18	0.434
Female	15	6	
<i>Site:</i>			
Nose& nasal sinuses	11	8	0.855
Nasopharynx	11	7	
Tonsils	13	3	
Oropharynx	7	4	
Oral cavity	8	2	
<i>Histopathology:</i>			
<i>B cell:</i>			
Diffuse large (CD20,CD45,CD79acy)	22	12	0.081
Burkitt's (CD45,CD10,CD20,CD79acy)	5	2	
MALT (CD45,CD20,CD79acy)	2	0	
Small lymphocytic (CD45,CD20,CD79acy,CD5)	2	1	
<i>T cell:</i>			
Peripheral T (CD45,CD45-RO,CD3,CD4,CD8)	10	5	
Anaplastic large (CD45,CD45-RO,CD3,CD5,CD4,CD8)	8	3	
Lymphoblastic (CD45, CD45-RO, CD3,CD5,CD4,CD8)	1	1	
<i>Immunophenotyping:</i>			
B-cell	31	9	< 0.001
T-cell	19	15	
<i>Lymph node involvement:</i>			
Present	20	17	< 0.001
Absent	30	7	
<i>Stage:</i>			
I&II&III	32	10	< 0.001
IV	18	14	
<i>Performance status:</i>			
1	4	1	0.543
2	26	6	
3&4	19	14	

* p.value is significant < 0.05, Numbers are too small for a valid statistical significance.

Table (2): Presence of EBV infection in relation to expression level of Ki-67, p53, bcl-2 and bax proteins.

EBV	Ki-67		p53		bcl-2		Bax	
	Low (n = 15)	High (n = 35)	Normal (n = 26)	Overexpression (n = 24)	Normal (n = 40)	Overexpression (n = 10)	Normal (n = 41)	Absent (n = 9)
Positive (n = 45)	10 (66.7%)	35 (100%)	22 (84.6%)	23 (95.8%)	35 (87.5%)	10 (100%)	36 (87.8%)	9 (100%)
Negative (n = 5)	5 (33.3%)	0	4 (15.4%)	1 (4.2%)	5 (12.5%)	0	5 (12.2%)	0
<i>p</i> -value	< 0.001		< 0.001		0.065		0.072	

* *p*.value is significant < 0.05.

Table (3): Overall survival in relation to prognostic factors and the tested markers.

Predictive variables	Univariate analysis				Multivariate analysis		
	MS	HR	CI	<i>p</i>	HR	CI	<i>p</i>
<i>Age:</i>							
< 18	16						
≥ 18	18	1	0.99-1.02	0.851			
<i>Sex:</i>							
Female	15						
Male	12	1.1	0.74-1.67	0.614			
<i>Site:</i>							
Nasopharynx (ref.)	20						
Nose and sinuses	18						
Tonsils	16						
Oral cavity	14						
Oropharynx	18	1.08	0.75-1.57	0.675			
<i>Immunophenotyping:</i>							
B	20						
T	9	1.54	1.04-2.28	0.033	2.546	0.907-7.150	0.0760
<i>Lymph nodes:</i>							
Negative	15						
Positive	10	1.1	0.731-1.55	0.650			
<i>Stage:</i>							
I, II, III	23						
IV	6	1.96	1.66-2.32	< 0.001	7.331	2.696-19.940	< 0.001
<i>Performance status:</i>							
1,2	14						
3,4	18	0.96	0.27-1.76	0.445			
<i>IMPI:</i>							
Non deleted	19						
Deleted	4	1.34	1.20-1.50	< 0.0224	13.88	1.012-190.57	< 0.001
<i>P53:</i>							
Normal	22						
Overexpression	8	1.36	2.67-19.94	< 0.002	7.331	2.696-19.940	< 0.001
<i>Ki-67:</i>							
Low	20						
High	10	7.82	1.01-60.12	0.0481	0.696	0.274-1.766	0.4449
<i>bcl-2:</i>							
Normal	12						
Overexpression	10	4.63	0.85-2.519	0.075			
<i>Bax:</i>							
Normal	14						
Absent	11	1.82	0.636-2.243	0.261			

MS: Median survival.
HR: Hazard ratio.CI: 95% confidence interval.
* *p*.value is significant < 0.05.

Table (4): Clinicopathological features, EBV infection and LMP1 deletion in relation to the clinical outcome of 30 patients with PLEHN.

No.	Age/ sex	Site	Histology	Stage	EBV	Del-LMP1	Treatment	Follow-up/ OAS	DFS
1	65/M	NP	DL	IIA	+	+	RT-P	DP/1M	0
2	65/M	NP	DL	IB	-	-	RT-C	CR/A	18M
3	47/M	T	DL	IA	+	+	CT&RT-C	PR/ 7M	0
4	11/M	N&NP	Burkitt's	IVB	+	+	CT-C	DP/3M	0
5	6/M	NP	Burkitt's	IVB	+	+	CT-C	DP/1M	0
6	29/M	NP	DL	IVA	+	+	CT&RT-C	CR/12M	6M
7	59/M	NS	DL	Relapsing	+	-	CT&RT-C	CR/A	12M
8	43/F	NS	DL	IIEA	+	+	CT-C	PR/9M	4M
9	35/M	OC	DL	IA	-	-	CT-C	PR/27M	12M
10	7/M	NS	Burkitt's	IVA	-	-	CT-C	PR/22M	20M
11	50/F	T	DL	IVB	+	+	CT&RT-P	CR/ 3M	3M
12	58/F	T	MALT	IA	+	+	CT-C	DP/3M	0M
13	52/F	OC	DL	IB	+	+	CT-C	DP/1M	0M
14	65/F	T	DL	IIIB	+	+	CT-C	DP/1M	1M
15	45/M	OP&NP	Peripheral T	IVA	+	+	CT&RT-P	CR/3M	3M
16	36/M	OC	DL	IB	+	+	CT-C	PR/15M	8M
17	61/M	NP	DL	IA	+	+	CT&RT-P	CR/2M	0
18	49/M	T	DL	IIIA	+	+	CT-C	DP/4M	1M
19	15/m	NP	DL	IVA	-	-	CT&RT-C	CR/A	20M
20	60/	N&S	DL	IIIB	+	+	CT-C	PR/19M	12M
21	40/F	NS	DL	IB	+	+	CT-C	DP/7M	3M
22	45/M	OP	Peripheral T	IA	+	+	CT-C	DP/2M	0M
23	70/F	T	DL	IIIB	+	+	CT&RT-P	DP/2M	1M
24	28/F	T	DL	IIA	+	+	CT-C	CR/A	5M
25	60/F	OP	DL	IIIA	+	-	CT&RT-C	PR/A	6M
26	35/	OC	DL	IVB	-	-	CT&RT-P	CR/A	25M
27	58/M	T	DL	IIIB	+	+	CT-C	DP/6M	2M
28	39/M	N&S	DL	IIIA	+	+	CT&RT-C	CR/19M	14
29	27/M	N&S	Peripheral T	IIIB	+	+	CT-C	PR/11M	5M
30	43/F	T	MALT	IA	+	-	CT-C	PR/A	33M

CR: Complete remission.
CT: Chemotherapy.

PR: Partial remission.
PT: Radiotherapy.

DP: Disease progression.
C : Curative.

A: Alive.
P: Paliative.

DISCUSSION

Data regarding the association between EBV and different types of lymphoma in immunocompetent patients is still scarce and controversial. In 1999, Calzolari et al. [13] demonstrated that EBV infection is highly associated with NHL of the head and neck in HIV-infected patients and claimed that the situation in non-infected patients is less clear. Similarly, Leong et al. [16] reported all immunosuppressed patients with large B-cell oral lymphoma had EBV infection compared to 9% only of immunocompetent patients. However, Solomides et al. [19] reported a high frequency of EBV infection in oral lymphoma in immunocompetent patients (14% of B-cell and 36% of pleomorphic T-cell lymphoma).

In Egypt, this controversy was similarly recognized. Abdel-Wahab and Abul-Ela, [20] reported the presence of EBV capsid antigen in 13.3% and 10% of Egyptian Burkitt's and non-Burkitt's lymphoma respectively but in none of the reactive lymphoid hyperplasias. A comparable frequency (7.7%) was reported by Mokhtar et al. [21] in 52 cases of Egyptian NHL not including Burkitt's lymphoma, whereas a much higher frequency was reported by Anwar et al. [22] and Assem et al. [23] in cases of Egyptian Burkitt's lymphoma (73%) and HD (97%), respectively.

Several studies tried to give an explanation for this controversy in the frequency of EBV infection in lymphoma patients. Among the mentioned causes were the sensitivity of the detection method, the way of sampling, the nature of the studied samples (fresh or paraffin), the clinicopathological features of studied cases (including type, grade, stage and site of lymphoma) as well as the frequency of EBV in the normal population under study [8].

In the present study EBV infection was detected in 90% and 70% of the studied cases using two highly sensitive techniques; EBER-ISH and PCR, respectively. The relatively high frequency of EBV infection in normal Egyptian population could provide an explanation since EBV DNA was detected in 40% of the normal samples.

The difference between the results of EBER ISH and PCR has been previously reported and

attributed to the difficulty in obtaining a good quality, amplifiable DNA from paraffin-embedded tissues, the heterogeneity of tumors, the ratio of tumor/normal cells, the presence of EBV particles in normal lymphocytes and the way of interpretation of the results [8].

Our results regarding the presence of EBV infection in all cases of nasal NK/T cell lymphoma are in agreement with previous studies which also showed that the virus is monoclonally integrated in the tumor cells. This confirms the etiological role of EBV in this type of lymphoma [7,19].

The frequency of del-LMP1 gene in the present study (68.6%) is within the universally-reported range. del-LMP1 gene was detected in 86% of NHL cases from Taiwan [4], 61% and 100% of Danish and Malaysian peripheral T cell lymphoma [24] and in 71% of aggressive NHL from USA [11]. In this work, del-LMP1 was also detected in normal tissues obtained from healthy individuals; however the frequency was lower than in tumor tissues and was usually mixed with the wild type variant. This finding is in agreement with Kingma et al. [11] and Chiang et al. [8]. It could therefore be postulated that, del-LMP1 may have a more potent tumor promoting activity than the full-size gene which confirms the occurrence of strain selection in neoplastic tissues. A recent *in vitro* study showed that, del-LMP1 is associated with 30% to 70% prolongation of the half life of the mutant protein [25]. Since LMP1 expression within human B lymphocytes induces DNA synthesis, abrogates apoptosis and plays an important role in the initiation and/or maintenance of the immortalized state, it is possible that the effects of LMP1 may be accentuated through loss of a genome segment required for its rapid turnover. Alternatively, cells with del-LMP1 may express an altered protein product with decreased immunogenicity for EBV-specific cytotoxic T cells. These EBV-infected cells may escape immunosurveillance thereby acquiring a survival advantage and the possibility for further mutagenic events [8]. However, Chang et al. [4] and Khanim et al. [17] mentioned that, there is no strain-selection in EBV-associated lymphoma since the frequencies of del-LMP1 in their studied cases were comparable. The difference in the sources of normal tissues was mentioned as an explanation for these conflicting results [8].

Khanim et al. [17] used lymphoblastoid cell lines (LCLs) established from healthy virus carriers by spontaneous outgrowth of peripheral B lymphocytes whereas normal NPT and TW were directly analyzed in this study and that of Chiang et al. [8]. The use of LCLs may underestimate the extent of EBV diversity in normal virus carriers due to selection of the dominant or the more transforming virus strains [10].

However, an important question whether EBV strains with del-LMP1 are preferentially associated with neoplastic transformation is still to be answered. The frequency of del-LMP1 variants in normal population is well established and it is still unclear whether its association with EBV-related malignancies is due to the predominance of del-LMP1 carrying EBV strains or is related to an increased transformation function. Khanim et al. [17] have mentioned that, although EBV isolates carrying del-LMP1 are found in a similar proportion of healthy EBV carriers and EBV associated tumors, it remains possible that this variant may influence the efficiency of malignant transformation leading to the increased incidence of EBV-associated tumors in regions where this gene polymorphism is common. Therefore, analysis of EBV genome in tumors and normal tissues will highly aid in solving this problem.

The present work demonstrates that *p53* overexpression is a frequent event in PELHN with a significant impact on the clinical outcome of patients. Similarly, Calzolari et al. [13] reported frequent inactivation of *p53* in EBV-associated head and neck lymphoma. They demonstrated that EBV infection and/or LMP1 expression lead to loss of function of the *p53* gene even in the absence of mutations either through enhanced degradation of the cellular protein or increased half-life by a viral protein-*p53* interaction. Also, Petit et al. [15] and Quintanilla-Martinez et al. [12] showed that *p53* overexpression is common in nasal NK/T cell lymphoma and is usually associated with large cell morphology, advanced disease stage and poor response to therapy [13-15].

Data regarding the expression of apoptosis regulatory genes in EBV-associated lymphomas is still conflicting. Assem et al. [23] failed to find any association between *bcl-2* expression and the presence of EBV in Egyptian cases of HD. In contrast, Preciado et al. [26] and Quin-

tanilla-Martinez et al. [12] reported *bcl-2* overexpression in HD-Reed Sternberg (RS) cells in Russia, Mexico and Argentina. Moreover, Bousset et al. [27] showed a significant association between *bcl-2* and Bax overexpression in Hodgkin's RS cells demonstrating that the absence of Bax may confer a growth advantage and play a central role in the pathogenesis of HD. Our results show a significant association between *bcl-2* and Bax as well as between the expression level of these two proteins and B phenotype. However we did not find an association between the expression level of these two proteins and the presence of EBV.

This controversy in the data could be explained by the heterogeneity of the studied samples, the detection method for both EBV and apoptosis regulatory genes, the degree of competence and clinical status of the patients, an existing genetic and/or racial difference as well as the strain typing of EBV [23]. It was mentioned that, as the two EBV strains differ in the level of BHRF1, the EBV homologue of the human *bcl-2* gene, they also differ in the EBV nuclear antigen, EBNA 2,3,4,6. This viral antigen contributes to the immortalizing function of EBV and the biological difference between type 1 and type 2 [23]. Previously published studies showed an obvious discordance between subtype 1 and *bcl-2*. Whereas Japanese patients with HD were exclusively of type 1 with del-LMP1, patients from Argentine did not show any prevalent strain. It was shown that, all the Japanese patients demonstrated discordant expression between *bcl-2* and EBV compared to 40% of the Argentine patients [26,28]. Since all our cases are of the type 1, this could explain the lack of any significant relationship between the presence of EBV and the expression level of *bcl-2* and/or Bax proteins.

In addition, recent studies show that, EBV targets apoptosis not only through the *bcl-2* pathway but also via other unidentified pathways [25]. Also a subset of EBV latent gene products can inactivate cell cycle checkpoints in G2/M for monitoring the fidelity and timing of cell division and genomic integrity. The *bcl-2* is not involved in this pathway since its level does not change significantly during apoptosis. Recently, cyclins and cyclin dependent kinases were mentioned as possible candidates in this pathway [29,30].

We conclude that, EBV infection is frequent in Egyptian PELHN. EBV was detected in all cases of nasal NK/T cell lymphoma which confirmed its etiological role in this type of lymphoma. del-LMP variants were preferentially selected over wt-variants and *p53* was found to be a poor prognostic factor in PELHN since its overexpression was highly associated with an advanced disease stage, the presence of other lymph node metastasis as well as with a higher tendency for recurrence and reduced survival rates. Similarly, an advanced disease stage and the presence of del-LMP variants are poor prognostic factors that are significantly associated with reduced survival rates.

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Table 1: Association between p53 Overexpression and Clinicopathological Features of Patients.