Detection of Epstein-Barr virus Latent Membrane Protein-1 in Pediatric Lymphoma: A Report from South Egypt Cancer Institute

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Abstract:

Background and objectives: Pediatric lymphoma is known to be associated with Epstein–Barr virus (EBV) infection. The objectives were to detect the frequency of EBV infection in pediatric lymphomas in our locality and to study its relationship with the clinicopathological characteristics of the patients.

Methods: In this descriptive cross-sectional study, we examined 78 consecutive cases of pediatric lymphomas for the presence of EBV in tumor cells by real-time Polymerase Chain Reaction on paraffin blocks for latent membrane protein-1 (LMP-1) over 3 years (January 2012 –January 2015). We collected data of the patients with pathology-proven primary lymphoma including age, sex, histologic subtype, and risk stratification.

Results: The most common subtype of pediatric lymphomas was NHL (52/78, 66.5%), while HL was diagnosed in 33.5%. EBV LMP-1 gene detection was found in 38.5% of HL, and 28.8% of NHL cases. EBV infection was significantly related to age, gender, and histological subtype in NHL cases; however, it was related to age only in those with HL. The overall difference in EBV LMP-1 gene detection was statistically significant regarding the age at presentation ($P < 0.001$).

Conclusion: The frequency of EBV infection in pediatric lymphomas in our locality is higher compared to Western countries, but it is lower than in endemic areas. Younger age at diagnosis was the most significant factor associated with EBV infection.

Keywords: Pediatrics, Epstein–Barr virus, Lymphoma, South Egypt Cancer Institute.

Introduction:

Epstein Barr Virus (EBV) belongs to the genus Lymphocryptoviridae, the gamma 1 subtype of the subfamily Gammaherpesvirinae and is one of the most common viruses in humans. It infects more than 95% of all individuals within the first four decades of life. The age of the primary infection is influenced by socioeconomic and developmental factors [1]. In developing countries, infections occur very early in life causing no specific characteristics other than the general symptoms of acute viremia. However, in developed countries, the infection is usually delayed till adolescence or early adulthood period where it causes a benign self-limiting lymphoproliferative disorder known as infectious mononucleosis (IM) [2].

The virus has been demonstrated to be implicated in the development of many malignancies with the list of such malignancies progressively increasing [2]. The

worldwide estimated number of new cases of Burkitt lymphoma (BL) and Hodgkin lymphoma (HL) was about 92,000, 51% of which have an association with EBV infection [3]. During EBV latent infection, EBV expressed proteins are mostly immunogenic. They can induce potent immune responses in immunocompetent individuals [4] The virus in the target cells expresses various patterns of latency genes and these determine the type of cancer that would be developed. The establishment of latent infection by EBV has been involved in several malignancies due to the expression of different sets of latent proteins, shown to play various biological roles. Of those, the LMP-1 gene is an essential oncogene, which is expressed as a constitutively active receptor in a majority of EBV-associated tumor cells [1]. Persistent EBV infection can serve as a perfect target for the treatment of EBV-associated lymphomas and the improvement of patient outcomes [3]. The frequency of this linkage varies both between countries as well as within the same country. It also varies within each histologic subtype of lymphoma, gender, and age at the time of diagnosis. The scarcity of studies on the association between EBV infection and lymphoma among children in our locality prompted us to conduct this study to detect the frequency of EBV infection in pediatric patients with lymphoma and the association between it and clinicopathological features of those patients at South Egypt Cancer Institute (SECI), Assiut University.

Methods:

This descriptive cross-sectional study was conducted at Pediatric Hematology and Oncology Department, Pathology Department, and Clinical Pathology Department, SECI, Assiut University. The study included 78 pediatric patients (≤ 18 years) diagnosed with lymphoma [HL or non-Hodgkin lymphoma (NHL)] during the period from January 2012 to January 2015. The study was approved by SECI institutional ethical committee.

The patients' hospital files were reviewed to collect the required data including demographics (age and gender), clinical presentation, histopathology subtype, staging, and risk stratification of the patients. The study will clarify EBV LMP-1 positivity and its relationship to the clinical characteristics of the patients included.

DNA extraction and real-time PCR

The genomic DNA was extracted from fixed paraffin-embedded tissues using the QIAamp DNA Mini kit (Qiagen, CA, USA) according to the manufacturer's instructions. The paraffin-embedded tissues were cut from each block and deparaffinized with xylene followed by ethanol washing. The samples were suspended in 50 µl TE buffer, containing 10 mM Tris-HCL (pH 8.0) and 1 mM EDTA (pH 8.0). The Real Star® EBV PCR Kit 1 .2 (Altona Diagnostics GmbH, Hamburg, Germany) consists of two master reagents A and B which contain PCR buffer, DNA polymerase, magnesium salt, primers, and probes. The amplicon 104 bp of EBV conserved LMP1 segment was targeted by the primers (LMP1-forward 5'-CAGTCAGGCAAGCCTATGA-3' and LMP1-reverse 5'-CTG GTT CCG GTG GAG ATGA-3') and probe (5'- (6FAM)GTC ATA GTA GCT TAG CTG AAC (TAMRA)-3') according to the established method [5]. The working master mix is composed of 15ul (5ul of master A and 10ul of master B) and 10ul of extracted DNA or the controls (quantification standards (QS1-QS4), positive or negative control) added to the Light Cycler® capillary. Thoroughly mix the samples and controls with the master mix by pipetting up and down then close the capillary using the appropriate lid and centrifuge at 2000 rpm/30sec. The real-time PCR thermal profile was programmed in Light Cycler® 2.0 machine (Roche, Germany) as following one cycle of initial incubation at 95°C/2min after that applying 45 cycles of 95°C/5sec, 60°C/30sec (data collection at this step) and 72°C/10sec. The result interpretation was carried out in the kit manual of instruction.

Statistical analysis

Data were verified, coded by the researcher, and analyzed using SPSS version 24 [6]. Descriptive statistics: means, standard deviations, medians, ranges, and percentages were calculated. Test of significances: for categorical variables: Chi-square/Monte Carlo Exact (MCE) test was used to compare the frequency between groups. For continuous data, an independent t-test analysis was carried out to compare the means of normally distributed data. A p-value equal to or less than 0.05 was considered significant.

Results:

Seventy-eight cases of pediatric lymphoma were included in this study; the median age at diagnosis was 6 years (ranging between 1.3-16 years). Male to female ratio of 1.5. Most cases (66.5%) were NHL, with the most common clinical presentation was abdominal mass (53.8%) followed by mediastinal and peripheral lymphadenopathy (26.9 and 15.4% respectively). Eighty-eight percent of NHL cases had advanced stages versus 34.6% of HL cases. The nodal disease was documented in all cases of HL (64.7% cervical, 41.2% axillary, 29.4% abdominal, and mediastinal involvement in 14.7%) with only 5/26 (19.2%) of them had an extra nodal involvement.

Regarding NHL cases, the EBV LMP-1 gene was detected in 15/52 cases (28.8%). The median age was 5 years, with a highly significant difference between LMP positive (3.5 years) and negative cases (6 years) (P= < 0.001). Furthermore, we noticed male predominance with male to female ratio was 1.7; with a high ratio in EBV LMP-1 positive cases (6.5) in comparison with negative cases (1.2), the difference was significant (P= < 0.04). Forty-four percent of BL and 36.4% of DLBL had positivity to EBV LMP-1 gene; however, no cases of T cell NHL were EBV positive; with a high statistical difference (P= < 0.001) (Table 1).

As demonstrated in (Table 2), the median age of classical Hodgkin lymphoma (CHL) cases was 8.5 years. Male to female ratio was 1.2, and the most

common histological subtype was mixed-cellularity (MC) (17/26) followed by nodular-sclerosis (NS) subtypes (7/26). Most cases were stratified as intermediate- risk (IR) (50%) and high-risk (HR) (26.9%) groups. Of twenty-six HL cases, 38.5% were screened positive for EBV LMP-1 (n=10) with a median age was significantly lower than negative cases (6 versus 9.5 years) (P= < 0.003). EBV LMP-1 gene was positive in 41.2% of MC, 28.6% of NS, and 50% of lymphocytic predominant (LP) histological subtypes, with no significant statistical difference.

For all pediatric lymphoma patients, EBV LMP-1 gene positive cases show a highly significant association with younger age at presentation (P= < 0.001), but not for gender and type of lymphoma (P= 0.221 and 0.445 respectively) (Table 3).

Table 1: Clinical characteristics of non-Hodgkin Lymphoma patients and their relation to Epstein–Barr virus infection (n=52)

	Total (%) (n = 52)	**LMP-1 positive (n = 15)**	**LMP-1 negative (n = 37)**	**P value**
Age/years				
• **Mean ± SD**	5.81 ± 2.6	4.13 ± 2.7	6.84 ± 2.1	**< 0.001***
• **Median (Range)**	5 (1.3 – 14)	3.5 (1.3 - 13)	6 (3.5 - 14)	
Sex				
• **Male**	33 (63.5%)	13 (39.4%)	20 (60.6%)	**= 0.040****
• **Female**	19 (36.5%)	2 (10.5%)	17 (89.5%)	
• **Male/Female Ratio**	1.7	6.5	1.2	
Histology				
• **BL**	25 (48.1%)	11 (44%)	14 (56%)	
• **DLBL**	11 (21.2%)	4 (36.4%)	7 (63.6%)	**= 0.001****
• **Anaplastic L**	2 (3.8%)	0 (0%)	2 (100%)	
• **LBL**	14 (26.9%)	0 (0%)	14 (100%)	
Tumour Stage				
• **II**	6 (11.6%)	2 (33.3%)	4 (66.7%)	= 0.575**
• **III**	35 (67.3%)	12 (34.3%)	23 (65.7%)	
• **IV**	11 (21.1%)	1 (9.1%)	10 (90.9%)	
Risk Stratification				
• **Intermediate**	41 (78.8%)	13 (86.7%)	28 (75.7%)	= 0.216**
• **High**	11 (21.2%)	2 (13.3%)	9 (24.3%)	

*Independent Sample t-test was used to compare the difference in mean between groups

**Chi-square test was used to compare the difference in frequency between groups

BL; Burkitt's lymphoma, DLBCL; Diffuse large B cell lymphoma, LPL; Lymphoblastic lymphoma, LMP; Latent membrane protein**.**

Table 2: Clinical characteristics of Hodgkin Lymphoma patients and their relation to Epstein–Barr virus infection (n= 26)

	Total (%) (n = 26)	**LMP-1 positive (n = 10)**	**LMP-1 negative (n = 16)**	**P value**
Age/years				
• **Mean ± SD**	8.67 ± 2.8	6.45 ± 2	9.8 ± 2.2	**< 0.003***
• **Median (Range)**	8.5 (5 – 16)	6 (5 - 12)	9.5 (5 - 16)	
Sex				
• **Male**	14 (53.8%)	5 (35.7%)	9 (64.3%)	= 0.461**
• **Female**	12 (46.2%)	5 (41.7%)	7 (58.3%)	
• **Male/Female Ratio**	1.2	1	1.3	
Histology				
• **MC**	17 (65.4%)	7 (41.2%)	10 (58.8%)	= 0.419***
• **NS**	7 (26.9%)	2 (28.6%)	5 (71.4%)	
• **LP**	2 (7.7%)	1 (50%)	1 (50%)	
Tumour Stage				
• **I**	4 (15.4%)	1 (25%)	3 (75%)	
• **II**	11 (42.3%)	5 (45.5%)	6 (54.5%)	= 0.575***
• **III**	8 (30.8%)	3 (37.5%)	5 (62.5%)	
• **IV**	3 (11.5%)	1 (33.3%)	2 (66.7%)	
Risk Stratification				
• **Low**	6 (23.1%)	2 (33.3%)	4 (66.7%)	= 0.528**
• **Intermediate**	13 (50%)	6 (46.1%)	7 (53.8%)	
• **High**	7 (26.9%)	2 (28.6%)	5 (71.4%)	

*****Independent Sample t-test was used to compare the difference in mean between groups
**Chi-square test was used to compare the difference in frequency between groups
***MCE test was used to compare the difference in frequency between groups
MC; Mixed-cellularity, ***NS***; Nodular-sclerosis, ***LP***; Lymphocytic-predominance**, *LMP*; *Latent membrane protein*.**

Table 3: Epstein-Barr virus latent membrane protein-1 in relation to clinicopathological criteria of pediatric lymphoma (n= 78)

	LMP-1 positive (n = 25)	**LMP-1 negative (n = 53)**	**P value**
Histopathology			
• **HL**	10 (38.5%)	16 (61.5%)	= 0.445*
• **NHL**	15 (28.8%)	37 (71.2%)	
Age/years			
• **Mean ± SD**	5.2 ± 2.3	7.5 ± 2.8	**< 0.001****
• **Median (Range)**	5 (1.3 - 13)	7 (3.5 - 16)	
Gender			
• **Male**	18 (37.5%)	30 (62.5%)	= 0.221*****
• **Female**	7 (23.3%)	23 (76.7%)	

*Chi-square test was used to compare the difference in frequency between groups
**Independent Sample t-test was used to compare the difference in mean between groups
HL; Hodgkin lymphoma, NHL; Non- Hodgkin lymphoma, LMP; Latent membrane protein

Discussion:

Lymphomas, a diverse group of immune-cell malignancies, constitute

the third most common cancer in childhood and adolescents, accounting

for approximately 15% of all malignancies. They have been grouped into two histological subtypes; HL and NHL with varying distribution geographically [7, 8]. In our institute, lymphoma was the second most common malignancy after leukemias accounting for 17.3% (10.5% for NHL and 6.8% for HL) [9].

EBV is one of the factors linked to the multistep pathogenesis of these neoplasms. It can express different patterns of latency genes which can subsequently determine the type of cancer that would be developed [1].

In the current study, most of the cases were diagnosed as NHL, which occurred in 66.5% of the cases, with BL being the predominant histological subtype, which is comparable to other studies in Africa and the Middle East [10–12]. HL constituted about one-third of pediatric lymphomas, MC was the most frequent histological subtype reported which agrees with the studies of Ghazaly et al and El Badawy et al in Egypt [13, 14].

The frequency of NHL EBV positive samples was 28.8%, which is consistent with many studies [2, 15], while it was lower than reports from endemic areas [12, 16]. EBV association in NHL varied according to age and sex, and the virus was statistically distributed in boys < 5 years old, which is similar to other studies [11, 17].

The major types of NHL linked to EBV are BL and DLBCL [18]. One of the most striking characteristics of pediatric BL is the variation in the frequency of EBV positivity in different geographic regions [16]. BL can be classified into three subtypes, endemic BL, sporadic BL, and Human Immunodeficiency Virus (HIV)-associated BL. The “high incidence” endemic BL (eBL) found in equatorial Africa and New Guinea is about 95% EBV-positive, whereas, in other parts of the world, sporadic BL (sBL) varies from 20%–to 80% EBV-positive depending on the geographical region with the lowest virus association occurred in Western countries [19]. HIV-BL (iBL) was quite different in pediatrics that showed an interesting 100% EBV association, higher than the typical 30-40% reported for adults [11]. In the current study, 44% of BL samples were LMP-1 positive which agrees with many studies by Ismail et al and Chabay et al [11, 20].

DLBCL makes up 10-20% of pediatric NHL [21]. Like BL, the pediatric form of EBV LMP-1 +ve DLBCL has marked geographical differences which are profoundly influenced by socioeconomic conditions. Sporadic cases were described in some Asian, African, and Latin American countries, while, it is extremely rare in Western countries and is usually associated with immunodeficiency [22]. In this study, 4/11 (36.4%) of the samples of DLBL had an EBV association which is concurrent with the observations of Peh et al (38%) and Cohen et al (40%) [23, 24], however, it is less than reported by the study of Nomura et al (25%) [25].

On screening of EBV LMP-1 in HL tumor samples, it was higher than reported in NHL cases, which coordinates with the studies of Sughayer et al and Bağır et al [15, 26]. This association was higher than reports from Europe and the United States and much lower than the underdeveloped countries in Africa and Latin America which may reach 100% of cases [27]. This higher incidence of EBV-positive HL may be due to underlying immunosuppression or may be associated with the timing of EBV infection which occurs very early in life in developing countries with an increased risk of developing HL 2 to 5 times compared to individuals who had no prior history [2].

The median age of HL EBV positive cases was significantly lower than negative cases, the same was observed by Chabay et al in Brazil, with the majority of EBV +ve cases occurring in boys < 10 years of age [11].

EBV is detected in Hodgkin Reed-Sternberg (HRS) cells in ∼40% of CHL in the Western world, mostly in cases of MC and lymphocyte-depleted subtypes, and less frequently in NS and LP classical subtypes [28]. Although many studies reported a high association between EBV+ ve cases with the prevalence of MC subtype reaching high frequencies up to 94% [29, 30], EBV +ve cases in our study were found to have no significant difference regarding histological subtypes distribution, which is similar to what was reported by Barros et al [31]. Furthermore, for clinical stages and risk groups, there was no significant relation to LMP-1 gene positive cases which is in agreement with findings reported by Claviez et al [32].

Finally, documented EBV infection had no significant relationship with either gender or the type of lymphoma, only younger age at presentation was found to have a strong relation to EBV positive cases, which agrees with Zareifar et al [17]. These findings may be related to early exposure to EBV infection in developing countries and its association with lower socioeconomic status [33].

Conclusion:

This study was the first to report the association between pediatric lymphoma and EBV in our region. The frequency of EBV infection in pediatric lymphoma patients in our center was as high as in developing countries but still lower than in endemic areas. Certain factors are associated with EBV infection such as age, gender, and histological subtype. Further prospective epidemiological studies will be needed involving different centers and different genetic viral assessments to investigate the evidence of the presence of EBV in lymphoma tissues because its presence may have a significant impact on prognosis and response to therapy, and may aid in targeted therapy.

List of Abbreviations

EBV	Epstein-Barr Virus
LMP-1	Latent Membrane Protein-1
HL	Hodgkin Lymphoma
NHL	Non- Hodgkin Lymphoma
BL	Burkitt's Lymphoma
DLBCL	Diffuse Large B-cell Lymphoma
MC	Mixed- Cellularity
NS	Nodular-Sclerosis
LP	Lymphocytic Predominant

Authors' Contributions

All authors made substantial contributions to the conception or design of the work, acquisition, analysis, or interpretation of data.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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