Role of Epstein Barr Virus in the Attack of Malignant Lymphoma

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Abstract

Objectives Epstein Barr Virus (EBV) infection is closely associated with occurrence of lymphoma; however, its pathogenesis remains unclear. The study investigates the relationship between EBV infection and chromosomal abnormalities in lymphoma and immunophenotyping. We also evaluated the effect of EBV on the prognosis of lymphoma. Methods Fluorescence quantitative polymerase chain reaction was used to detect the copy numbers of EBV-DNA from bone marrow in 14 cases of Hodgkin Lymphoma (HL), 136 cases of patients with Non-Hodgkin Lymphoma (NHL), and 37 cases of hematologically healthy control subjects. Results EBVDNA copies were found in 49 of 150 (32.7%) patients with lymphoma and 2 of 37 healthy control subjects (P<0.001). The EBV infection rate of NHL patients, HL patients and healthy controls were 32.4% (44/136), 35.7% (5/14), and 5.4% (2/37), respectively. The positive rates of EBV in the NHL and HL groups was higher than that in healthy subjects (P<0.05). Chromosome karyotype analysis indicated that the EBV infection rate in NHL patients with chromosomal abnormalities was 44.4% (4/9). No statistical significance was found between chromosome abnormality and EBV infection (P>0.05). In addition, EBV+ - lymphoma exhibited higher relapse and mortality rates, compared with EBV-lymphoma. Conclusion we conclude that EBV infection is correlated with the incidence of lymphoma and the infection rate of EBV shows no statistical significance in B-lymphoma and T-lymphoma. No statistical correlation between EBV infection and chromosomal abnormalities is indicated. EBV positive patients showed an unfavorable prognosis.

Keywords: Malignant lymphoma; Chromosome; Epstein Barr virus; FQ-PCR; Immunophenotyping

Introduction

In 1964, Epstein and Barr first discovered the Epstein Barr virus, an omnipresent human herpes virus [1]. More than 90% of adults and about 50% to 89% of children have been infected with EBV [2]. Generally, EBV infects B lymphocytes (B cells), and subsequently persists in a quiescent state in resting memory B cells to establish long-term infection [3,4]. Numerous studies have indicated that EBV is associated with various human diseases, such as, Infectious Mononucleosis (IM), Nasopharyngeal Carcinoma (NPC), and gastric cancer [5-7]. An increasing number of studies also suggest that EBV can infect T lymphocytes (T cells) and Natural Killer lineage cells (NK cells) during primary infection, such as Chronic Active EBV infection (CAEBV) and EBV-associated Hemophagocytic Lymphohistiocytosis (HLH) [7,8].

Malignant Lymphoid neoplasms (ML) comprise a group of distinct entities with widely varying clinical features, histology, immune phenotypes, and genetic abnormalities. The WHO classification of lymphoid neoplasm encompasses Hodgkin lymphoma and Non-Hodgkin Lymphoma (NHL) [9]. Malignant lymphoma comprises 3.37% of all malignancies worldwide [10]. Despite its largely undetermined etiology, lymphoma is suggested to stem from interactions between various exposures, inherited susceptibility, and opportunistic infections. The same cell type in lymphoma and lymphocytic leukemia lead to the same disease but with different clinical manifestations and stages; in addition, they share similar biological characteristics but have different lesion sites [11]. Thus, studies have consistently focused on the relationship between EBV infection and lymphoma. Specifically, the close correlation between Burkitt's Lymphoma (BL) and EBV has been recognized; however, the relationship with other lymphomas remains uncertain [12]. No literature has been reported on the causal relationship between EBV infection and immunophenotyping of lymphoma and chromosomal aberrations. Therefore, we distinguished EBV-DNA copies to investigate EBV
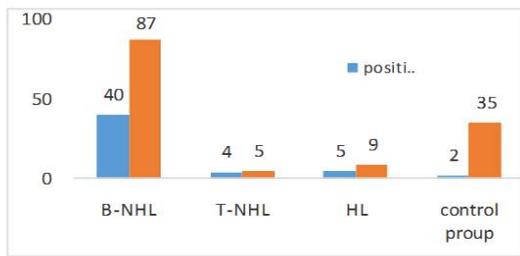
Figure 1: Distribution of EBV infection in different malignant lymphoma subtypes.
EBV infection in B-NHL, T-NHL, HL and control group was 31.5%, 44.4%, 35.7%, 5.4%, respectively. EBV infection in NHL and HL groups were higher than in healthy subjects. There was no statistical correlation between B-NHLs and T-NHLs ($\chi 2$=0.6, p>0.05).
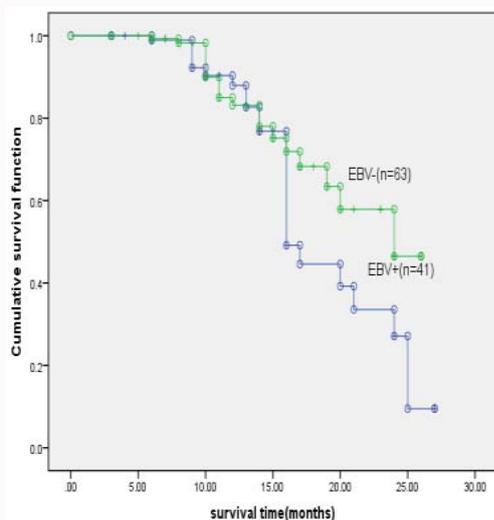

Figure 2: Kaplan-Meier survival curves in follow-up patients with ML according to EBV-DNA copies. Patients with EBV infection show a significantly shorter survival time from those without EBV infection. Analysis by Kaplan-Meier technique and log-rank test.

infection in the immunophenotyping of lymphoma and explored whether EBV infection is associated with chromosomal aberrations.

Materials and Methods

Patients

We obtained bone marrow samples from 150 patients (72 males and 78 females), including 136 patients with NHL and 14 patients with HL. All patients came from the Affiliated Hospital of Qingdao University (Qingdao, China), from January 2013 to December 2016. The diagnosis of lymphoma was established based on recent diagnostic criteria of WHO (2008), including morphology, immunology, cytogenetic and clinical characteristics. In addition, 37 cases of healthy individual bone marrow were collected as normal control. In the present study, "health" is defined as the absence of any type of blood system disease and other cancers. The mean age of the patient was 51 years (range 15 years to 79 years), and the mean age of the control group was 43 years (range 17 years to 73 years). The male: female ratios in the study and control groups were 1:1.08 and 1:1.06, respectively. The study was approved by the Ethics Committee of the Qingdao University. All recruited cases were informed of the study, and a content letter was signed before the sample was collected.

DNA extraction

Proteinase K digestion and phenol chloroform methodology were used extract DNA from bone marrow samples. At room temperature, 2 mL of bone marrow was rinsed with PBS 3 times in 15 mL reaction tubes. Subsequently, 1 mL of SENT lysis buffer (150 mM NaCl, 10 mM Tris HCl, 10 mM EDTA, 0.1% SDS) and 35 µL proteins K (100 µg/mL) were added, and the sample was digested for 2 hat 55°C. The list was extracted twice with phenol-chloroform. DNA was precipitated with cold anhydrous ethanol and then transferred to a 1.5 mL Eppendorf tube, dried at room temperature and then resuspended in 80 µL double-distilled water. The purity and quantity of extracted DNA were determined using a nucleic acid protein detector. The purified DNA was either used immediately or stored at -80°C.

Determination of the copy number of EBV-DNA

The copy number of EBV-DNA was measured by real-time Fluorescence Quantitative Polymerase Chain Reaction (FQ-PCR) (Shanghai Hongshi Medical Technology Co., Ltd, China) using an EBV-PCR Fluorescence Quantitative diagnostic kit (DaAn Gene, China). During PCR, the specific Bam HIW sequence of the EBV genome was amplified. The key to real-time PCR specific reaction were primers that included two chains, one forward 5'-CCCAACACTCCACCACACC-3', and one reverse: 5'-TCTTAGGAGCTGTCCGAGGG-3'. The dual-labeled fluorescent probe was as follows:5'-(FAM)-CACACACTACACACACCCACCCGTCTC-(TAMRA)-3'. The detection process was conducted strictly in accordance with the instructions provided by the manufacturers. The standard curve was constructed by using different concentration gradients of positive quantitative standards (1×10^4 copies/ml, 1×10^5 copies/ml, 1×10^6 copies/ml, and 1×10^7 copies/ml). The dynamic curve and the fluorescence standard curve of EBV-DNA nucleic acid amplification were automatically displayed by the detection system. All specimens were assayed in duplicate. The lower limit of detection was 1000 copies/ml, and EBV-DNA copy numbers under this limit was considered EBV negative.

Cytogenetic analysis

The karyotype of bone marrow cells was analyzed using the R-band technique after a culturing period of 24 hrs. We analyzed 20 cells in metaphase. In addition, the karyotype was described using the International System for Cytogenetic Nomenclature (1995).

Immunophenotyping

The immunophenotype in bone marrow samples was determined by Flow Cytometry (FC 500 MPL, Beckman Coulter, USA). Analysis of the immunophenotype by FCM with reference to the World Health Organization (2000) Classification of malignant lymphoma [13]. The gating strategy was the use of CD45 plot versus side scatter, and the expression of various surface and cytoplasm markers were analyzed in the population. The expression of antigens in less than 20% was considered negative; in addition, the expression of antigen in more than 50% was considered positive, and the expression of antigen in 20% ~ 50% was considered suspicious positive [14]. The kappa/lambda ratio of greater than 3 or less than 0.5 is monoclonal,

Table 1: Markers of B and T lineage NHL.

Lineage	Markers
B	CD10,CD19,CD20,CD23,cCD79a
T	CD2,CD3,CD4, CD5,CD7,CD8

Table 2: Clinical features and biological characteristics of malignant lymphoma patients [n(%)].

Patients [n(%)]			
	EBV+	EBV-	*P*-value
Total number of ML case	49	101	<0.001
Age (years)			
Mean	50.1	52	NS
Range	15-79	18-72	NS
Gender			
Male	29 (19.3)	61 (40.7)	NS
Female	20 (13.3)	40 (26.7)	
EBV-DNA copies/ml DNA (n)			
Median	33540	-	
Range	5640 - 48700000	-	
Mean ± SD	1372992 ± 6988691	-	
Karyotypic analysis (n)	49	101	
NHL group(n)	44	92	NS
Normal	40 (29.4)	87 (64.0)	
Abnormal	4(3.0)	5 (3.7)	
HL(n)	5	9	NS
Normal	5 (35.7)	9 (64.3)	
Abnormal	0	0	
White blood cell count, WBC(×10⁹/L)			
Median	11.5	8.9	NS
Range	2.2-70.1	1.8- 69.7	
Mean ± SD	14.0 ± 11.5	10.8 ± 8.9	
Platelets, PLT (×10⁹/L)			
Median	56	51	NS
Range	13-231	13-263	
Mean ± SD	65.2 ± 44.4	67.2 ± 52.5	
Hemoglobin, Hb (g/L)			
Median	64	61	NS
Range	30.0 -139.0	29.0 -160.0	
Mean ± SD	68.7 ± 25.5	67.4 ± 28.0	
Lymphocyte count (×10⁹/L)			
Median	14.5	9.8	NS
Range	1.02-109.5	1.2-106.7	
Mean ± SD	18.6 ±19.4	14.6 ± 14.8	
Clinical follow-up in ML	41	63	
Deaths	3 (7.3)	2 (3.2)	NS
Relapse	23 (56.1)	20 (31.7)	<0.05
Hepatomegaly	4 (9.8)	2 (3.2)	NS
Splenomegaly	9 (22.0)	4 (6.3)	NS

supporting B-NHL. The panel of markers used is listed in Table 1 [15,16].

Statistical analysis

Experimental data were analyzed using SPSS ver. 21.0 (IL, Chicago); P<0.05 indicates statistical significance. The Kolmogorov-Smirnov (K-S) test checks whether the continuous variable obeys the normal distribution. Comparison of quantitative data was analyzed using two nonparametric tests (Mann-Whitney U-test) on independent samples. Pearson's χ2 test or Fisher's exact test was used to analyze quantitative data. The relationship between EBV infection and survival rate of patients with lymphoma were determined using the log rank test. The statistical characteristics of continuous variables were described using medians, arithmetic means, standard deviations, and minimum and maximum values.

Results

EBV infection of lymphoma

EBV infection was detected in 49 of 150 patients with lymphoma, comprising an infection rate of 32.7% (49/150). It was also detected in 2 of 37 patients from the control groups, comprising an infection rate of 5.4% (2/37). The incidence of EBV in patients with lymphoma was significantly higher than that in the normal healthy group ($\chi2=9.8$, $p<0.05$). In different types of lymphoma, the distribution of EBV-DNA copy numbers is as follows: 44 of 136 (32.4%) NHL, 5 of 14 (35.7%) HL (Figure 1). The incidence of EBV in NHL and HL groups was significantly higher than that in the healthy control group ($\chi2=10.8$, $\chi2=7.9$, $p<0.05$). The distribution of EBV-DNA copy numbers followed a positively skewed distribution (skewness of 6.748 and kurtosis of 46.461 by K-S test). Patient characteristics are shown in Table 2.

EBV infection and chromosome karyotype

Chromosome analysis or karyotyping indicated that 6% (9/150) of patients with lymphoma had an abnormal karyotype. Moreover, 4 of 49 patients with EBV-positive lymphomas and 5 of 101 patients with EBV-negative lymphomas were found to have chromosomal abnormalities. No statistical correlation between EBV infection and chromosomal abnormalities was indicated ($\chi2=0.6$, $p>0.05$) (Table3).

EBV infection and immuophenotyping of NHL

The 136 cases of NHL included 127 B-NHLs (40 for EBV+) and 9 T-NHLs (4 for EBV+) according to flow cytometry results. No statistical correlation between B-NHLs and T-NHLs was indicated ($\chi2 = 0.6$, $p>0.05$). The distribution of EBV infection in NHL subtypes was as follows: 40 of 127 (31.5%) B-NHLs and 4 of 9 (44.4%) T–NHLs (Figure 1).

Clinical follow-up and other related factors

At the end of 2016, 104 patients (41 EBV+ and 63 EBV-) can be used for clinical follow-up in this study. The results indicated that the relapse rates of the EBV+ and EBV- groups were 56.1% (23/41) and 31.7% (20/63), respectively. Mortality rates were 7.3% (3/41) and 3.2% (2/63) in the EBV+ and EBV- groups, respectively. The EBV+ group had a higher relapse than the EBV- group ($\chi2=6.1$, $p<0.05$), and the mortality of both groups exhibited no statistical correlation ($\chi2=0.9$, $p>0.05$). The study also showed a significant correlation between EBV infection and survival of patients with lymphoma (Figure 2).

Discussion

In 1964, Epstein and Barr first discovered EBV in children with Burkitt's lymphoma in Africa; several studies on EBV were then implementing [1]. EBV belongs to the gamma herpes family and maintains lifelong infection in immune competent individuals, which can induce a broad spectrum of malignant tumor through multiple mechanisms. Malignant lymphoma can be divided into two categories: Hodgkin's Lymphoma (HL) and Non-Hodgkin's Lymphoma (NHL). HL is one of the most important types of lymphatic hematopoietic tumor. It often occurs in Western developed countries in Europe and North America. Studies have shown that more than 30% HL is associated with EBV infection in Europe [4]. In 1987, Weiss et al. [17] first detected EBV-DNA in HL tissues, and the detection rate of EBV-DNA was 20% to 50%. Wu [18] confirmed the relationship between EBV and HL by immunohistochemistry and EBER in situ hybridization, which detected the EBV virus in R-S cells. However, the detection rate of EBV-positive HL varies in different regions; for

instance, 65% in Japan, about 50% in the United States [19, 20], and 48% to 57% in China [21]. The results indicate that the detection rate of EBV in HL by FQ-PCR is 35.7%. The incidence of EBV in the HL group was significantly higher than that in the normal control group. This study suggests that EBV infection plays an important role in HL, which is consistent with previous studies. However, the functional mechanism of EBV in HL is currently undetermined. Recent research demonstrated that the pathogenesis of EBV [22] is as follows: (1) EBV infection causes the chromosomal mutation or translocation of lymphocytes to induce the activation and over expression of c-myc oncogenes, ultimately leading to lymphoma; (2) EBV infection causes the apoptosis of Fast-mediated apoptosis in lymphocyte lines and the expression of the apoptotic inhibition gene bcl-2; lymphocyte apoptosis becomes inhibited, and lymphoma occurs. In this experiment, no abnormal chromosomes were detected in HL. Thus, no relationship between EBV infection and chromosomal abnormalities in HL can be established. This lack of relationship might be attributed to the sample capacity of HL being too small and the possibility that the bone marrow might not have been invaded by the lymphoma cells.

In this study, we found that the rate of positivity to EBV in NHL was 32.4%; no significant difference in the incidence of EBV between NHL and HL was indicated. This result varies to a certain degree from those of other studies [23]. This difference may be attributed to the low amount of HL collected. In the subsequent experiment, more HL samples will be collected. EBV mainly infects B lymphocytes via the receptor CD21 [24], but increasing evidence that EBV can also infect T lymphocytes, natural killer cells, and mononuclear macrophages [7]. In the current study, FCM was used to type NHL, and the results showed that the EBV expression rates were 31.5% in B-NHLs and 44.4% in T-NHLs; no significant difference was observed between the two. Thus, no difference in the pathogenicity of EBV was observed between the B and T cell types of NHL. Studies show that specific chromosomal aberrations play a vital role in the occurrence and development of NHL. Cytogenetic analysis has become a highly useful method to determine acquired chromosomal abnormalities in the diagnosis of NHL. Approximately 90% of NHL has chromosomal abnormalities [25]. BL was first described by Burkitt in 1958 as an aggressive lymphoma [26]. Approximately more than 90% of endemic BL is associated with EBV infection [5]. Chromosomal translocation between chromosome 8 and either chromosomes 2, 14, or 22 are reportedly the important characteristics of BL. Translocation occurs when the c-myc that lies on the long arm of chromosome 8 transfers to chromosomes 2, 14, or 22, resulting in over expression of c-myc [27]. Recent research using Fluorescence in Situ Hybridization (FISH) and molecular genetics techniques suggests that EBV infection contributes to the genomic instability of BL and NPC. In addition, chromosomal abnormalities have been observed in EBV-associated HLH and CAEBV by FISH and cytogenetic analysis [28]. However, in the current study, we failed to find evidence of EBV influence on chromosomal aberrations in patients with NHL. Some subtypes of genetic changes may not be diagnosed by conventional cytogenetic analysis. Thus, cytogenetics, combined with FISH and molecular genetics, is required in new experiments with larger sample sizes to elucidate the possible role of EBV infection in NHL-associated chromosomal aberration. In addition, lymphoma cells may not invade the bone marrow.

EBV infection is reported as a potential factor in the progression of Acute Lymphoblastic Leukemia (ALL) [29]. The current study

Table 3: Karyotype and EBV infection in patients with NHL.

Sno	Gender	Age	Type	Abnormal karyotype	EBV
1	M	73	B-NHL	46, XY, t(9; 22) (q34; q11) [8]/53, XY, idem, +4, +5, +6, +8, +14, +22, +der(22) t (9; 22) [2]	+
2	F	59	B-NHL	complex karyotypes aberrations [4]/46, XX [16]	+
3	F	66	B-NHL	47, XX, del(9) (q11, q12),+18 [3]/46, XX [7]	-
4	M	64	B-NHL	45, X, -Y [5]/46, XY [15]	+
5	M	63	B-NHL	45, X, -Y [9]/46, XY [11]	-
6	M	74	B-NHL	45, XY, -4 [8]/46, XY [12]	+
7	F	66	B-NHL	46, XX, del(15) (q21, q22) [2]/46, XX [18]	-
8	F	48	B-NHL	Karyotype of near tetraploid with structural abnormalities [3]/46, XX [17]	-
9	M	44	B-NHL	47, XY, +X [10]	-

demonstrated a higher relapse rate and poorer prognosis in lymphoma patients with EBV infection. These findings are similar to those in previous studies in which the EBV-DNA load in CLL is an independent predictor of clinical course and survival [30]. The study also showed significant correlation between lymphoma-associated death and EBV-DNA copies. These findings are consistent with the conclusions by Grywalska et al. [31], which suggested that high viral loads of EBV-DNA in the peripheral blood of patients with CLL is associated with an unfavorable prognosis. Regardless, more detailed clinical and biological materials are needed to determine whether EBV infection is associated with poor clinical outcomes in patients with malignant lymphoma.

In conclusion, EBV is the main pathogenic factor for malignant lymphoma; however, its pathogenesis remains unclear. No apparent relationship is exhibited between EBV infection and chromosome abnormalities in lymphoma and immunophenotyping. In addition, EBV infection leads to poor prognosis of lymphoma. EBV-positive patients showed an unfavorable prognosis. To understand the pathogenesis of EBV in malignant lymphoma, further studies have to be conducted.

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