

The study of correlation between EB virus with Chronic lymphoblastic leukemia

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Abstract: To investigate the relationship between EBV infection and the occurrence and development of chronic lymphoblastic leukemia (CLL), and to provide a reliable basis for revealing the close relationship between EBV infection and CLL. Fluorescence quantitative polymerase chain reaction (FQ-PCR) was used to detect the copy numbers of EBV-DNA from the bone marrow of 80 CLL patients. 40 healthy persons were control group and 40 cases for clinical follow-up. The results showed that the EBV positive rates (EBV+) of CLL patients and healthy controls were 25% (20/80) and 7.5% (3/40) respectively. Chromosome analysis showed that the rate of EBV+ in CLL patients with chromosome abnormalities was 18.75% (3/16), while the rate of EBV- was 81.25% (13/16), and no significant difference was observed between chromosome abnormality and EBV infection ($2=0.21$, $p>0.05$). Clinical follow-up showed that the relapse rate mortality rate of EBV+ and EBV- groups within 5 years were 55.6% (10/18), 28.6% (12/42) and 44.4% (8/18), 4.7% (2/42), respectively ($p<0.05$). ML-EBV+ had higher relapse and mortality rates compared with ML-EBV-. 6 patients with EBV-positive CLL progressed to diffuse large B-cell lymphoma (DLBCL). EBV infection may be related to the occurrence and development of CLL. EBV infection is not conducive to the prognosis of CLL. And EBV may participate in the transformation of CLL to DLBCL.

Keywords: Chronic lymphoblastic leukemia; Chromosome; Epstein-Barr virus; FQ-PCR; Immunophenotyping

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1. Introduction

The Epstein-Barr virus (EBV) was first identified 40 years ago in cultured Burkitt's lymphoma cells when no human lymphoid cell had ever been maintained in culture[1]. It is also known as the human herpes virus 4, is a ubiquitous DNA virus belonging to the gamma subfamily of herpes viruses[2]. More than 90% of the adults and about 50%-89% of children worldwide are infected with EBV[3]. Primary infections are usually asymptomatic, and only when it is delayed into adolescence or adulthood, a benign lymphoproliferative disorder of infectious mononucleosis (IM) can occur. The main site in which EBV persists in the body is late-infected B cells[4], which are characterized by resting memory B lymphocytes. Under normal circumstances, EBV can establish a persistent infection in the body without affecting the behavior of B lymphocytes[5]. To do this, the virus has evolved an elegant strategy based on subtle development of almost all aspects of B cell physiology. The end result of the interaction of EBV with the infected host is the establishment of a non-pathogenic latent infection of memory B lymphocytes that allows the virus to last a lifetime. Studies have shown that, in particular, the presence of the EBV genome and the sustained expression of viral proteins strongly support the involvement of EBV in the pathogenesis of a wide range of human malignancies. Several studies showed the association of EBV infection with nasopharyngeal carcinoma (NPC), Burkitt's lymphoma (BL), Hodgkin's

lymphoma (HL), EBV-associated hemophagocytic lymphohistiocytosis (HLH) and chronic active EBV infection (CAEBV)[6-9]. EBV can be considered as a prototype of a carcinogenic virus as a direct transformant. In fact, in classical EBV-associated tumors, the viral genome is present in almost all tumor cells, which display viral RNA and protein expression, which have different effects on the induction of the transformed phenotype. Based on these characteristics and strict association with different tumor types, EBV is classified as a Class I carcinogen. Another convincing factor was the detection of a homogeneous (clone) EBV epistasis using viral end-sequencing in several EBV-associated tumors (HL, NPC, BL) and some pre-neoplastic lesions. These findings indicate that these tumors are developing from a single cell infected with EBV prior to growth in vitro and are consistent with the role of EBV in the early stages of tumor development.

Chronic lymphocytic leukemia (CLL) is a mature B lymphocyte clonal proliferative tumor characterized by the accumulation of lymphocytes in peripheral blood, bone marrow, spleen and lymph nodes[10]. CLL is the most common adult leukemia in Europe and the United States, accounting for nearly 30% of all leukemias. CLL occurs mainly in the elderly. At the time of initial diagnosis, 85% of patients are over 55 years old, with a median age of 72 years. CLL is relatively rare in Asian countries including China, with a median age of onset of about 65 years. Genetic factors play a more important role in the pathogenesis of CLL than environmental factors. The incidence rate of males is

higher than that of females, and the ratio of male to female is 1.5~2:1. CLL also is a disease characterized by the accumulation of non-differentiated small lymphocytes, most of which are in the G0/G1 phase of the cell cycle due to defects in apoptosis[11]. CLL cells are small lymphoid B cells with a cytoplasm that is sparse and has a regular outline. The nucleus contains clusters of chromatin, which usually does not exist. Although chronic lymphocytic leukemia (CLL) is defined as chronic, it is characterized by significant heterogeneity[12,13]. Only 30% of patients survived within 10-20 years[14]. The remaining CLL patients develop end-stage within 5-10 years, although the incidence is lower. Individuals with invasive CLL survive no more than 2-3 years[15]. The reason for this uneven natural history is unclear. Also, the etiology of CLL remains unclear.

Thus, the aim of this study was to define a role of EBV in the etiopathogenesis of CLL. The detailed objectives included the determination of the EBV-DNA copy number in mononuclear cells and isolated B lymphocytes from peripheral blood of CLL patients, healthy individuals, the analysis of association between this parameter, prognostic factors, stage of the disease, and its clinical manifestation. Our work was to study the relationship between EBV infection and CLL by detecting EBV-DNA content in patients' bone marrow cell.

2. Materials and Methods

2.1. Patients

Bone marrow samples were obtained from 80 CLL patients (52 males and 28 females) admitted to the Affiliated Hospital of Qingdao University from January 2013 to December 2018. NHL diagnosis was established according to the latest diagnostic criteria of WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues (2008). In addition, 40 bone marrow samples were collected from healthy controls. The "healthy" was defined as the absence of any type of hematological or other cancers. The mean age of the patient group was 56 years (range 45-78 years), and the control group was 43 years (range 17-73 years). The male-female ratio in the study group was 1.86:1, and that in the control group was 1.32:1. The study was approved by the Ethics Committee of Qingdao University. All patients were informed about the study, and consent was obtained before sample collection.

2.2. DNA extraction

Proteinase K digestion and phenol-chloroform method were used to extract DNA from the bone marrow samples as previously described. 2 ml of each bone marrow sample was rinsed thrice with PBS at room temperature, and digested with 1ml SENT lysis buffer (150mM NaCl, 10mM TrisHCl, 10mM EDTA,

0.1% SDS) and 35 µl proteinase K (100µg/ml) for 2 h at 55°C. The lysates were extracted twice with phenol-chloroform, and the DNA was precipitated with cold anhydrous ethanol, dried at room temperature and re-suspended in 80 µl double-distilled water. The purity and quantity of the extracted DNA were determined using a nucleic acid protein detector, and the samples were either analyzed immediately or stored at -80°C.

2.3. 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 (Da An Gene, China) according to the manufacturer's instructions. The specific BamH I-W sequence of the EBV genome was amplified and the standard curve was plotted using different concentrations of the positive controls (1×10⁴, 1×10⁵, 1×10⁶ and 1×10⁷ copies/ml). The dynamic and the fluorescence standard curves of EBV-DNA amplification were automatically plotted by the detection system. All samples were assayed in triplicates. The lower limit of detection was 1000 copies/ml, and samples with EBV-DNA copy numbers below this limit were considered EBV negative (EBV-).

2.4. Immunophenotyping

The immunophenotype of the bone marrow cells was analyzed by flow cytometry (FC 500 MPL, Beckman Coulter, USA, FL), and characterized in accordance with the WHO 2000 classification of lymphoma[16]. The expression levels of various surface and cytoplasmic markers (Table 1) was analyzed in the CD45+ leucocyte population. Samples with < 20%, 20-50% and >50% positivity for a marker were respectively considered negative, suspicious positive and positive for that antigen [17].

2.5. Cytogenetic analysis

The karyotype of bone marrow cells was analyzed after 24 h culture using the R-band technique according to standard procedures[18] described by the International System for Human Cytogenetic Nomenclature 2009. At least 10 metaphases were analyzed for each sample. All cytogenetic analyses were performed prior to the MDS treatment. A structurally complex karyotype was defined as more than or equal to 3 chromosomal aberrations, including at least one structural aberration.

2.6. Statistical analysis

Experimental data were analyzed using SPSS version 21.0 (IL, Chicago), and $p < 0.05$ was set as the statistical significance. The Kolmogorov-Smirnov (K-S) test was used to check normal distribution of the continuous variables. Quantitative data were compared using two nonparametric tests (Mann-Whitney U-test) on independent samples, and Pearson's χ^2 test or Fisher's exact test was used to analyze descriptive data.

3. Results

3.1. EBV infection status of the CLL patients

EBV infection was detected in 20 of the 80 (25%) NHL patients, and in 3 of 40 (7.5%) healthy controls, indicating significantly higher incidence of EBV in the former ($\chi^2 = 4.02, p < 0.05$) (Figure 1).

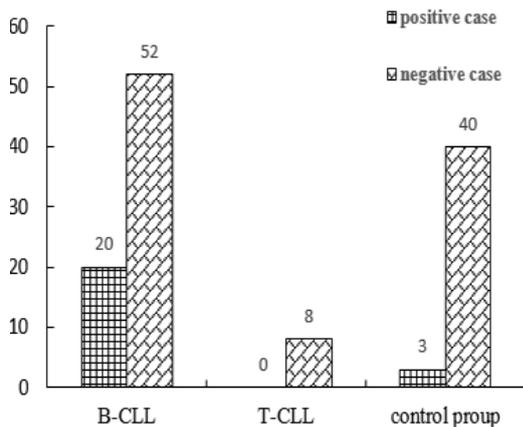


Figure 1 EBV infection rates in different CLL subtypes. Longitudinal coordinates represent the number of patients and the abscissa axis represents the grouping. EBV infection rate in B-CLL and control groups were 27.8% and 7.5%. EBV-DNA was not detected in T-CLL. EBV infection in the B-CLL groups were higher than in the healthy subjects ($\chi^2 = 4.02, p < 0.05$).

3.2. EBV infection and CLL immunophenotyping

Surface immuno-phenotyping classified the 80 cases of CLL into 72 B-CLLs and 8 T-CLLs. The EBV infection rate in the B-CLL was 27.8% (20/72) and EBV-DNA was not detected in T-CLL (Figure 1).

3.3. EBV infection and karyotype

Chromosomal analysis showed that 20% (16/80) of the MDS patients had an abnormal karyotype (Table 3). In addition, chromosome abnormalities were observed in 3 of the 20 EBV+ patients and in 17 of the 60 EBV- patients, and no significant correlation was determined between EBV infection and chromosomal abnormalities ($\chi^2 = 0.21, p > 0.05$).

Table 1. Markers of B and T lineage CLL

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

3.4. Clinical follow-up and other related factors

At the end of 2018, 60 patients (18 EBV+ and 42 EBV-) were followed-up to monitor survival and relapse. The relapse rates in the EBV+ and EBV- groups were 55.6% (10/18) and 28.6% (12/42) respectively. The mortality rates were 44.4% (8/18) and 4.8% (2/42) (Table 2). The EBV+ patients showed higher relapse ($\chi^2 = 3.95, p < 0.05$) and the mortality rates compared to that of the EBV- patients ($\chi^2 = 11.57, p < 0.05$). Within the EBV+ group, ML-associated deaths were correlated with higher EBV-DNA copy numbers at diagnosis, compared to the surviving patients (Mann-Whitney U-test, $P < 0.05$) (Table 4). Moreover, 6 of patients with CLL develop diffuse large B-cell lymphoma (DLBCL), and all of them were EBV positive.

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

	EBV+	EBV-	P-value
Total number of CLL case	20	60	<0.05
Age(years)			
Mean	56	57	NS
Range	45-75	55-78	NS
Gender			
Male	13 (65.0%)	39 (65.0%)	NS
Female	7 (35.0%)	21 (35.0%)	

EBV-DNA copies/ml DNA(n)			
Median	102000	-	
Range	36700-2430000	-	
Mean ± SD	425773.5±7253067.0	-	
Karyotypic analysis (n)	20	60	
B-CLL group(n)	20	52	NS
Normal	17 (35.1)	39 (64.9)	
Abnormal	3 (45.5)	13 (54.5)	
T-CLL(n)	0	8	-
Normal	-	8 (35.7)	
Abnormal	-	-	
Clinical follow-up in CLL	18	42	
Deaths	8 (44.4)	2 (4.7)	<0.05
Relapse	10(55.6)	12 (28.6)	<0.05

Table 3. Karyotypes and EBV infection status in CLL patients

Number	Gender	Age	Type	Abnormal karyotype	EBV
1	M	58	B-CLL	46, XY, t(3;17)(q26;q22), i(8)(q10), del(12)(p12) [4]/ 46, XY [6]	-
2	F	65	B-CLL	46, XX, t(13;17)(q12;q21) [2] / 46, XX [8]	-
3	M	73	B-CLL	46, XY,t(9;22)(q34;q11) [8]/53, XY, idem, +4, +5, +6, +8, +14, +22, +der(22)t(9;22) [2]	+
4	F	59	B-CLL	complex karyotype [4]/46,XX [10]	+
5	M	60	B-CLL	46,XY,+12,del(14)(q22-24),+3,del(6q),del(11)(q14-23) [10]	-
6	M	63	B-CLL	complex karyotype [4] / 46, XX [10]	-
7	M	70	B-CLL	46,XY,13q- [10]	+
8	F	81	B-CLL	47,XX,+12 [10]	-
9	M	69	B-CLL	46,XY,11q- [10]	-
10	F	70	B-CLL	46,XX,17p- [10]	-
11	M	72	B-CLL	46,XY,14q+ [10]	-
12	F	73	B-CLL	46,XX,6q- [10]	-
13	F	66	B-CLL	46,XX,del(17p13) [10]	-
14	M	68	B-CLL	46,XY,del(13q14) [10]	-
15	M	75	B-CLL	46,XY,t(11;14)(q13;q32) [10]	-
16	M	77	B-CLL	46,XY,t(14;19) (q32;q13) [10]	-

M: male, F: female, +: positive, -: negative

Table 4. EBV-DNA copy numbers (copies/ml) and survival of ML patients

	Median	Range	Mean ± SD	p-value
CLL-associated deaths	266400	114500-2430000	874544.4±914603.7	<0.05
Survival patients	38260	36700- 39820	38260.0±2206.2	

4. Discussion

CLL is a low-grade, monoclonal B/T lymphoproliferative disease with great heterogeneity in clinical and pathology. The cause of disease is mostly related to heritage, but environmental factors have little influence on it. EBV is a member of the human herpesvirus family. It exists all over the world. The vast majority of adults have evidence of EBV infection in the past. EBV enters a new host through saliva. It infects B lymphocytes in the oropharynx and persists in B cells throughout an individual's life. The virus can selectively immortalize B cells in vitro. Some studies have found that there is a certain correlation between CLL and EBV, such as Laytragoon-Lewin et al. described two CLL patients who had EBV-derived latent membrane proteins in blood mononuclear cells. In Hermouet et al. study showed that 2 of 21 patients with B-cell CLL had high EBV viral load in peripheral blood lymphocytes detected by real-time PCR. However, no other association between EBV and CLL/SLL has been reported so far. In this study, FQ-PCR was used to detect EBV-DNA copy number in 80 CLL patients. The results showed that 20 of CLL patients had EBV infection, but EBV was not detected in any of the 60 normal bone marrows. And the activity of EBV replication in CLL patients was significantly higher than that in normal control group. Therefore, EBV sensation maybe play a certain role in the pathogenesis of CLL.

As a malignant clonal disease, CLL development is driven by chromosomal abnormalities, which have been observed in approximately 80% of CLL patients[19,20], the most common cytogenetic abnormalities was del (13q14), +12, 11q-, del (17p13), 6q- and 14q+. In this work, the overall incidence of chromosomal abnormalitie with MDS was 20%, this result was not consistent with previous studies, probably because of the conventional chromosome banding techniques used in this study, and some minor genetic changes could not be detected[20]. And, in this study, the common cytogenetic abnormalities were del (13q14), +12, 11q-, del (17p13), 6q- and 14q+. This result was very similar to the previous series. But the mechanism that caused CLL chromosomal abnormalities was unclear. Previous studies had found that EBV-DNA could incorporate into the chromosomes of cells, causing chromosomal instability[21,22]. Several studies have found that EBV infection was also associated with chromosomal abnormalities in malignant tumor. For instance, fluorescence in situ hybridization (FISH) and molecular genetics showed that EBV infection contributed to the genomic instability of BL and NPC[8,23-25], and chromosomal abnormalities caused by EBV infection were also observed in HLH and CAEBV [26, 27]. However, we had not find any association between EBV infection status and

chromosome aberrations in CLL patients. This could be due to the fact that conventional cytogenetics like karyotyping cannot detect minute genetic alterations, and also because the CLL cohort was small. Therefore, the role of EBV infection in CLL-related chromosomal aberrations should be analyzed in a larger cohort by combining karyotyping, FISH and molecular genetics.

EBV infection is also a potential risk factor in the progression of acute lymphoblastic leukemia (ALL)[28]. The current study demonstrated a higher relapse rate and poorer prognosis in CLL patients with EBV infection. EBV-DNA load in chronic lymphocytic leukemia (CLL) was an independent predictor of clinical course and survival[29]. We also found a significant correlation between CLL-associated deaths and the EBV-DNA copy numbers. These findings were consistent with literature[30], high viral loads of EBV-DNA in the peripheral blood of CLL patients was associated with an unfavorable prognosis. Previous study found that a small percentage (about 2-8%) of CLL patients developed DLBCL[31]. In this study, 6 of patients with CLL develop DLBCL, and all of them were EBV positive. Therefore, EBV could promote the development of CLL to DLBCL. However, more clinical parameters need to be analyzed to validate the association of EBV infection with poor clinical outcomes in CLL patients.

5. Conclusion

EBV infection may be related to the occurrence and development of CLL. EBV infection is not conducive to the prognosis of CLL. And EBV may participate in the transformation of CLL to DLBCL.

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