Original Study

Determination and Role of Epstein-Barr Virus in Patients With Lymphoproliferative Disorders

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Abstract

Many studies describe an association between Epstein-Barr virus and lymphoproliferative disorders, especially lymphomas and acute lymphoblastic leukemia (ALL). Because of the delay or lack of adequate in immune response among these patients, serologic tests can lead to false-negative results. Real-time PCR testing of patients being admitted with lymphoproliferative disorders, especially those diagnosed as ALL and lymphomas, is useful during follow-up and treatment.

Introduction: Epstein-Barr virus (EBV) is associated with different types of human malignancies, including Burkitt lymphoma, nasopharyngeal carcinoma, and lymphomas. We retrospectively investigated the presence of EBV-DNA by real-time PCR in clinical samples of patients diagnosed as having hematologic malignancies while investigating the cause of lymphoproliferative disorders, and investigated its relationship to clinical manifestations. **Patients and Methods:** Fifty clinical samples sent to Gazi University's hematology clinics between November 2013 and March 2018 were included. EBV-DNA was investigated by real-time PCR method, and EBV-IgM and EBV-IgG antibodies were investigated by ELISA. **Results:** Fifty serum samples were investigated, and 10% (5/50) EBV-DNA positivity was determined in patients. Of the 5 patients with EBV-DNA positivity, 2 had acute lymphoblastic leukemia, 1 lymphoma, 1 T-cell lymphoma, and 1 B-cell lymphoma. Concomitant EBV-DNA and viral capsid antigen (VCA)-IgM positivity was not detected. The VCA-IgM test results of the all EBV-DNA–positive patients were negative and VCA-IgG positive (except for 1 patient). Regarding virus load, of the 5 samples, 2, 1, 1, and 1 of the samples had a virus load of 10^2, 10^3, 10^4, and 10^5 copies/mL, respectively. **Conclusion:** EBV infection is threatening in patients with hematologic malignancies and are diagnosed by serologic and molecular methods. As a result of the study, we suggest that the detection of EBV-DNA by real-time PCR in patients being admitted with lymphoproliferative diseases and diagnosed as acute lymphoblastic leukemia and lymphomas may be useful in follow-up and treatment.

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Introduction

Epstein-Barr virus (EBV) is a gamma herpesvirus, which belongs to the Herpesviridae family. EBV, also called human herpesvirus 4 (HHV-4), was first isolated in 1964 by the researchers Epstein, Barr, and Achong from the Burkitt lymphoma tumor samples from African children.[1]

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The first contact with EBV usually occurs during childhood. EBV infects the oropharyngeal and nasopharyngeal epithelial cells, salivary glands, and B lymphocytes. Acute EBV infection can cause infectious mononucleosis, and EBV persists in the B lymphocytes. EBV does not cause cytopathic effect in the infected cell, and infection is carried lifelong. EBV is common worldwide; roughly more than 90% of the adult population is infected by EBV, where it is carried as a latent asymptomatic infection in the vast majority of individuals.[2]

EBV has been associated with a remarkably diverse range of cancer types such as nasopharyngeal carcinoma, Burkitt lymphoma, Hodgkin lymphoma, non-Hodgkin lymphoma (NHL), B-cell lymphoma, and natural killer (NK)/T-cell lymphoma, as well as hematologic malignancies such as acute leukemia.[3,4] Although the etiology is unknown, there are many studies describing an association between EBV and lymphomas and

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acute lymphoblastic leukemia (ALL). EBV-associated malignancies, especially ALL and lymphomas, are important causes of mortality and morbidity.[5-7]

Diagnosis, follow-up, and prognosis of EBV infection are important for these patients. EBV-specific serologic tests are used for the diagnosis of EBV infections. EBV immunoglobulin (Ig) G and EBV-IgM formation against the major antigens of the EBV viral capsid antigen (VCA), early antigen (EA), and nuclear antigen (EBNA) can be investigated. While the presence of VCA-IgM and VCA-IgG in the absence of EBNA-IgG indicates an acute infection, the presence of VCA-IgG and EBNA-IgG when VCA-IgM was negative indicates past infections. EBV infections can also be diagnosed by molecular methods, especially real-time PCR.

Because EBV appears to be one of the etiologic factors in lymphoma and hematologic malignancies such as acute leukemia, more studies are needed to investigate the relationship between EBV and ALL and lymphoma. Because of the delay in immune response or lack of adequate immune response among these patients, serologic diagnosis of EBV infection is difficult, and serologic tests can lead to false-negative results. As little as 10 copies/mL of EBV-DNA can be detected, and the virus load can be quantitatively determined by real-time PCR. Serial quantitative monitoring of EBV-DNA levels by the real-time PCR method is advantageous for evaluating the changes in early diagnosis and clinical course.

The primary aim of this study was to retrospectively investigate the presence and virus loads of EBV-DNA to provide an overview of clinical manifestations and diagnosis; and secondarily to emphasize that the adoption of EBV-DNA real-time PCR monitoring in ALL and lymphoma patients may reduce related complications.

Patients and Methods

The study samples were collected between November 2013 and March 2018 in Gazi University, Faculty of Medicine, Department of Hematology. Fifty clinical samples obtained from 40 patients, 20 each of male and female subjects, were included in the study. Clinical samples were analyzed by serologic and molecular methods. The serologic profile of patients for EBV was analyzed by enzyme-linked immunosorbent assay (ELISA), and the presence and virus loads of EBV-DNA was analyzed by real-time PCR.

Collection of Blood Samples

Blood samples were centrifugated at 3000 rpm for 5 minutes, and the serum was separated and stored at −80°C until the time of the study.

Serologic Analysis

The EBV VCA-IgG, VCA-IgM, EBNA-IgG, EBNA-IgM, and EA-IgG antibodies were analyzed by ELISA, and serologic profiles of patients were determined according to the manufacturer's instructions (DIA.PRO, Milan, Italy). EBV antibodies were analyzed spectrophotometrically. Absorbances were read at 450 nm (Tecan, Zurich, Switzerland), and the obtained optical density (OD) results were evaluated. According to the manufacturer's instructions, if the negative control was OD < 0.1 for the VCA-IgM, VCA-IgG, EBNA-IgG, and EA-IgG, and the positive control (calibrator 100 arbU/mL) was OD > 1.0, then the test was considered to be correct. The method used in this study does not show cross-reactivity, and its sensitivity and specificity are both > 98% according to the instruction manual.

Molecular Analyses

Molecular analyses included nucleic acid isolation and DNA amplification. EBV-DNA was investigated by the real-time PCR method. DNAs were extracted from the samples with QIAamp DSP Virus Kit in EZ1 Advanced (Qiagen, Germantown, MD) device. Total nucleic acid suspensions were prepared and vortexed, and were recovered in 60 µL of nuclease-free water and stored at −80°C until amplification. The amplification of the isolated virus DNAs was performed by the real-time PCR method using the artus EBV RG PCR Kit in Rotor-GeneQ (artus, Altona, Germany) device, and the results were evaluated quantitatively. Primers amplifying the 97 bp portion of the EBV genome were used for the amplification. In real-time PCR for EBV analysis, as little as 10 copies/mL of EBV-DNA can be detected, and the virus load can be determined quantitatively. One negative control and 4 standards each containing a different quantitation of EBV-DNA (10^2 to 10^6 copies/mL) were used for EBV analysis. The method used does not show cross-reactivity with the other human herpesviruses, and its sensitivity is 95%.

Statistical Analysis

Data were analyzed by SPSS 20.0 (IBM, Armonk, NY). The Mann-Whitney *U* test and Fisher exact test were used to evaluate the data, and a cutoff *P* value of < .05 was considered significant.

Ethical Review and Patient Consent

The research proposal was approved by the ethical review board of Gazi University and was conducted in accordance with the ethical principles of the 2013 Helsinki Declaration.

Results

Forty patients, 20 each (50%) male and female, between the ages of 19 and 75 years were included in the study. The numbers and age ranges of pediatric patients included in the study are shown in Table 1.

Of the 40 patients, 30% (12/40) were diagnosed as NHL, 15% (6/40) ALL, 10% (4/40) acute myeloid leukemia, 10% (4/40) multiple myeloma, 2.5% (1/40) Hodgkin lymphoma, 2.5% (1/40) B-cell lymphoma, and 2.5% (1/40) T-cell lymphoma; the others included things such as myelodysplastic syndrome, thrombocytopenia, and leukocyte disorders.

Clinical samples were analyzed by serologic and molecular methods, and the results of the patients were evaluated together.

Table 1 Gender and Age of Study Patients

Gender	N	Age (Y), Median (Min-Max)	Age (Y), Mean ± SD
Male	20	43 (22-75)	43.5 ± 17.8
Female	20	40 (19-67)	38.0 ± 19.7
Total	40	40 (19-75)	41.5 ± 17.6

Abbreviation: SD = standard deviation.

Table 2 Relationship Between EBV Serology and Real-Time PCR Results

Patient No.	VCA-IgM	VCA-IgG	EBNA-IgM	EBNA-IgG	EBV-DNA	Diagnosis	*P*
1	−	+	−	+	+	ALL	.62
2	−	−	−	−	+	T-cell lymphoma	
3	−	+	−	+	+	ALL	
4	−	+	Gray zone	+	+	B-cell lymphoma	
5	−	+	−	+	+	NHL	

Abbreviations: ALL = acute lymphoblastic leukemia; EA = early antigen; EBNA = nuclear antigen; EBV = Epstein-Barr virus; Ig = immunoglobulin; NHL = non-Hodgkin lymphoma; VCA = viral capsid antigen.

Serologic results were available for all EBV-DNA–positive patients. VCA-IgM, which is an indication of acute infection, was found to be negative in all the EBV-DNA–positive patients. In 4 patients with NHL, T-cell lymphoma, B-cell lymphoma, and ALL, VCA-IgM negativity was detected in the presence of VCA-IgG and EBNA-IgG. An EBNA-IgM result was negative in 4 patients and in the gray zone for 1 patient, but the patient did not participate in the treatment and follow-up process. The relationship between EBV serology and real-time PCR results is shown in Table 2.

EBV-DNA was positive in 10% (5/50) of samples from 40 patients by real-time PCR of all clinical samples. Of the 5 patients with EBV-DNA positivity, 2 patients were diagnosed with ALL, 1 lymphoma, 1 T-cell lymphoma, and 1 B-cell lymphoma.

Considering all patients included in our study, 33% (2/6) of patients diagnosed with ALL and 20% (3/15) of patients diagnosed with lymphoma (NHL [n = 1], T-cell lymphoma [n = 1], and B-cell lymphoma [n = 1]) were positive for EBV-DNA. Table 3 shows the clinical diagnosis and real-time PCR results of the patients.

EBV-DNA positivity was highest in patients diagnosed with lymphoma than in ALL patients, but no statistically significant difference was found ($P > .05$).

EBV-DNA was found between 10^2 and 10^5 copies/mL. In 2 of 5 positive samples, EBV-DNA was 10^2 copies/mL and disease was diagnosed as ALL and T-cell lymphoma, 10^3 copies/mL was found in 1 sample of ALL, 10^4 copies/mL in 1 sample of B-cell lymphoma, and 10^5 copies/mL in in 1 sample of NHL The quantitation of DNA detected in EBV-DNA–positive samples and the distribution according to underlying diseases are shown in Table 2.

Regarding the relationship between the amount of the EBV-DNA and the diagnosis of the patients, the highest EBV-DNA quantitation ($> 10^5$) was detected in patients diagnosed with NHL, but there was no statistically significant difference ($P > .05$).

Discussion

EBV has been associated with a remarkably diverse range of cancer types such as nasopharyngeal carcinoma, Burkitt lymphoma, Hodgkin lymphoma, NHL, B-cell lymphoma, and NK/T-cell lymphoma, as well as hematologic malignancies such as acute leukemia. In our study, the prevalence and virus load of EBV-DNA in patients who were diagnosed with lymphoproliferative disorders were investigated. Although our small sample size means that the percentage values may not reflect a true finding, it is conceivable that EBV infection is also related to lymphoproliferative disorders. EBV-DNA was positive 10% of all patients analyzed by real-time PCR. After acute EBV infection, EBV persists in the B lymphocytes and can cause major types of B-cell malignancies such as Burkitt lymphoma, NHL, and B-cell lymphoma.[7] Dojcinov et al[8] investigated and classified EBV-associated lymphoid proliferations in 122 EBV-positive patients, and 25% were diagnosed as B-cell lymphoma. The results of the study support routine EBV molecular testing in patients, especially those older than 50 years who are suspected of having B-cell lymphoma or B-cell lymphoproliferative disorders. Abadi et al[9] investigated the prevalence of EBV infection in patients with NHLs, and EBV-DNA was detected as 26.5%. The researchers maintained that EBV might be an etiologic factor in NHLs, especially in B-cell lymphoma. In the present study, 3 of 5 EBV-DNA–positive patients were diagnosed as having lymphoma, which included B-cell lymphoma, T-cell lymphoma, and NHL. Our study results lead us to conclude that EBV may be an etiologic factor in lymphomas, and therefore routine EBV tests should be offered to patients who are suspected to harbor an EBV-associated lymphoma.

Studies mainly focus on the association between EBV infection and leukemia. Several studies were performed that indicate that EBV may play a role in the pathogenesis of ALL.[4-6] EBV infection is of small importance for patients with hematologic malignancies

Table 3 Quantitation of DNA Detected in EBV-DNA–Positive Samples and Their Clinical Relationship

Diagnosis	Variable	Patient No.	EBV-DNA Positive	EBV-DNA (Copies/mL)	*P*
Acute lymphoblastic leukemia	ALL	1	33% (2/6)	10^2	.55
	ALL	2		10^3	
Lymphoma	NHL	3	20% (3/15)	10^4	
	B-cell lymphoma	4		10^5	
	T-cell lymphoma	5		10^2	

Abbreviations: ALL = acute lymphoblastic leukemia; EBV = Epstein-Barr virus; NHL = non-Hodgkin lymphoma.

receiving standard chemotherapy, and no routine diagnosis for EBV is recommended in this group of patients, either before and after therapy. In our study, 2 patients with ALL had undergone allogeneic hematopoietic stem-cell transplantation. Also, all of the patients were immunosuppressed and had concomitant cytomegalovirus (CMV) reactivation, which manifested as CMV retinitis in 1 patient. Also, the use of medication might have caused a delay in EBV-specific T-cell response and increased viral infections.[10] Guan et al[6] investigated EBV infection in patients with acute leukemia and hematologically healthy control subjects. EBV-DNA was found to be positive in 40.9% (45/110) of patients with ALL and 5.4% (2/37) of hematologically healthy controls. In this study, the positivity rate of EBV in the ALL group was higher than in the control group ($P < .05$). They therefore focused on the relationship between EBV infection and ALL, and the study results indicated that there is EBV infection in ALL patients. In the present study, similarly, EBV-DNA positivity was found in 33% of patients with ALL. In our opinion, it is highly likely that EBV by itself may not cause ALL, but may be an important cofactor, at least in some patients.

EBV-specific serologic tests are also used for the diagnosis of EBV infections. In immunosuppressed patients, the immune response is inadequate, so the EBV serology cannot be interpreted. For these patients, molecular methods are required.[11,12] Karadağ et al[11] reported that in immunosuppressed patients, serologic tests on their own may be insufficient, and the diagnosis should be supported by VCA-IgG avidity and real-time PCR test results. Soylu et al[12] studied EBV-specific antibodies in 7363 serum samples. Overall, 70% of samples showed past infection, 13% were negative for EBV, 1.5% showed acute infection, and 15% were doubtful. The researchers suggested that when there is a concern about EBV diagnosis, both clinical and serologic follow-up must be done together or EBV-DNA must be investigated in samples. Similarly, in present study, VCA-IgM as an indicator of acute infection was negative in all EBV-DNA−positive patients. Also, EBNA-IgM, an indicator of latent infection, was negative in all EBV-DNA−positive patients. Our study has shown that in immunosuppressed patients such as those with ALL and lymphoma, serologic tests may be inadequate in detecting the EBV infection, and molecular methods such as real-time PCR is required.

In some studies, the relationship between the amount of EBV-DNA and clinical manifestations was investigated. Gartzonika et al[13] reported that the virus load ranged between 10^2 and 10^4. Visco et al[14] detected $\geq 10^3$ copies/mL EBV-DNA in approximately 20% of patients with leukemia. Likewise, in our study, the total virus load of EBV-DNA ranged between 10^2 and 10^5 copies/mL. Morishima et al[15] investigated the EBV virus load in patients with EBV-positive B-cell lymphoma. The authors indicated that EBV-DNA is a valuable diagnostic and prognostic marker for EBV-positive patients, and their results suggest that plasma EBV-DNA level is a useful diagnostic biological marker in patients with EBV.

In the present study, the patients were diagnosed with EBV-associated B-cell lymphoproliferative disorders, which were defined as B-cell lymphoma. One patient received 4 cycles of anti-CD20 monoclonal antibody (rituximab) therapy, and clinical response was achieved. After 6 cycles of R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, prednisolon) chemotherapy, complete response was obtained in the second patient. The patients with T cell lymphoma and ALL were immunosuppressed and had concomitant CMV reactivation. Therefore, low levels of EBV-DNA were a sign of a possible cross-reaction, which may be frequently observed during viral infections. These patients were treated properly against CMV infection, but specific treatment for EBV reactivation was not considered because of clinical discordance. Nevertheless, high EBV-DNA levels with concurrent clinical findings such as lymphadenopathy and organomegaly may refer to a posttransplant lymphoproliferative disorder in hematopoietic stem-cell transplant recipients, which deserves further attention because a targeted therapy modality is essential in this setting.[16-19]

Gartzonika et al[13] investigated the quantification and diagnostic utility of EBV-DNA detection as an adjunct to serologic diagnosis of EBV infection. Sera from 118 patients referred for suspected primary EBV infection were studied for EBV-specific antibodies and by real-time PCR. EBV-DNA was detected by real-time PCR in the plasma of patients with a sensitivity and a specificity of 95.7% and 100%, respectively. In the same study, EBV-DNA was detectable in all samples drawn until 12 day after onset of symptoms; 20 days after onset, all samples were negative. It seems that PCR is more sensitive than serology, especially at the beginning of the disease course, when an immunologic response may not be detected. Therefore, the use of real-time PCR for the detection of the virus DNA should be more frequent, especially in immunosuppressed patients.

Conclusion

EBV infection is threatening in patients with hematologic malignancies, especially ALL and lymphoma. As a result of the inadequate immune response in immunosuppressed patients with ALL and lymphoma, the results of EBV serology can be incorrectly interpreted. Because real-time PCR can detect the virus DNA before the symptoms occur and enables preemptive therapy, quantitative EBV-DNA testing in such patients is crucial in determining the presence and also the amount of the virus DNA, which in turn helps with the patient's prognosis and follow-up.

Clinical Practice Points

- Although the etiology is unknown, there are many studies describing an association between Epstein-Barr virus (EBV) and lymphomas and acute lymphoblastic leukemia (ALL). Diagnosis, follow-up, and prognosis of EBV infection are important for these patients.
- EBV infections can be diagnosed by serologic tests and molecular methods, especially by real-time PCR. However, because of the delay in immune response or lack of adequate immune response among these patients, serologic diagnosis of EBV infection is difficult, and serologic tests can lead to false-negative results.
- As few as 10 copies/mL of EBV-DNA can be detected, and the virus load can be quantitatively determined by real-time PCR.
- Serial quantitative monitoring of EBV-DNA levels by real-time PCR method is advantageous for evaluating the changes in the early diagnosis and clinical course.

- As a result of this study, it seems that PCR testing is more sensitive than serologic testing, especially at the beginning of the disease course, when an immunologic response may not be detected.
- The use of real-time PCR for the detection of virus DNA should be more frequent, especially in immunosuppressed patients.

Disclosure

The authors have stated that they have no conflict of interest.

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