Journal of Clinical Virology 52 (2011) 231–235

Contents lists available at ScienceDirect

Journal of Clinical Virology

journal homepage: www.elsevier.com/locate/jcv

Lytic and latent EBV gene expression in transplant recipients with and without post-transplant lymphoproliferative disorder

Jing Kroll[a,*], Shaobing Li[b], Marilyn Levi[a], Adriana Weinberg[b]

[a] Department of Internal Medicine, University of Colorado Denver, CO, USA
[b] Department of Pediatrics, University of Colorado Denver, CO, USA

ARTICLE INFO

Article history:
Received 17 June 2011
Accepted 24 June 2011

Keywords:
Epstein–Barr virus
Post-transplant lymphoproliferative disorder
Hematopoietic stem cell transplant

ABSTRACT

Background: Epstein–Barr virus (EBV) is associated with post-transplant lymphoproliferative disorder (PTLD), which has significant morbidity and mortality in transplant recipients. To devise prophylactic measures, we need predictors of PTLD and a better understanding of the physiopathogenesis of the disease.
Objectives: To identify a molecular pattern of EBV gene products in blood that is specific to PTLD and can be used for the diagnosis of this disease.
Study design: We evaluated the ratio between latent and replicating EBV nucleic acids in individuals with PTLD by comparison with transplant recipients without PTLD and immunocompetent hosts with EBV DNA-emia. Subjects were prospectively identified between July 2009 and October 2010 at the University of Colorado Hospital. EBV DNA, LMP-2A Latency III and BZLF1 Lytic genes mRNA were quantified using real-time PCR.
Results: We found that PTLD subjects ($N=7$) had significantly higher EBV DNA-emia compared with non-transplant immunocompetent subjects ($N=69$; $p<0.0001$), and transplant recipients without PTLD ($N=105$; $p<0.0001$). The ratios between LMP-2A and BZLF1 mRNA in transplant recipients were significantly lower than in non-transplant subjects ($p=0.04$). However, PTLD and non-PTLD transplant recipients displayed similar ratios.
Conclusions: These results suggest that EBV replication makes a larger contribution to the circulating EBV DNA in transplant recipients compared with immunocompetent hosts. Transplant recipients seem to lose control over EBV replication, which may contribute to the development of PTLD.
Published by Elsevier B.V.

1. Background

Post-transplant lymphoproliferative disorders (PTLD) are the most serious and potentially fatal complications of the EBV reactivation in transplant individuals. In hematopoietic stem cell transplant (HSCT) recipients, the incidence of PTLD varies from 0.07% in autologous HSCT recipients to 29% in T-cell depleted HSCT recipients.[1–7] The incidence of PTLD in solid organ transplant (SOT) recipients varies from 0.7% to 11.4%.[8–10] The treatment of PTLD and its success varies with the clinical manifestations of the disease. Early PTLD may be reversed by reducing the immunosuppression, whereas advanced PTLD/lymphoma commonly requires antineoplastic chemotherapy. Immunotherapy with the anti-CD20 monoclonal antibody rituximab has shown promising results.[11–19] In spite of therapy, PTLD has a high mortality rate of 50–80%[20–22] and doubles the risk of graft loss.[23]

The hallmark of EBV infection of B lymphocytes is the establishment of latency. The172 kbp EBV genome encodes approximately 100 genes, but only a small fraction is expressed during latency including two latent membrane proteins (LMP-1 and 2). Latency can be disrupted by a variety of cellular activators, resulting in the expression of BZLF-1, which induces the switch from latency to lytic replication. The EBV life cycle provides important information, namely that the latent and lytic cycles involve the expression of unique, non-overlapping genes, which can potentially differentiate between these 2 stages of infection.

To date, the diagnosis of PTLD relies on histology, but many studies have looked for non-invasive methods to diagnose PTLD. Although patients with PTLD invariably have circulating EBV DNA and generally in high titers, other transplant and non-transplant patients who do not develop PTLD may also have circulating EBV DNA.[24,25] Moreover, a threshold value of EBV DNA load in blood associated with the development of PTLD has not been established. Attempts were also made to identify latent transcripts

* Corresponding author at: Division of Infectious Disease, Department of Medicine, University of Colorado Denver, 12700 E 19th Avenue, Room 11121, Aurora, CO 80045, USA. Tel.: +1 303 274 4484; fax: +1 303 724 4485.
E-mail addresses: Jing.Kroll@ucdenver.edu, JInglukroll@yahoo.com (J. Kroll).

1386-6532/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.jcv.2011.06.013

predictive of PTLD development but the results were inconclusive.[26,27]

Rising circulating EBV DNA in blood can potentially represent two phenomena: (1) increasing viral replication; or (2) proliferation of latently infected B cells. We hypothesized that in patients with PTLD, circulating EBV DNA predominantly derives from latently infected proliferating B cells with a minor participation of actively replicating virus.

2. Objectives

The purpose of this study is to identify a molecular pattern of EBV gene products in blood that is specific to PTLD by comparing the ratio of latent and replicating EBV nucleic acids in individuals with PTLD with those in transplant recipients without PTLD and immunocompetent hosts with EBV DNA-emia.

3. Study design

3.1. Subjects

The study was approved by the local IRB. Between July 2008 and October 2010, patients at University of Colorado Hospital who had ≥1 positive whole blood EBV DNA PCR results were prospectively identified. Subjects were classified as transplant recipients with histology-proven PTLD; transplant recipients who did not develop PTLD for ≥6 months after the blood sample was obtained; and immunocompetent hosts without any known acquired or congenital immunosuppressive condition including EBV-associated neoplasm. For subjects who had ≥2 blood samples containing EBV DNA, the sample with the highest viral load was chosen for further testing and analysis.

3.2. Real-time quantitative EBV DNA PCR

Viral DNA was extracted from 200 μl of frozen whole blood using the MagNA Pure apparatus (Roche). 10 μl of extracted DNA were added to 10 μl of EBV DNA PCR master mix containing LightCycler FastStart DNA reaction mix (Roche), EBNA-1 gene primers GAGGGTGGTTTGGAAAGC and AACAGACAATGGACTCCCTTAG (0.5 μM each) and probes AGTCGTCTCCCCTTTGGAATGGC-fluorescein and LC Red640 – CTGGACCCGGCCCACAACCTG-phosphate elongation block (0.2 μM each) and $MgCl_2$ (3.5 mM). A PCR run was considered valid if all the controls (high and low positive, negative and extracted) performed within their pre-specified ranges. The dynamic range (linear portion of the curve) spans from 500 to1,000,000 DNA copies/ml. Arbitrary values of 250 and 2,000,000 c/ml were ascribed to samples that resulted 100 to 500 and >1,000,000, respectively.

3.3. Total RNA extraction from frozen whole blood

A combination of Trizol LS Reagent with MaXtract High Density (QIAGEN) and QI-Amp columns-denominated TriMax was used for RNA extraction. Briefly, 750 μl Trizol LS Reagent were combined with 125 μl of blood for a single extraction. After adding chloroform, the homogenized sample was added to MaXtract High Density tube for phase separation. After centrifugation, the aqueous phase was transferred to QIAamp RNA mini column followed by RNase-free DNase for on column digestion. RNA was then eluted in 30 μl RNase-free water.

3.4. mRNA reverse transcription real time quantitative PCR

5 μl of RNA obtained as described above were reverse transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche). RNA was added to 15 μl of master mix containing 0.5 μM of each primer, 0.2 μM of each probe and 3.5 mM $MgCl_2$. Primers and probes for LMP-2A real time PCR amplification were as follows: forward primer: GATTGAGGACCCACCTT; reverse prime: CGCTAGTATCGAGAGCA; Probe 1: TTTGCATTGCTGGCCGC – fluorescein; and Probe 2: LC Red640 – CTGGCGGACTACAAGGCA-phosphate. Primers and probes for the BZLF-1 real time PCR amplification were as follows: forward primer = TCCTCGTGTTAAAACATCTGG (89,938–89,957); reverse primer = ATAAAGCGATACAAGAATCGG (90,194–90,215); Probe 1 = CCTCACGGTAGTGCT – fluorescein (90,049–90,052/90,138–90,151); and Probe 2 = LC Red640 – AGCAGTTGCTTAAACTTGGCC-phosphate (90,154–90,173). GAPDH was used as housekeeping control gene as previously described.[28]

Table 1
Characteristics of the subjects in the study.

Subjects	PTLD	non-PTLD	non-Transplant
Number of subjects (%)	7	105	69
Sex			
Males	5 (71%)	61 (58%)	41 (60%)
Female	2 (29%)	44 (42%)	28 (40%)
Age median	55 y/o (37–73)	48 y/o (21–82)	53 y/o (23–81)
Race			
Caucasian	5 (70%)	66 (63%)	50 (72%)
Hispanic	1 (15%)	20 (19%)	14 (21%)
African American	1 (15%)	13 (12%)	5 (7%)
Other	0	6 (6%)	0
Type of transplant			
Heart	2 (28%)	13 (12%)	
HSCT	0	23 (23%)	
Kidney	4(57%)	41 (39%)	
Liver	1 (15%)	13 (12%)	
Lungs	0	15 (14%)	

3.5. Statistical analysis

Data were log transformed to normalize their distribution and analyzed using unpaired *t*-test. All tests were two-tailed. We conducted the analyses using Prism software, version 5 (GraphPad).

4. Results

4.1. Characteristics of the study population

A total of 181 subjects with EBV viremia were identified, including 7 subjects with PTLD, 105 transplant recipients without PTLD and 69 non-transplant subjects (Table 1). Sex, age, race were similar in all three groups. Among the 7 PTLD subjects, kidney transplant patients accounted for the majority of cases. This reflects the higher number of kidneys compared with other organs transplanted at our institution.

4.2. Development and validation of mRNA quantitative PCR

The primers of the LMP-2A and BZLF1 mRNA reverse transcription PCR were chosen to span intron-containing areas, which allowed us to assess potential DNA contamination. Gel migration of the PCR-generated amplicon in DNase treated samples invariably yielded single bands of 194 and 106 bp for BZLF1 and LMP-2A, respectively, whereas undigested samples also showed the higher molecular weight amplicon generated by DNA amplification (Fig. 1).

To determine the lower limit of detection of reverse transcription PCR for targeted genes, we spiked EBV DNA-negative human blood with the EBV-producing marmoset B-cell line (B95-8 cells) at different concentrations from 10^6 to 10^1 cells/ml and, subsequently, performed mRNA quantitative PCR assays on triplicate