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ORIGINAL ARTICLE

Epstein-Barr virus load in whole blood is associated with immunosuppression, but not with post-transplant lymphoproliferative disease in stable adult heart transplant patients

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Summary

Development of Epstein-Barr virus (EBV)-associated post-transplant lymphoproliferative disease (PTLD) is a serious complication following heart transplantation (HTX). This study investigates EBV DNA load in adult heart transplant recipients, its association with immunosuppression, and its potential as a marker for development of PTLD. EBV DNA load was measured prospectively by quantitative real-time polymerase chain reaction (PCR) in 172 stable HTX patients. Sixty-seven patients (39.0% of total) had a positive EBV PCR at initial examination [median 4.9 (range 1.1–16.9) years post-HTX]. In follow-up testing of 67 positive patients 6 months later, 36 patients continued to have a positive EBV PCR. Overall incidence of EBV DNA was significantly associated with calcineurin inihibitors, azathioprine medication, and with the absence of mycophenolate mofetil (MMF) treatment. In patients with positive EBV DNA levels at initial examination and negative levels at retesting, cyclosporine A levels were found to be significantly higher at initial examination (148.4 ± 70.2 vs. 119.6 ± 53.5 ng/ml, $P < 0.05$). Three patients (1.7%, 3/172) were diagnosed with PTLD during the course of the study (mean follow up 4.0 years). EBV DNA viral load determination does not appear to be useful for risk prediction or early diagnosis of PTLD in adults after HTX, but an association of EBV DNA load with qualitative and quantitative immunosuppression is demonstrated.

Introduction

Post-transplant lymphoproliferative disease (PTLD) is often a fatal complication after heart transplantation (HTX) with a cumulative incidence of approximately 5% [1–4]. HTX follow-up patients show a 120-fold higher relative risk when compared with the general population [5]. When diagnosed within the first 5 years after transplantation, tumors are often Epstein-Barr virus (EBV) DNA positive [6]. It is widely accepted that immunosuppressive drugs cause a defective T-cell tumor surveillance, enabling clonal expansion of EBV-infected B cells [7]. Negative EBV serologic status prior to transplantation and primary EBV infection post-transplant have been identified as risk factors for development of PTLD [8,9]. Elevated EBV DNA load has been linked to the develop-

ment of late-onset PTLD in children after HTX or bone marrow transplantation [10–12]. Using quantitative real-time polymerase chain reaction (PCR), it is possible to monitor EBV DNA load in peripheral blood. Because of the lack of published data on adult heart recipients, the present study investigates EBV DNA load in stable adult heart transplant recipients, its association with immunosuppression, and the prognostic and diagnostic potential of EBV DNA detection as a marker for development of PTLD.

Patients and methods

The study population consisted of 172 stable HTX follow-up patients of whom 20.9% (36/172 patients) were female. Detailed patient characteristics are given in Table 1. All patients were on stable doses of immunosuppression and free from acute infection or rejection for at least 3 months before entry into the study. All patients gave written informed consent prior to inclusion in the study. Mean patient age was 51.1 (range 19.7–69.4; SD ± 10.5) years. Median time after HTX at initial screening was 4.9 years (range 1.1–16.9), retesting was performed 6 months after the initial assessment. All patients received post-transplantation induction therapy using anti-thymocyte globulin (ATG) according to the local standard of care (Heidelberg Heart Transplantation Center). Dosage and duration of therapy were adjusted according to CD4 T-cell counts monitored daily during the first week post-HTX by flow cytometry, aiming at absolute CD4 T-cell numbers below 50/µl [13]. Most patients were on a dual immunosuppressive regimen, consisting of cyclosporine A (CsA) and mycophenolate mofetil (MMF) or azathioprine (AZA). Detailed distribution of immunosuppressive regimens and doses/blood levels are shown in Fig. 1 and Table 2c. A rejection score was determined by scoring points for the degree of rejection according to ISHLT

Table 1. Patient characteristics.

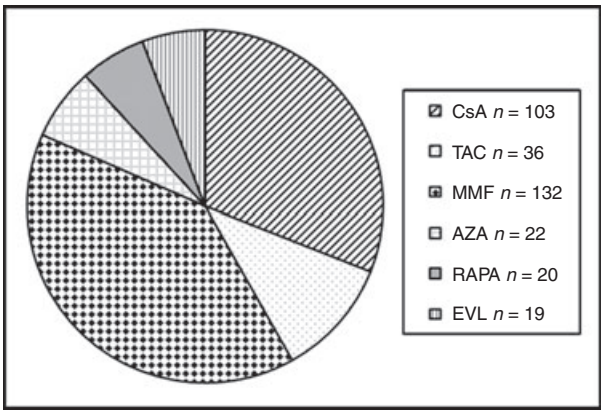
	Baseline (n = 172)	Follow-up* (n = 67)
Mean patient age (years, SD, range)	51.1, 10.5 (19.7–69.4)	
Median time after HTX (years)	4.9	
Creatinine (mg/dl)	1.75 ± 1.68	1.49 ± 1.2
Urea (mg/dl)	61.6 ± 29.1	58.3 ± 29.0
GOT/AST (U/l)	22.8 ± 14.3	28.5 ± 37.9
GPT/ALT (U/l)	28.7 ± 22.7	27.7 ± 18.4
GGT (U/l)	98.9 ± 156.2	87.4 ± 213.1
Hemoglobin (g/dl)	13.3 ± 9.4	12.9 ± 2.0
Leukocyte count (10^9/l)	6.3 ± 2.4	6.7 ± 1.9
Thrombocyte count (10^9/l) (10^9/l)	247.2 ± 82.1	245.3 ± 65.2
Serum cholesterol level (mg/dl)	184.4 ± 38.5	184.3 ± 43.2
Serum HDL level (mg/dl)	46.0 ± 13.8	51.3 ± 12.0
LDL level (mg/dl)]	104.5 ± 32.3	103.5 ± 28.4

ALT, alanine aminotransferase; AST, aspartate aminotransferase; DNA, deoxyribonucleic acid; EBV, Epstein-barr virus; GGT, gamma glutamyl transferase; GOT, glutamic-oxaloacetic transaminase; GPT, glutamic-pyruvic transaminase; HDL, high density lipoprotein; HTX, heart transplantation; LDL, low density lipoprotein; SD, standard deviation.

Values are expressed as mean ± SD; 172 stable adult HTX patients were studied at initial testing (baseline). All EBV DNA$^+$ (n = 67) patients were re-tested 6 months later (follow-up).

*Level of significance: all P = n.s.

(a)



(b)

Immunosuppressive regimens	n / % of total (172 patients)
RAPA	2 / 1.2
CsA	8 / 4.6
MMF	1 / 0.6
TAC	1 / 0.6
AZA/CsA	20 / 11.6
AZA/TAC	2 / 1.2
RAPA/MMF	15 / 8.7
RAPA/CsA	2 / 1.2
RAPA/TAC	1 / 0.6
EVL/MMF	16 / 9.3
EVL/CsA	3 / 1.7
TAC/MMF	31 /18.0
TAC/CsA	1 / 0.6
MMF/CsA	69 / 40.1

Figure 1 Immunosuppressive drugs. (a) Immunosuppressive drugs of all patients (332 drugs in 172 patients, because of monotherapies in 12 patients): cyclosporine A (CsA, n = 03 in 59.9% of patients), tacrolimus (TAC, n = 36 in 20.9% of patients), mycophenolate mofetil (MMF) (n = 132 in 76.7% of patients), azathioprine (AZA) (n = 22 in 12.8% of patients), Sirolimus (RAPA, n = 20 in 11.6% of patients), Everolimus (EVL, n = 19 in 11.0% of patients), and steroids (n = 54 in 31.4% of patients). CsA and TAC target levels varied according to time after transplantation [CsA: 1–2 months post-HTX 225–275 ng/ml, months 3–6 175–225 ng/ml, months 6–12 120–150 ng/ml, months 12–24 100 ng/ml, >24 months 50–60 ng/ml [local CsA reduced schedule from month 6 on for enhanced nephroprotection); TAC: months 1–6 post-HTX 10–14 ng/ml, months 6–12 7–10 ng/ml, months 12–24 5–8 ng/ml, >24 months 5 ng/ml (local TAC reduced schedule from month 12 on for enhanced nephroprotection)]. (b): list of immunosuppressive regimens (n/% of total).

classification (1990 version) [14]: ISHLT 0, 1A = 0 points, 1B = 1 point, 2 = 2 points, 3A = 3 points, 3B = 4 points, 4 = 5 points. A cumulative point score was calculated for all biopsies prior to entry into the study and divided, first, by the number of biopsies performed and then by the number of years post-HTX to yield the rejection index [15]. A mean rejection index of 0.5 (range 0.0–12.4, SD ± 1.6) and a mean number of treated rejections prior to initial study entry of 1.6 (range 0.0–17.0, SD ± 2.3) were observed.

EBV PCR

Quantitative real-time PCR was performed as previously described [16], for detection of EBV DNA and the subsequent determination of viral DNA load in whole blood (formal detection limit 10^2 copies/ml, some variation in exact quantification between 10^2 and 10^3 copies/ml). 10^2 copies/ml were used as the qualitative detection limit [positive EBV PCR results (EBV^+) versus negative EBV PCR results (EBV^-)]. Nucleic acid was extracted from 200 µl of whole blood.

Drug measurement and EBV monospot testing

Cyclosporine A trough levels were measured locally by a validated immunoassay (Dade-Behring Marburg GmbH, Marburg, Germany) in human EDTA blood. Plasma mycophenolic acid (MPA) trough concentrations were measured locally by a validated Emit® MPA Assay (Dade-Behring Marburg GmbH, Marburg, Germany), a homogeneous enzyme immunoassay for the quantitative analysis of MPA in human plasma. Separation of plasma was performed immediately in a centrifuge at +4 °C. CsA and MMF total daily dose was administered as a usually equal morning-and-evening dose; no midday dose was given.

The EBV monospot testing was performed using the Avitex IM latex slide agglutination test, based upon the reaction between infectious mononucleosis antibodies and a latex reagent sensitized with a bovine red cell mononucleosis antigen. Fifty microliter of fresh EDTA plasma samples were used according to manufacturer's guidelines (Omega Diagnostics LTD., Hillfoots Business Village, Alva, Scotland, UK).

All human studies have been reviewed by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 2000 Declaration of Helsinki.

Statistics

Statistical analyses were performed with Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA). Because of limited sample size and lack of normal distribution, nonparametric testing (two way: Wilcoxon signed rank test, chi-squared test; three-way: Friedman test) was applied for comparisons between groups. Z-test was applied for comparison of proportions between groups. Univariate analysis of risk factors for dichotomous, categorical results of EBV DNA testing (positive/negative) was performed. *P*-values of ≤0.05 were considered as statistically significant.

Results

The EBV^+ PCR results in the initial screening examination were found in 39.0% (67/172) of patients with a median copy number of 13×10^3 copies/ml (range 0.1×10^3–850.0×10^3 copies/ml, Fig. 2). In follow-up testing of these 67 EBV^+ patients 6 months later, 36/67 (20.9% of total) patients continued to have a positive EBV PCR result ($EBV^{+/+}$), while 31/67 (18.0% of total) patients had a negative retest result ($EBV^{+/-}$). Of all 172 patients, 170 (98.8%) were found to be EBV IgG positive prior to HTX. EBV monospot testing was performed to exclude acute infection. All patients tested for EBV viral load were negative for CMV antigen (pp65) 0–6 months previously (routine annual tests). Two male patients were tested negative for EBV; one of these patients remained always EBV IgG negative during follow up and the other seroconverted (IgG) during an assumed clinically unapparent acute infection post-HTX. These two patients remained free of lymphoma throughout the study and subsequent follow up (12/2007), equivalent to more than 6.0 years after HTX. All 36 patients with positive EBV DNA-levels on both testing occasions ($EBV^{+/+}$) post-transplantation showed positive pretransplantation EBV serology. Thirty out of 31 patients (96.8% of this subgroup) with undetectable EBV DNA upon re-testing ($EBV^{+/-}$) were EBV seropositive prior to HTX; in one patient acute EBV infection was suspected, despite normal

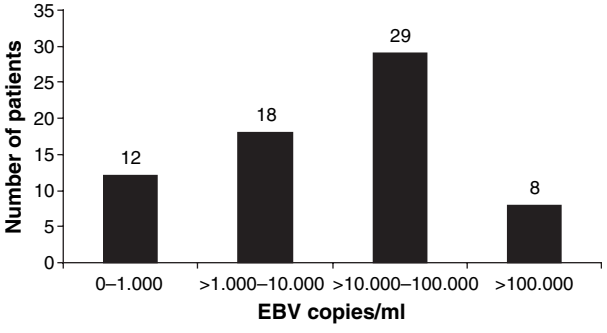


Figure 2 Distribution of EBV load. Distribution of EBV load at initial examination in copies/ml.

Table 2a. Type of immunosuppressive drugs and EBV PCR findings at initial and follow-up testing.

Immuno-suppressant:	EBV^{+} patients at initial testing (n = 67)	$EBV^{+/-}$ follow-up (n = 31)	$EBV^{+/+}$ follow-up (n = 36)
ATG			
+ (n = 172)	39.0%	18.0%	20.9%
– (n = 0)	n.d. (all patients received ATG)	n.d. (all patients received ATG)	n.d. (all patients received ATG)
CNI			
+ (n = 139)	45.3%	20.1%	25.2%
–(33)	12.1% ($P < 0.01$)	9.1% (P = n.s.)	3.0% ($P < 0.01$)
AZA			
+ (n = 22)	90.9%	31.8%	59.1%
–(n = 150)	31.3% ($P < 0.01$)	16.0% (P = n.s.)	15.3% ($P < 0.01$)
MMF			
+ (n = 132)	25.8%	15.2%	10.6%
–(n = 40)	82.5% ($P < 0.01$)	27.5% (P = n.s.)	55.0% ($P < 0.01$)
Corticosteroids			
+ (n = 54)	46.3%	20.3%	25.9%
–(n = 118)	35.6% (P = n.s.)	16.9% P = n.s.	18.6% (P = n.s.)

ATG, antithymocyte globulin; AZA, azathioprine; CNI, calcineurin inhibitor; EBV^{+}, initially EBV DNA positive; $EBV^{+/-}$, EBV DNA positive at initial testing and negative at follow-up testing; $EBV^{+/+}$, EBV DNA positive at initial and follow-up testing; MMF, mycophenolate mofetil.
Frequency of qualitative EBV DNA findings as % of total number of patients in respective medication subgroups.

Table 2b. Risk factors for various EBV PCR results.

Patient characteristics:	$EBV^{+/+}$ (n = 36)	$EBV^{+/-}$ (n = 31)	EBV^{-} (n = 105)
Recipient age (years, ±SD)	49.7 (11.6)	52.3 (8.9)	51.3 (10.5) P = n.s.
Time post-HTX (years, ±SD)	5.6 (4.8)	6.7 (5.3)	6.0 (5.1) P = n.s.
Female	22.2%	22.6%	20.0% P = n.s.
Rejection index (±SD)	0.6 (1.7)	0.5 (1.4)	0.5 (1.6) P = n.s.

$EBV^{+/+}$, EBV DNA positive at initial and follow-up testing; $EBV^{+/-}$, EBV DNA positive at initial and negative at follow-up testing; EBV^{-}, EBV DNA negative at initial testing; HTX, heart transplantation; SD, standard deviation.
Various demographic characteristics as mean ± SD or % of subgroup are shown for each EBV PCR subgroup.

monospot testing. In comparison, 99.0% (104/105) of transplant recipients with negative EBV PCR results (EBV^{-}) were EBV sero-positive prior to transplant. This difference was not statistically significant (P = n.s.). There was no clear association between positive EBV DNA load with respect to time after transplantation, gender, recipient age, recipient diagnosis, number of previous rejection episodes, or induction therapy (all patients received ATG according to the local standard of care [13] (Table 2a,b).

Overall incidence of EBV DNA detection (all patients ever positive for EBV) was significantly higher in patients on immunosuppressive regimens including calcineurin inhibitors (CNI) or AZA as well as in patients not on MMF medication ($P < 0.01$). Interestingly, this association was markedly stronger in the subgroup of patients that were positive for EBV DNA on both testing occasions ($EBV^{+/+}$) (Table 2a).

In patients with positive EBV DNA levels at initial examination and negative EBV DNA load at retesting ($EBV^{+/-}$), mean CsA levels were found to be significantly higher at initial testing when compared with retesting (148.4 ± 70.2 ng/ml vs. 119.6 ± 53.5 ng/ml, $P < 0.05$). In these patients ($EBV^{+/-}$), concomitant immunosuppression was not significantly altered (Table 2c). Patients undergoing a qualitative change in immunosuppressive regimen were not included in this analysis.

In contrast, in patients with two positive EBV DNA results ($EBV^{+/+}$), mean CsA levels at initial EBV PCR examination and at retesting were identical (96.7 ± 42.3 ng/ml vs. 106.9 ± 50.9 ng/ml, P = n.s.) (Table 2c). In this subgroup ($EBV^{+/+}$), 10 patients showed a change of EBV DNA levels of at least one order of magnitude (10-fold change; higher n = 7, lower n = 3). Eight of these 10 patients (80.0%) had a concordant change in CsA trough levels of more than 25%, while concomitant immunosuppression remained constant. In the remaining two patients, who had more than a 10-fold increase in EBV DNA load, CsA levels remained unchanged (less than 25.0% change), while concomitant immunosuppression had been significantly increased (doubling of MMF dose). In $EBV^{+/+}$ patients with changes in EBV DNA levels of less than one order of magnitude, CsA levels and concomitant immunosuppressive medication remained unchanged (less than 25.0% difference) in 10 patients remaining on the same qualitative immunosuppression protocol. In 16 patients, qualitative alteration of immunosuppressive regimen between EBV DNA tests precluded this type of analysis.

Table 2c. Characteristics of immunosuppressive drugs as found in various EBV PCR groups at initial and follow-up testing.

Immunosuppressive drug	Baseline	Follow-up	Level of significance
EBV$^{+/-}$	**Positive *n* = 31**	**Negative**	**versus baseline**
CsA level, SD (dose, SD) (μg/ml)	148.4, 70.2 (179.5, 53.9)	119.6, 53.5 (163.5, 39.9)	$P < 0.05$, $n = 14$
TAC level, SD (dose, SD) (μg/ml)	7.3, 0.7 (4.5, 3.9)	9.7, 4.4 (8.0, 6.4)	P = n.s., $n = 4$
MPA level, SD (MMF dose, SD) (mg/l)	2.0, 0.8 (2615.4, 858.3)	1.6, 0.6 (2423.1, 916.6)	P = n.s., $n = 18$
EVL level, SD (dose, SD) [ng/ml]	3.8, 1.5 (1.4, 0.1)	4.7, 2.2 (1.8, 0.9)	P = n.s., $n = 4$
RAPA level, SD (dose, SD) (ng/ml)	5.2, 0.0 (1.0, 0.0)	5.5, 0.0 (1.0, 0.0)	P = n.s., $n = 2$
AZA dose, SD (mg)	62.5, 12.5	50.0, 0.0	P = n.s., $n = 4$
Patients on corticosteroids in %	35.5	19.4	P = n.s. (0.3)
EBV$^{+/+}$	**Positive *n* = 36**	**Positive**	
CsA level, SD (dose, SD) (μg/ml]) $n = 19$	96.7, 42.3 (170.4, 60.2)	106.9, 50.9 (147.9, 61.7)	P = n.s., $n = 20$
TAC level, SD (dose, SD) (μg/ml) $n = 2$	4.3, 0.0 (5.0, 0.0)	6.9, 0.0 (7.0, 0.0)	P = n.s., $n = 2$
MPA level, SD (MMF dose, SD) [mg/l] $n = 9$	2.7, 1.8 (2166.7, 1280.2)	3.1, 2.2 (1833.3, 1105.5)	P = n.s., $n = 10$
EVL level, SD (dose, SD) (ng/ml)	8.7, 0.8 (1.8, 0.3)	n.a.	n.a., $n = 2$ (initial)
RAPA level, SD (dose, SD) (ng/ml)	7.9, 0.0 (2.0, 0.0)	5.8, 1.8 (1.7, 0.5)	P = n.s., $n = 1$ (initial) $n = 3$ (follow-up)
AZA dose, SD (mg)	62.5, 16.6	47.5, 16.6	P = n.s., $n = 7$
Patients on corticosteroids in %	38.9	22.2	P = n.s.
EBV^{-}	**Negative *n* = 105**		
CsA level, SD (dose, SD) (μg/ml)	119.7, 68.5 (178.7, 73.3)		
TAC level, SD (dose, SD) (ng/ml)	10.6, 3.7 (5.3, 2.6)		
MPA level, SD (MMF dose, SD) (mg/l)	2.7, 2.4 (2162.5, 923.6)		
EVL level, SD (dose, SD) (ng/ml)	6.8, 2.5 (1.8, 0.6)		
RAPA level, SD (dose, SD) (ng/ml)	6.1, 2.1 (1.7, 0.6)		
AZA dose, SD [mg]	62.5, 12.5		
Patients on corticosteroids in %	27.6		

AZA, azathioprine; CsA, cyclosporine A; EBV, Epstein-Barr Virus; EVL, everolimus; MMF, mycophenolate mofetil; MPA, mycophenolic acid; PCR, polymerase chain reaction; RAPA, sirolimus; TAC, tacrolimus.

In patients negative for EBV DNA on first and only testing (EBV$^-$), mean blood concentration and doses of immunosuppressants were similar to that of patients ever positive for EBV DNA (Table 2c); the lack of a second EBV DNA determination precludes statistical assessment of the association of EBV DNA level and change in immunosuppression as in the EBV$^+$ patients. Immunosuppressive regimens in all patients, directly related to EBV DNA results are shown in Table 2a,c and Fig. 1.

PTLD

Out of the studied patients, three patients (1.7%, 3/172) were diagnosed with PTLD during the course of study (mean follow up 4.0 years). Two lymphomas were diagnosed as EBV negative (one male and one female patient), one lymphoma (male patient) was EBV positive by immunohistology (LMP-1 positive, EBNA-2 positive, ZEBRA not tested). This patient had been positive for EBV DNA on both tests with viral load quantities in the upper range, but comparable to patients without lymphoma (1.4×10^5 and 6.4×10^5 copies/ml). Details of clinical course are summarized in Table 3 and Fig. 3.

Discussion

Within this study, quantitative real-time PCR was used to demonstrate consistently elevated EBV DNA in whole blood in 20.9% of adult HTX patients with considerable individual fluctuations in quantitative viral load. In another 18.0% of patients, EBV DNA detection was only temporary. This was either attributable to acute infection in one patient tested negatively prior to transplantation (despite a normal monospot testing before the first EBV PCR), or more commonly, temporary reactivation of viral replication.

In line with previous studies regarding polyomavirus (BK virus) infections [17], overall incidence of EBV DNA detection in patients ever positive for EBV DNA (EBV$^+$) was significantly higher in patients on immunosuppressive regimens including a CNI or AZA as well as in patients not on MMF medication. This correlation becomes markedly stronger upon selective analysis of patients positive for EBV DNA on both testing occasions (EBV$^{+/+}$). This finding appears to implicate CNI treatment per se and its qualitative degree (see below) as a potential risk factor for reoccurrence of EBV DNA in

Table 3. Characteristics, clinical course and management of PTLD patients.

	Patient 1	Patient 2	Patient 3
Sex	Male	Male	Female
EBV serostatus prior to HTX (IgG)	Positive	Positive	Positive
EBV load at time of diagnosis of PTLD (DNA copies/ml)	640×10^3	Negative	Negative
Clinical features	Highly malignant B-NHL stage IV EBV positive (LMP-1 positive, EBNA-2 positive, ZEBRA not tested)	Highly malignant B-NHL stage IV EBV negative	Suspected B-NHL with cerebral and spleen lesions EBV negative
Chemotherapy [time for therapy (month/year)]	Prednisone, Vincristine (12/04) 8 series of Rituximab-CHOP-14 (1/05–6/05) Prednisone, Gemcitabine, Oxaliplatinum (6/05)	4 series of Rituximab-CHOP- 21 (10/06–2/07)	reduction of immunosuppression (2/02)
Complications and outcome (month/year)	Mucositis (stage III), Herpes-stomatitis, sepsis (during pancytopenia), polyneuropathy (1/05–6/05) Early relapse of highly malignant B-NHL (6/05) exitus (7/05)	Restaging MRI: complete Cru (3/07)	Focal cerebral and spleen lesions (2/02) Complete remission (8/03)
Immunosuppression (daily dose)	Cyclosporine A, 175 mg Azathioprine, 50 mg ↓ Cyclosporine A, 50 mg	Tacrolimus, 6 mg Azathioprine, 100 mg ↓ Everolimus, 4 mg Prednisone, 5 mg	Cyclosporine A, 250 mg Azathioprine, 100 mg ↓ Cyclosporine A, 200 mg Prednisone, 5 mg

CHOP, cyclophosphamide, hydroxydaunorubicin (adriamycin), oncovin (vincristine), prednisone/prednisolone; CRu, unconfirmed complete response; EBNA: Epstein-Barr Virus nuclear antigen; EBV, Epstein-Barr Virus; HTX, heart transplantation; IgG, immunoglobulin G; LMP, latent membrane protein; MRI, magnetic resonance imaging; NHL, non-Hodgkin Lymphoma; PTLD, post-transplant lymphoproliferative disorder; ZEBRA, z Epstein-Barr Virus replication activator.

seropositive individuals. The present data suggest that treatment with AZA seems to increase the frequency of EBV DNA load in patients (EBV reactivation), whereas MMF immunosuppression might have a protective effect. These findings correspond to recent data in pediatric patients [18], although it needs to be acknowledged that the mechanisms and implications of EBV (re)activation differ widely in these patient populations. The association of elevated EBV DNA levels with the absence of MMF co-medication is largely a direct consequence of positive association with AZA medication, as these two drugs are used mutually exclusive in combination regimens. Given the more recent introduction of MMF, patients still on AZA medication tend to continue with the same after transplantation, possibly explaining the larger postoperative time in $EBV^{+/+}$ and $EBV^{+/-}$ patients (P = n.s.).

Significantly higher CsA levels were seen in the initial assessment (EBV^{+}) when compared with the reassessment values (EBV^{-}) in patients with one positive and one negative EBV DNA test ($EBV^{+/-}$), whereas CsA levels in $EBV^{+/+}$ positive patients were not significantly different upon retesting. Additionally, in the $EBV^{+/+}$ subgroup, 80% of patients with a change of EBV DNA levels of at least one order of magnitude (10-fold change) had a concordant change in CsA trough levels of more than 25%, while concomitant immunosuppression remained unchanged. This data, because of the association established with qualitative and quantitative immunosuppression, would suggest EBV DNA testing might be a useful tool for monitoring the individual degree of immunosuppression in adult patients after HTX [19].

No further correlation of EBV DNA presence with demographic or clinical characteristics was found, includ-

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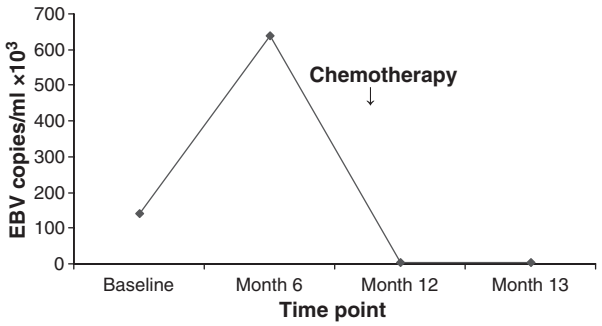

Figure 3 EBV load in EBV-positive lymphoma. Two months after the second testing for EBV DNA, another male patient developed PTLD with lymph node manifestation and soft tissue infiltration (stage IV). This patient had a positive EBV pre transplantation status. This patient's lymphoma was EBV positive by immunohistology (LMP-1 positive, EBNA-2 positive, ZEBRA not tested). In addition, EBV DNA levels were 1.4×10^5 copies/ml at initial examination [1], rising further to 6.4×10^5 copies/ml upon retesting [2]. After treatment with eight series of Rituximab/CHOP the viral load decreased to 4.0×10^3 copies/ml [3] and remained on this level [4]. Despite initial marked treatment response, the patient died from early lymphoma recurrence.

ing recipient age, gender, and time post-transplantation, which is in contrast to previous studies in liver transplant recipients [20]. Unlike OKT-3, ATG has not been linked with an increased incidence of lymphomas after HTX [5]. All patients received ATG according to the local standard of care [13], therefore effects of ATG, if any, cannot be evaluated in this cohort of stable HTX patients. Association of EBV DNA detection and clinical events (rejection, infection, malignancy other than PTLD, etc.) were precluded by the lack of longitudinal data >6 months, but should be addressed in future studies.

Symptomatic CMV infection has been implicated to trigger EBV infection/reactivation [21]. Only one possible case of new EBV infection was seen in the present cohort. In addition, all patients were negative for CMV antigen (pp 65) 0–6 months prior to EBV viral load determination, making a strong association of CMV/EBV co-infection to be less likely in the current study.

Although evidence supports elevations of EBV load in the peripheral blood of patients after solid organ transplantation as an adjunct to making the diagnosis of EBV-induced PTLD [16,22,23], our data in adult heart transplant patients show no clear association between EBV load and PTLD development. Because of the low absolute incidence of PTLD in this study population and two cases (66.7%) of EBV negative PTLD, the value of EBV DNA testing for PTLD development appears limited. EBV DNA levels in the one patient developing EBV-positive PTLD were elevated, but were not significantly higher when compared with the other EBV-positive patients after HTX free from lymphoma. In contrast to previous studies, suggesting a threshold value of 1.0×10^4 EBV copies/ml plasma [16,22–24], no cut-off values for therapeutic interventions to prevent lymphoma can be defined because of this limited association and the large overlap of EBV loads with apparently disease-free transplant patients.

Limitations of the current study include the short longitudinal assessment of EBV DNA loads (6 months) and the comparatively low number of patients developing PTLD (three patients out of 172, 1.7%) when compared with previous studies [1], which may be attributed to the relatively short time from transplant (median 4.9 years) and generally low level of immunosuppression [19]. The present univariate analysis of association of EBV DNA with immunosuppressive regimens excludes numerous other demographic and clinical variables that may also influence EBV reactivation. Although gender, age, and time post-HTX did not correlate with EBV status, multivariate analysis might provide additional information, but was precluded by small sample size. Currently published data regarding EBV load and clinical course are discrepant [25], which might be as a result of the therapy chosen to treat PTLD (reduction of immunosuppression, chemotherapy, rituximab or antiviral agent) [26], and as a consequence, this variable would require further investigation in future large multi-center trials.

Conclusions

After HTX, the diagnostic sensitivity/specificity of EBV load determination by quantitative real-time PCR for the occurrence of PTLD is low. In contrast to previous studies, especially from pediatric bone marrow transplantation patients [12], where patients with excessive EBV DNA levels in plasma ($>1.0 \times 10^4$ copies/ml plasma) or a drastic increase in EBV viral load appear to be at an increased risk to develop PTLD [27], our data show no association of EBV DNA levels in whole blood with PTLD development. Therefore, serial EBV DNA testing is unlikely to be a useful diagnostic tool for early detection of PTLD in adult heart transplant patients, but might be a useful adjunct in the assessment of individual degree of immunosuppression [19]. Individual longitudinal follow up of the presence of EBV DNA and correlation with clinical events in (heart) transplant recipients appear to be warranted.

Authorship

AOD: designed study, collected and analyzed data, draft of manuscript; MK: collection/interpretation of data; SC: collected data; AK: collected data; LF: collected data; FUS: data interpretation, PS: data interpretation; PS: data interpretation, HAK: final approval; and TJD: designed study, data interpretation, final approval.

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