Epstein–Barr Viral Load is Associated to Response in AIDS-Related Lymphomas

Paula Yurie Tanaka · Kouichi Ohshima · Masao Matsuoka · Ester Cerdeira Sabino · Suzete Cleusa Ferreira · Anna Shoko Nishya · Renata de Oliveira Costa · Edenilson Eduardo Calore · Nilda Maria Perez · Juliana Pereira

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Abstract AIDS-related lymphoma (ARL) development is associated to immunodeficiency state with proliferation of B-cells driven by HIV itself and EBV infection. However, Epstein–Barr DNA is not detected in malignant cells of all ARL subtypes. A prospective and controlled study to analyze EBV viral load (VL) in plasma and peripheral blood mononuclear cells (PBMC) of ARL patients was performed to analyze if Epstein–Barr VL could be related to response in these patients. Fifteen patients with ARL were included in this study with measurement of EBV VL at three different periods of time: at lymphoma diagnosis, upon completion of chemotherapy, and 3 months after. Two control groups composed by HIV-negative and HIV-positive patients were also evaluated for EBV VL comparison. In situ hybridization for EBER was performed on diagnostic samples of all ARL patients. Median EBV VL in PBMC and plasma had a significant decrease ($p = 0.022$ and $p = 0.003$, respectively) after ARL treatment. EBER was positive in 7 (46.7 %) cases. Median EBV VL in

PBMC before lymphoma treatment in patients positive for EBER was significantly higher compared to EBER negative cases ($p = 0.041$). Reduction of EBV viral load during treatment of lymphoma could be predictive of response. EBER expression was associated to advanced stages of disease and worse immune status. Our study suggests that measurement of EBV VL during ARL treatment could be used as a marker for response, but further studies are needed to validate this association.

Keywords Aids-related lymphoma · HIV · EBV

Short Communication

Human immunodeficiency virus (HIV) infection increases the risk for development of non-Hodgkin lymphoma by 100-fold compared to general population [1]. Most acquired immunodeficiency syndrome-related lymphomas

P. Y. Tanaka · J. Pereira
Faculdade de Medicina – Programa de Ciências Médicas,
Universidade de São Paulo, Av. Dr. Arnaldo, 455, São Paulo,
Brazil
e-mail: pyt@uol.com.br

K. Ohshima
Department of Pathology, School of Medicine, Kurume
University, 67 Asahi-machi, Kurume-shi, Fukuoka-ken, Japan

M. Matsuoka
Institute for Virus Research, Kyoto University, 53 Asahi-machi,
Kurume-shi, Fukuoka-ken, Japan

E. C. Sabino · S. C. Ferreira · A. S. Nishya
Laboratório de biologia molecular –Fundação pró-sangue, Av.
Dr. Enéas de Carvalho Aguiar, 155, São Paulo, Brazil

R. de Oliveira Costa · J. Pereira
Instituto do Câncer do Estado de São Paulo, Av. Dr. Arnaldo,
251, São Paulo, Brazil

E. E. Calore · N. M. Perez
Pathology Section - Instituto de Infectologia Emílio Ribas, Av.
Dr. Arnaldo, 165, São Paulo, Brazil

J. Pereira (✉)
Laboratório de Imunopatologia, Prédio de Ambulatórios do
Hospital das Clínicas, Av. Dr. Enéas de Carvalho Aguiar, 255,
1º andar, bloco 12 sala 61, Cep: 05403 – 001, São Paulo, Brazil
e-mail: julianapereira29@hotmail.com

(ARL) are diffuse large B cell lymphomas (DLBCL) and Burkitt lymphomas (BL). After introduction of highly active antiretroviral therapy (HAART) the incidence of ARL seems to be decreased mainly for primary central nervous system lymphoma (PCNSL). However, ARL remains the main cause of death related to AIDS in adults infected with HIV. Adverse prognostic factors for ARL included a CD4+ count less than 100 cells/ μ L, poor performance status, previous AIDS diagnosis and high risk in international prognostic index (IPI) [2]. The pathogenesis of ARL is associated to many factors including a continuous stimulation of B-cells by other viruses as Epstein–Barr virus (EBV) and human herpes virus type 8 with a poor immune status. EBV infection has been described in 40–90 % of ARL. The role of the EBV in pathogenesis and prognosis in ARL remains unclear [3]. EBV is not always found in the malignant cells but in cases of PCNSL and primary effusion lymphomas [4]. EBV viral load and EBV encoded small RNA (EBER) expression has been studied as prognostic factors in ARL with contradictory results [5, 6]. In order to analyze the impact of EBV viral load in serum and peripheral blood mononuclear cells (PBMC), EBER expression in ARL patients we performed a prospective and controlled study. Fifteen HIV-positive patients with DLBCL and BL according to the World Health Organization classification treated at Instituto de Infectologia Emilio Ribas and Hospital das Clínicas - FMUSP from January 2009 to March 2010 were studied. Patients were staged by Ann Arbor system and were treated with standard dose of CHOP (cyclophosphamide 750 mg/m² D1, doxorubicin 50 mg/m² D1, vincristine 1.4 mg/m² D1 and prednisone 60 mg/m² D1–D5) for 6–8 cycles. Central nervous system prophylaxis was performed for all cases using four intrathecal injection of cytarabine 50 mg plus dexamethasone 2 mg weekly in the first 4 weeks of treatment. In case of a cerebrospinal fluid involvement, intrathecal chemotherapy was administered twice-weekly, until total disappearance of the malignant cells. Granulocyte colony stimulating factor at dose of the 300 μ g/day was indicated for all patients from day 5 of the chemotherapy cycle until the neutrophil counts exceeded 1.5×10^9 /L. All patients received prophylaxis for *Pneumocystis carinii* and HAART was used for all patients. Zidovudine use was avoided due to hematological toxicity. The response to lymphoma treatment was assessed after cycles 4, 6 and 8 and was classified as complete response (CR), partial response, unconfirmed CR (CRu) or progressive disease (PD) according to International Workshop criteria [7]. Patients with PD or relapsed disease (RD) were treated with the salvage chemotherapy regimen ICE (ifosfamide, carboplatin, etoposide). Peripheral venous blood was harvested for EBV viral load measure at lymphoma diagnosis, after completion of chemotherapy, and 3 months afterwards.

Two control groups composed by 26 HIV-positive patients without opportunistic infections and 30 HIV-negative individuals were also evaluated for EBV VL in PBMC and serum for comparison. Biopsy samples of lymphoma diagnosis were evaluated by situ hybridization for EBER analysis. This study was approved and informed consent was provided, according to the Brazilian Research Ethical Committee. Paraffin sections of tumor were processed for in situ hybridization using the EBER-PNA probe from DakoCytomation according to the manufacturer's instructions. Real-time polymerase chain reaction for EBV was performed using DNA extracted from 200 μ L from serum and from PBMC that were obtained from whole blood before stored at -20°C . For EBV viral load analysis the QIAamp DNA blood reactive from Qiagen was used. The detection value for EBV VL in serum was 68 copies/mL. For statistical analysis, the comparison between continuous variables in different groups was performed by Kruskal–Wallis test followed by Mann–Whitney's if results were significant. For discrete variables Fisher's exact test was done. Survival curves were estimated by Kaplan and Meier method. Wilcoxon's test was performed to compare EBV viral load in PBMC and serum in ARL patients at different times. To compare EBV viral load between serum and PBMC Spearman's correlation test was performed. For all analyzes significant results were considered for $p < 0.050$. Patient's clinical features are shown in Table 1, 13/15 (86.7 %) patients were male, with median age of 43 years (28–66 years). Risk factors for HIV infection included sexual contact was seen in 14/15 (93.3 %) and intravenous drug addict in 1/15 (6.7 %). DLBCL was found in 10/15 (66.7 %), BL in 4/15 (26.7 %) and PCNSL in 1/15 (6.7 %). Eleven (78.6 %) patients presented stage III or IV at diagnosis and the IPI score of high-intermediate and high-risk was observed in 7/15 (46.7 %) patients. Median CD4 T-cell count at diagnosis was 215 cells/ μ L (43–930) and 6/15 (40 %) patients showed HIV viral load above the cut-off point (50 copies/mL). Fourteen (93.3 %) patients were treated with CHOP and the patient with PCNSL received only radiotherapy. This patient acquired CR but died few months later. Nine (64.3 %) patients received full dose of CHOP and acquired CR. Five of nine patients (55 %) died later, two in relapsed disease. Other 2/15 patients died during chemotherapy and 2/15 presented PD and died, one of them after salvage chemotherapy. One patient abandoned treatment after the first cycle of chemotherapy and died later. All patients but one with relapsed disease died of sepsis. Four patients started HAART at the time of lymphoma diagnosis. Two patients received two nucleosides reverse transcriptase inhibitor (NRTI) plus one PI and two were treated with two NRTI plus 1 non-nucleoside reverse transcriptase inhibitor (NNRTI). Ten patients had been currently receiving HAART; four were using two NRTI plus

Table 1 Characteristics of patients ($n = 15$)

Age year, median (range)	43.0 (28–66)
Sex, n (%)	
Female	2 (13.3)
Male	13 (86.7)
Risk factors HIV, n (%)	
Homosexual	8 (53.3)
IVDU	1 (6.7)
Heterosexual	6 (40.0)
Prior aids, n (%)	
Yes	11 (73.3)
No	4 (26.7)
CD4 count cells/ μ L, n (%)	
<100	10 (66.7)
≥ 100	5 (33.3)
Pathologic type, n (%)	
DLBCL	10 (66.7)
BL	4 (26.7)
PCNSL	1 (6.7)
B symptoms, n (%)	
Present	13 (86.7)
Absent	2 (14.3)
Stage, n (%)	
I/II	3 (21.4)
III/IV	11 (78.6)
Extranodal sites, n (%)	
Yes	12 (80.0)
No	3 (20.0)
Number of extranodal sites, n (%)	
0–1	9 (60.0)
≥ 2	6 (40.0)
LDH level U/L, median(range)	386.0 (161–10,308)
LDH level, n (%)	
$\leq$ Normal	7 (46.7)
>Normal	8 (53.3)

IVDU intravenous drug abuse, AIDS acquired immunodeficiency syndrome, LDH lactic dehydrogenase, DLBCL diffuse large B-cell lymphoma, BL Burkitt lymphoma, PCNSL primary central nervous system lymphoma

one PI, and six patients two NRTI plus one NNRTI. One patient did not use HAART, although prescribed before lymphoma diagnosis and the use of two NRTI plus one PI was initiated. There were no differences between the type of antiretroviral used and EBV viral load. EBV viral load in PBMCs and serum was performed for all patients at diagnosis, in 10/15 (66.6 %) patients after treatment and in four patients 3 months afterwards. Median EBV viral load before treatment was 1.3 copies/ 10^6 cells (0.1–147.2) in PBMC and were 40 copies/mL (0–24,900) in serum. During treatment the EBV viral load decrease to 0.05 copies/ 10^6 cells

Fig. 1 Probability of survival among patients with ARL

(0–10.9) in serum and was undetectable in PBMC. EBV viral load was significantly reduced after the last cycle of chemotherapy in comparison to diagnosis ($p = 0.022$ and $p = 0.003$, serum and PBMC, respectively). The EBV viral load remained not detectable or in low levels in PBMC and serum of patients with CR. Overall survival at 12 months of follow-up was 21.4 % (Fig. 1). No significant correlation was found between EBV viral load in PBMC or serum with CD4 T-cell counts and HIV viral load at diagnosis of lymphoma ($r = 0.166$ $p = 0.554$). In the control groups, EBV viral load in serum was not detectable (HIV-negative) and was lower in HIV-positive than in ARL patients ($p < 0.01$). The median of EBV viral load in PBMC in HIV-positive and HIV-negative control groups was 0.1 copy/ 10^6 PBMC (0–1.25) and undetectable, respectively. EBV viral load (PBMC) in ARL patients at diagnosis was higher than in HIV positive and HIV-negative control groups ($p = 0.002$ and $p < 0.001$, respectively). EBER was positive in 7/15 (46.7 %) of ARL in cases with more than 1 extranodal site ($p = 0.041$) and T CD4 counts less than 100 cells/ μ L ($p = 0.026$). Median EBV viral load in PBMC before treatment among patients with positive EBER expression was significantly higher compared to those with negative expression ($p = 0.041$). This study suggests that higher levels of EBV viral load could be associated with more aggressive lymphoma and poor immune status (measured by absolute counts of T-CD4 lymphocytes). There was a significant correlation between EBV load in PBMC and EBER expression in tumor cells. The real significance of EBV VL in the setting of the ARL still remains unclear. There aren't studies providing relevant evidence of EBV sequences inserted in tumor cells. Usually patients included have different status of HIV infection and do not account the adherence to antiretroviral therapy neither the type of immune reconstitution [8]. The type of EBV infection

latency in ARL patients has not been elucidated. EBV viral load does not seem to be predictive for ARL development, but some studies suggest it may be related to lymphoma treatment outcomes. Fan et al., detected EBV viral load in 74 % cases of 35 ARLs patients. EBV VL was found in the serum of all patients whose samples were positive for EBER ($n = 17$). In agreement with our study these authors demonstrated that EBV viral load decrease at completion of lymphoma therapy [9]. Bonnet et al., measured EBV viral load in whole blood of 14 ARL patients, 19 HIV non-AIDS patients and 20 HIV seronegative patients, with significant difference in patients with ARL compared to HIV-negative patients. Patients with ARL achieving CR had significant decrease in EBV VL [10]. These data suggests that the decrease of EBV viral load during treatment of ARL could be related to response. EBER expression was present in 7/15 (46.5 %) of ARL patients and related to extranodal disease and CD4 T-cell count less than 100 cells/ μ L. These data indicates that EBER expression might be associated with more advanced disease and with severe immunodeficiency. Studies in patients with lymphoma without HIV infection have also correlated EBER expression with more advanced disease, similar to our results [11, 12]. In conclusion, our study suggests that measurement of EBV VL during ARL treatment could be a predictive factor for response, although further studies with a higher number of patients are needed to validate this association.

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