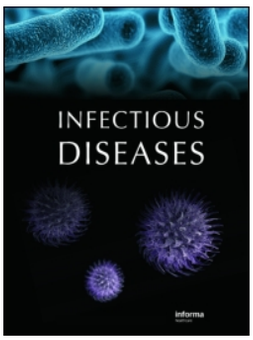

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A longitudinal and cross-sectional study ofEpstein-Barr virus DNA load: a possible predictor of AIDS-related lymphoma in HIV-infected patients

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ORIGINAL ARTICLE

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A longitudinal and cross-sectional study of Epstein-Barr virus DNA load: a possible predictor of AIDS-related lymphoma in HIV-infected patients

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ABSTRACT

Introduction: HIV-infected patients are more than 100-fold greater at risk for developing malignant AIDS-related lymphoma (ARL) compared to the general population. Most ARLs are EBV related. The main purpose of this study was to investigate whether a high peak EBV DNA load in HIV-infected patients is predictive of ARL, including classical Hodgkin lymphoma.

Methods: From an ongoing prospective HIV positive cohort study, we conducted a case-control study between 2004 and 2016 among patients from whom at least one EBV DNA load in serum or plasma was available. We compared peak EBV DNA load between patients with (49 cases) and without ARL (156 controls).

Results: The geometric mean of the peak EBV DNA load measured before diagnosis of malignant lymphoma was 52,565 IU/mL in EBER-positive lymphoma patients vs. 127 IU/mL in controls ($p < .001$). Patients with EBV DNA loads >100,000 IU/mL have an increased risk for diagnosis of malignant lymphoma compared to patients with EBV DNA loads ≤100,000 IU/mL (adjusted OR 12.53; 95%CI: 4.08; 38.42). In the longitudinal study, including 13 patients with at least three left-over plasma samples available for retesting, measurements of EBV-DNA during the preceding 12 months proved to be of poor value for predicting subsequent lymphoma diagnosis.

Conclusions: A EBV DNA load >100,000 IU/mL can be useful in clinical setting to accelerate time to diagnosis and treatment. EBV-DNA loads in samples taken during the preceding year of ARL diagnosis showed to be of poor predictive value.

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Introduction

Before the era of combined antiretroviral therapy (cART), HIV-infected patients were at more than hundred-fold greater risk of developing non-Hodgkin lymphoma and more than seven-fold greater risk of developing classical Hodgkin lymphoma compared to the general population [1]. Despite the declining incidence of AIDS-related lymphoma (ARL) since the introduction of cART [2,3], it constitutes >50% of all AIDS-related malignancies [4,5] and remains the main cause of AIDS-related deaths in HIV infected adults [5,6]. Epstein-Barr virus (EBV) is considered to be involved in the pathogenesis in around half of all ARL [7] and in nearly all cases of HIV-associated Hodgkin lymphoma [7–9]. Multiple studies reported on the value of EBV DNA load in plasma as a prognostic marker of ARL treatment [10–13]. The extent to which EBV-DNA is elevated in serum, plasma or blood before, at the time, and after ARL diagnosis is still insufficiently explored [12,13]. The main purpose of this study was to investigate whether a high peak EBV DNA load in HIV-infected patients is predictive of ARL, including classical Hodgkin lymphoma.

Methods

Study population

Stored EBV-DNA load samples from HIV-infected patients who were in care of our hospital from January 2004 to August 2016 were identified in the virology laboratory. We retrieved patients data from the HIV Monitoring Foundation ATHENA cohort database. This cohort includes data obtained from all HIV-infected patients in care from 1997 onward in the Erasmus Medical Center Rotterdam. Informed consent was obtained from all individuals [14]. Data that were not available were marked as missing data. $CD4^+$ cell count and plasma HIV RNA load assessed six months prior to or after peak EBV DNA were collected. Patients with missing quantitative EBV DNA and patients lost to follow-up were excluded. To improve power of the study, for each case four HIV positive controls without ARL were randomly selected from the database of the virology laboratory. In patients with at least three stored surplus serum or plasma samples available within 12 months before ARL diagnosis, we retested the samples for EBV-DNA in order to study the course of EBV viral load one year before ARL diagnosis.

Samples

EBV DNA load was measured using an internal controlled [15] quantitative real-time Taqman PCR based on Niesters et al. [16] and LC480-II (Roche Applied Science, Almere, The Netherlands).

Between January 2004 and October 2015, EBV DNA loads were measured in copies/mL. To reliably compare the EBV loads between different laboratories, from October 2015 onwards, the qPCR was standardized using the 1st WHO International Standard and validated according to the ISO15189:2003 norm, including sensitivity, specificity, LLOD and a clinical validation. From here on, the viral loads were measured in IU/mL.

The conversion factor between copies/mL and IU/mL was determined using positive samples from the clinical validation data ($n = 39$). The mean viral load difference between both methods was 0.16 log, SD = 0.28. Also, Bland and Altman plot and a paired, two-tailed *t*-test were performed. The results, $Y = 0.0192x + 0.147$, $R^2 = 0.0114$ and 0.00199, respectively, confirmed that there was no significant difference between the two methods and the conversion factor was stated as one.

When multiple samples were available for a patient, the highest EBV DNA load, i.e. peak EBV DNA load, measured before diagnosis of malignant lymphoma was taken into account. Samples with undetectable EBV DNA load were assigned half of the lower limit of detection (50 IU/mL) [17]. Plasma HIV-1 RNA loads ≥100,000 copy/mL were presented as 100,000 copy/mL and samples with undetectable HIV-1 RNA were assigned half of the lower limit of quantification (50 copies/mL).

EBV-encoded RNA (EBER)

To define whether malignant lymphomas were truly EBV-related, *in situ* hybridization targeting EBER was performed on lymphoma tissue in 38/49 of the patients (77.6%). If EBER examination was not performed on lymphoma tissue, patients were not included in the analysis evaluating the mean difference in EBER-positive lymphoma patients, EBER-negative lymphoma patients and controls.

Statistical analysis

We performed logarithmic transformation of EBV DNA loads in order to obtain a normally distributed variable. Mean differences in peak EBV DNA loads between EBER-positive malignant lymphoma patients, EBER-negative malignant lymphoma patients and controls were

measured using a parametric one-way analysis of variance (one-way ANOVA). If assumptions of equality of variances were not met, Welch's test was performed. Post hoc test was performed to evaluate which groups reached significant mean differences. Risk estimations were calculated with cross-tabulations and presented as odds ratios. Adjusted odds ratios and their 95% confidence interval were calculated using a binary logistic regression model. Age at peak EBV DNA load, sex, $CD4^+$ cell count assessed six months prior or after peak EBV DNA load and nadir $CD4^+$ cell count was considered confounders based on current literature. Furthermore, we performed receiver operating characteristic (ROC) analysis to estimate the area under the curve (AUC) and asses the accuracy of EBV DNA load as a predictive marker of ARL. An optimal EBV DNA load cut-off value predicting lymphoma in HIV-positive adults was determined from the ROC curve using the coordinates in which sensitivity and specificity were most valid. Positive predictive values were taken into account as well. Statistical analysis was performed using IBM SPSS Statistics version 21 (Armonk, NY).

Results

Patient characteristics

A total of 511 HIV-positive patients with plasma samples were available. Forty-nine patients with historically confirmed ARL were eligible. Randomly selected controls consisted of 156 patients. Patient characteristics are shown in Table 1. ARL patients were older compared to controls (median 47 years vs. 42 years), more likely to be male (88% vs. 68%), and more often originating from Europe (71% vs. 49.4%). Both groups had similar low $CD4^+$ cell counts at the time of ARL diagnosis and were comparable concerning cART usage and HIV RNA suppression when peak EBV-DNA loads were obtained. Median peak EBV DNA loads were substantially different between cases and controls (Mann–Whitney's test, $p < .001$), 11,100 IU/mL (interquartile range: 337; 258,500) and 25 IU/mL (interquartile range: 25; 675), respectively. Almost 35% of ARL patients had an EBV DNA load >100,000 IU/mL, compared to merely 3% of controls. Prior opportunistic infections occurred in 40% of controls in contrast to 16% in ARL patients (Pearson's chi-square, $p = .002$).

Lymphoma subtypes

Nearly half of all cases were diffuse large B-cell lymphoma (21 cases) of whom 52% were EBV-related. Lymphoma types with the highest percentage of EBER positivity were primary effusion lymphoma (75%) and Hodgkin lymphoma (66.7%).

EBER positive and negative ARL

Figure 1 shows median and interquartile ranges of logarithmic transformed EBV DNA loads of EBER-positive lymphoma patients ($n = 28$), EBER-negative lymphoma patients ($n = 10$) and controls ($n = 156$). In 11 ARL patients, EBER data were missing. The geometric mean of the peak EBV DNA load measured before diagnosis of ARL was 52.565 IU/mL in EBER-positive lymphoma patients and 127 IU/mL in controls ($p < .001$). The geometric mean in the EBER-negative group was 430 IU/mL, which was significantly different from EBV DNA loads in the EBER-positive group ($p = .001$). No significant difference was observed between EBER-negative lymphoma patients and controls ($p = .44$).

Table 1. Patient characteristics.

	Lymphoma patients ($n = 49$)	Controls ($n = 156$)
Age[a]	47 (41–56)	42 (34–50)
Male gender	87.8	67.9
EBER performed	57.1	–
Peak EBV DNA load, IU/mL	11,100 (337–258,500)	25 (25–675)
<50	12.2	61.5
50–100,000	53.1	35.3
>100,000	34.7	3.2
$CD4^+$ cell count, $\times 10^9$/L[b]	0.15 (0.03–0.22)	0.13 (0.03–0.34)
Nadir $CD4^+$ cell count, $\times 10^9$/L	0.07 (0.02–0.16)	0.05 (0.02–0.18)
HIV RNA load copy/mL[b]	250 (25–100,000)	6580 (55–100,000)
<50	32.7	24.4
≥50	67.3	75.6
cART >4 weeks	57.1	41.7
Prior opportunistic infection	16.3	40.4
European	71.4	49.4

Results are expressed as median (interquartile range) or %.
[a]Age at peak EBV DNA load measurement.
[b]$CD4^+$ cell count and HIV RNA load measured 6 months prior to or after EBV measurement.

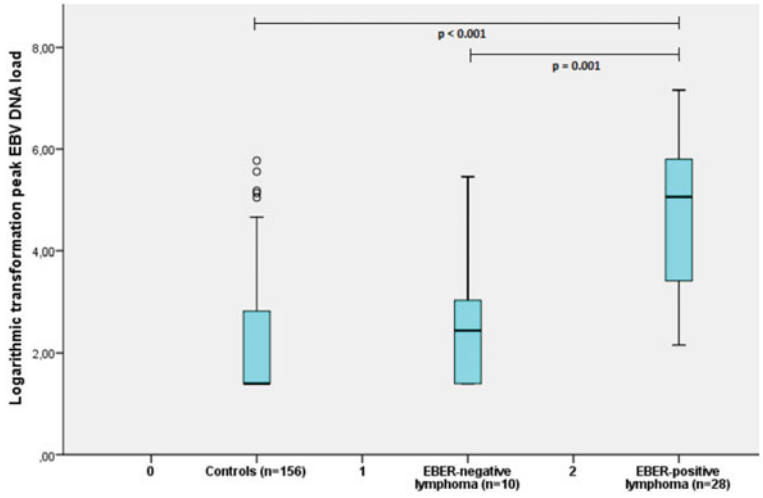

Figure 1. Log-transformed peak EBV DNA load distribution of cases and controls.

Risk of malignant lymphoma

HIV-infected adults with detectable EBV DNA loads in plasma or serum were at increased risk of ARL, compared to patients with undetectable EBV DNA loads (odds ratio 11.47; 95% confidence interval 4.60; 28.75). In addition, when using a cut-off of >100,000 IU/mL, the risk on ARL further increased (odds ratio 16.04; 95% confidence interval 5.52; 46.66). Adjusted odds ratios were 10.57 (95% confidence interval 3.99; 28.00, *R*-square 0.324) and 12.53 (95% confidence interval 4.08; 38.42, *R*-square 0.238).

Predictive biomarker

The ROC curve showed an AUC of 0.814 ($p < .001$). When a cut-off point of 367,500 IU/mL was used we achieved the highest specificity (71.2%) and sensitivity (75.5%), with a corresponding positive predictive value of 45%. When a cut-off point of 50 IU/mL (i.e. detectable EBV DNA load) was used, the corresponding positive predictive value was 41.7%, specificity was 61.5% and sensitivity was 87.8%. A cut-off point of 100,000 IU/mL showed a high specificity (96.8%) and positive predictive value (77.3%), but sensitivity (34.7%) was low.

Course of plasma EBV DNA load 12 months before ARL diagnosis

Thirteen patients were identified with at least three available plasma samples for testing of EBV DNA load during 12 months before ARL diagnosis (Figure 2). One patient never showed a detectable EBV-DNA (L-1), one patient showed several bursts of EBV DNA load already six years prior to ARL diagnosis (L-2). The other

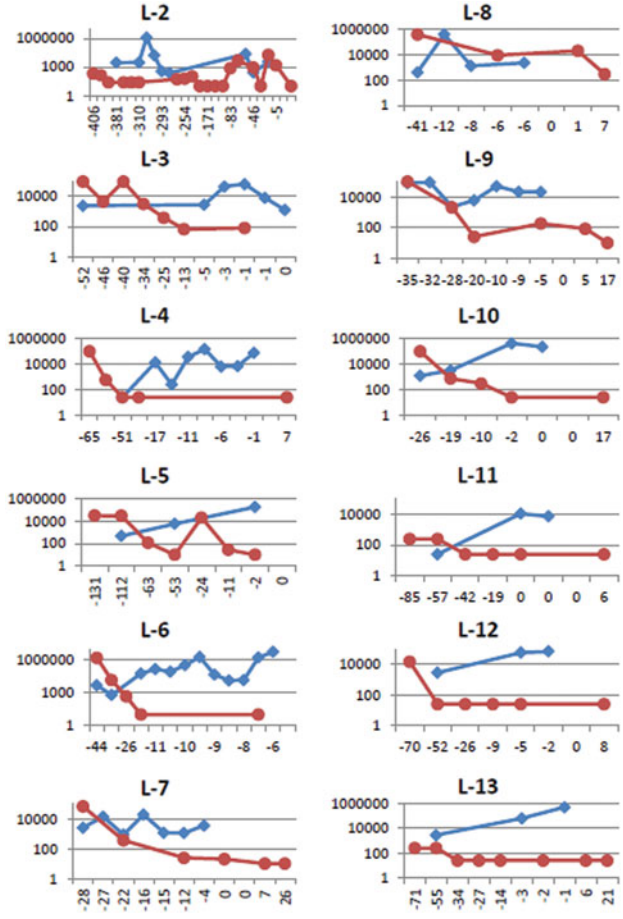

Figure 2. Plasma EBV DNA (squares) and HIV RNA (circles) load 12 months before ARL diagnosis.

11 patients (L3–L13), except one (L9), had detectable EBV-DNA loads (median 2900 IU/mL) which were lower than EBV-DNA loads at the time of ARL diagnosis (median 68,800 IU/mL). L9 showed a variable bursting course of EBV-DNA during the year before ARL diagnosis. In this longitudinal group of 13 patients, the median time of cART usage before ARL diagnosis was 14

months. In 10/13 patients, HIV-RNA was well suppressed. The nadir $CD4^+$ before start cART was median 70 cells/mm^3.

Discussion

EBV-DNA loads in cross-sectional study

In the present study, we investigated the predictive value of EBV DNA loads in plasma. We showed that HIV-infected adults with peak EBV DNA loads >50 IU/mL in plasma or serum are at increased risk of ARL. This is in agreement with Fan et al. who found in a study of 48 HIV-infected patients with ARL that EBV-DNA >2500 copies/mL strongly correlated with the presence of lymphoma [12]. A more recent study from Tanaka et al., showed that median EBV-DNA loads in peripheral blood mononuclear cells (PBMCs) at the time of ARL diagnosis in 17 HIV positive patients was significantly higher (1.3 copy/10^6 cells) compared to EBV DNA loads in HIV positive patients with opportunistic infections (0.1 copy/10^6 cells) ($p = .002$) or in HIV negative controls (EBV-DNA undetectable) ($p < .001$) [11]. Bonnet et al., who studied 14 HIV positive patients with ARL, showed that EBV DNA load in whole blood is of poor value for ARL diagnosis, but suggested it to be useful as a marker for ARL treatment response [13]. As reported in other studies [12,18–22], there is considerable overlap in EBV DNA loads between the cases and control which made it impossible to set a cut-off that completely distinguished lymphoma patients from non-lymphoma patients. In our study, it is reasonable to conclude that in patients with an EBV-DNA load >100,000 IU/mL, a search for ARL seems useful.

EBV-DNA in longitudinal study

Although EBV DNA loads shortly before diagnosis may be predictive of ARL, serial EBV DNA load measurements in the 13 HIV-infected patients in this study during the preceding 12 months before ARL diagnosis showed to be of poor predictive value for monitoring the development of ARL. EBV DNA load 12 months before ARL diagnosis was not different from those in HIV positive controls with opportunistic infections without ARL. The combination of a disturbed balance between virus and host, expressed as high EBV-DNA and low nadir $CD4^+$ cell count <200 mm^3, may increase the risk of development of ARL. Our control patients, also late presenters, with comparable low $CD4^+$ cells, rate of cART usage and HIV suppression, may have regained control on EBV replication, despite the occurrence of opportunistic infections, and not develop ARL. $CD4^+$ count is a crude marker of cell-mediated immune function, but they do not reflect the level of EBV-specific cytotoxic T cell immunity [12].

There are several limitations to this study. Consistent with a retrospective nature of the study, the timing of EBV DNA sample collection was not always optimal and there were missing data. The number of patients with longitudinal data was small. Follow-up after treatment of ARL is missing, which is a failure in understanding the pathogenic mechanism of ARL. Despite this, our results suggest that EBV DNA load may be a surrogate marker of ARL Diagnosis. EBV DNA levels have been shown to fluctuate over time with low levels, which in HIV-infected patients may increase rapidly, with an increased risk of developing ARL.

Conclusions

Our findings suggest that HIV-infected adults with peak EBV DNA loads above 100,000 IU/mL in plasma or serum are at approximately 13-fold increased risk of ARL compared to patients with peak EBV DNA loads equal or below 100,000 IU/mL. Peak EBV DNA load in plasma or serum is not a predictive biomarker on account of low sensitivity and specificity. However, with a cut-off point of 100,000 IU/mL and a corresponding positive predictive value of 77.3%, peak EBV DNA load can be useful in clinical setting to accelerate time to diagnosis and treatment. EBV-DNA loads in samples taken during the preceding year of ARL diagnosis showed to be of poor predictive value. Larger studies are required to confirm the diagnostic value of this marker and define its prognostic value in ARL treatment in HIV positive patients.

Disclosure statement

No potential conflict of interest was reported by the authors.

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