Clinical Cancer Research

The Viral Load of Epstein–Barr Virus (EBV) DNA in Peripheral Blood Predicts for Biological and Clinical Characteristics in Hodgkin Lymphoma

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Abstract

Purpose: The Epstein–Barr virus (EBV) is present in the malignant Hodgkin/Reed–Sternberg (HRS) cells of 20% to 40% cases of Hodgkin lymphoma (HL) in Western countries. We were interested in the detection and quantification of cell-free plasma EBV-DNA as an indicator of biological and clinical characteristics in EBV-associated HL.

Experimental Design: EBV was detected in peripheral blood compartments (whole blood, plasma, and mononuclear cells) at diagnosis by real-time PCR for the EBNA (EB nuclear antigen) region ($n = 93$) and in HRS cells by *in situ* hybridization for EBV-encoded small RNAs (EBER; $n = 63$). These data were correlated to histological and clinical characteristics, EBV serology, circulating cell-free DNA, and interleukin (IL)-6 levels.

Results: Detection of EBV-DNA in plasma had a high specificity (90%), but a relatively low sensitivity (65%) to predict for EBV association. The viral load was higher in patients with advanced stage disease, older age in the presence of B-symptoms, and international prognostic score more than 2. The presence of EBV in HRS cells and higher plasma EBV-DNA copy numbers correlated to an increased frequency of tumor-infiltrating CD68+ macrophages in lymph node biopsies. Plasma EBV-DNA load correlated to circulating cell-free DNA and IL-6 levels, and inversely correlated to lymphocyte counts and EBNA1 antibody titers.

Conclusion: Although the presence of EBV-DNA in peripheral blood cannot be regarded as a surrogate marker for EBER, the plasma EBV-DNA load at HL diagnosis is an indicator of disease activity and biological characteristics associated with negative prognosis. Moreover, the inverse correlation to EBNA1 antibody titers and lymphocyte counts may indicate a reduction in immunosurveillance, favoring the expansion of EBV-HRS cells in HL. *Clin Cancer Res; 17(9); 2885–92. ©2011 AACR.*

Introduction

Epstein–Barr virus (EBV) is a member of the herpes virus family preferentially targeting human B cells. Although it mainly persists as harmless passenger, EBV has tumorigenic potential, being able to transform normal B cells into lymphoblastoid cell lines *in vitro*. Furthermore, EBV has been associated with several lymphoma types (1–4). The contribution of EBV to Hodgkin lymphoma (HL) etiology differs according to immunostatus, age group, and geographic origin (5, 6). The large majority of HL arising in the setting of HIV infection are pathogenetically linked to EBV (7), whereas in immunocompetent patients, 30% to 40% of HL carry the EBV genome in the malignant Hodgkin and Reed-Sternberg (HRS) cells. In Western countries, EBV is infrequent in HL of young adults, although an association with delayed exposure to EBV and a history of infectious mononucleosis is recognized. In contrast, EBV association is more common in elderly HL, which might result from loss of the normal balance between latent EBV infection and host immunity (8).

Primary EBV infection triggers the production of antibodies against the viral capsid antigen (VCA) and the latent EB nuclear protein EBNA-2 (EB nuclear antigen). The resolution of infection is reflected by the appearance of anti-EBNA-1 antibodies and a decline of anti-EBNA-2 antibodies, resulting into a high anti-EBNA-1 to anti-EBNA-2 antibody ratio (9). A persistent reduced anti-EBNA-1 to

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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doi: 10.1158/1078-0432.CCR-10-3327

Translational Relevance

Circulating Epstein–Barr virus (EBV) DNA in peripheral blood is an indicator and biomarker for EBV-associated Hodgkin lymphoma (HL). We further explored associations of EBV-DNA plasma load to biological and clinical characteristics of HL. The strong correlations of circulating EBV-DNA to parameters of disease activity, such as stage, the international prognostic score, and new biomarkers, as cell-free circulating DNA and the number of tumor-infiltrating CD68+ macrophages, make EBV-DNA a meaningful marker for the biological and clinical presentation of EBV-associated HL. Since plasma EBV-DNA may be absent in patients with limited disease, EBV-DNA cannot be regarded as a surrogate marker for EBV-encoded small RNAs, and study of EBV in Hodgkin/Reed–Sternberg cells is still the gold standard recommended for screening for EBV association in HL. The inverse correlations of circulating EBV-DNA to lymphocyte counts and EB nuclear antigen titers point to a reduction in immunosurveillance in EBV-associated HL, favoring the expansion of EBV in HL.

anti-EBNA-2 ratio has been interpreted as a marker of defective host immunocontrol against latent EBV infection, and has been described in patients with HL (10).

The frequency of circulating EBV-infected cells seems to be higher in EBV-associated HL than non-EBV–associated cases (11). EBV genome is also detectable in the serum and plasma of EBV-associated HL patients, as ''naked'' DNA, rather than virions (12), consistent with the notion that cell-free viral DNA may be shed from malignant cells of the tumor tissue. The mechanism of release of viral DNA from the tumor tissue may be similar to the release of cellular DNA fragments that can be measured as circulating cell-free DNA in peripheral blood in patients with HL (13). By using conventional PCR, Gallagher and colleagues reported detection of EBV-DNA in 91% of serum samples from EBV-positive and in 23% of EBV-negative HL patients (12). Limited data on associations between EBV status at HL diagnosis and patients' biological and clinical characteristics are available (14–17).

EBV may modulate the cellular composition of the microenvironment and the production of inflammatory cytokines of prognostic relevance in HL (18, 19). Recent expression profiling studies indicated that the microenvironment also impacts on prognosis in HL (20, 21). In particular, the frequency of tumor-associated CD68+ macrophages in HL lymph nodes biopsies has been identified as an important prognostic marker in HL (21).

We studied the role of EBV-DNA copy number in different blood compartments (whole blood, mononuclear cell fraction, and plasma) in patients with HL at diagnosis, as predictive assay for the EBV status and as potential marker for disease activity. Other biomarkers as cytokine levels, cell-free circulating DNA, histological assessment of EBV-encoded small RNA (EBER) in HRS, and CD68 frequency in lymph nodes were also evaluated in addition to patient characteristics and other classical laboratory parameters.

Patients and Methods

Patient characteristics

Our analysis included 93 patients (median age 36 years, range 13–83 years; 53 women and 40 men), diagnosed with HL and treated at our Institute of Hematology, Catholic University S. Cuore, Rome, Italy, between March 2004 and May 2010. Patient characteristics, including the international prognostic score (IPS; ref. 22), are detailed in Table 1. Serological testing suggested previous exposure to hepatitis B virus (HBV) in 8/86 patients (antiHBcAg positive), with only 1 patient being positive for HB surface antigen (HBsAg), whereas no patient had a positive serology for HIV or HCV. Peripheral blood samples were obtained at the time of initial diagnosis with EDTA as an anticoagulant.

Chemotherapy consisted of ABVD in 55 patients, 6 patients were treated with MOPP or MOPP-like regimens, while 32 patients with advanced stage disease (stage IIB with bulky disease to stage IV), younger than 60 years, were treated with BEACOPP (cyclophosphamide, etoposide, adriamycin, procarbazine, vincristine, bleomycin, and prednisone). Radiotherapy was included for consolidation in patients with limited stage disease and initial bulky disease. Informed consent was obtained from patients according to institutional guidelines, and blood sample collection was approved by our institutional ethical committee.

EBV serology and EBV-DNA quantification

Serum samples were screened in the presence of immunoglobulin (Ig) G (IgG) antibodies to EBNA-1 and VCA by using commercially available enzyme immunoassays (Liason EBNA IgG and Liason VCA IgG, DiaSorin S.p.A.) according to the manufacturer's instructions.

EBV-DNA was measured in the peripheral blood of 93 HL patients at the time of initial diagnosis (whole blood, $n = 69$; plasma, $n = 75$; mononuclear cells, $n = 74$), using a commercial real-time PCR kit, amplifying a 191 bp region of the EBNA-1 gene (BioQuant EBV, Biodiversity), according to the manufacturer's protocol, and the ABI PRISM 7300 Sequencer Detection System (Applied Biosystems).

Immunohistochemical analysis and *in situ* hybridization for EBV

Immunohistochemical analysis for CD68 was done on 3 μm tissue slides by the antihuman mouse monoclonal antibody CD68 (1:100, clone PGM-1, Dako, High Glostrup) after proteolytic treatment (pronase 0.05% in tris buffer pH 7.6) for 10 minutes at room temperature. Immunodetection was done by an avidin–biotin–peroxidase complex solution (ScyTek), 3,39-diaminobenzidine as the chromogen, and Mayer's hematoxylin as the counterstain. We used the immunohistochemical score proposed by Steidl and colleagues with a cutoff at 5% CD68-positive cells (21).

Table 1. Patient characteristics

	Variable	All patients ($n = 93$)	Elderly patients >50 years ($n = 22$)
Age	Median, range, y	36 (13–83)	62 (50–83)
Age	>50 y	22 (24%)	22
Sex	Male	40 (43%)	8 (36%)
Histologic subtype	NS	64 (69%)	13 (59%)
	NS 1	40 (43%)	7 (32%)
	NS 2	16 (17%)	3 (14%)
	Mixed cellularity	4 (4%)	2 (9%)
	Lymphocyte rich	4 (6%)	2 (9%)
	Lymphocyte depleted	2 (2%)	1 (5%)
	Not classifiable	19 (20%)	4 (18%)
Immunohistochemical Staining			
	CD30 positive	88/91 (98%)	20/21 (95%)
	CD15 positive	88/91 (98%)	21/21 (100%)
	CD20 positive	20/75 (27%)	2/14 (14%)
Stage	Limited	44 (48%)	7 (37%)
	Advanced	48 (52%)	14 (63%)
Presence of B-symptoms	Yes	32 (35%)	12 (55%)
Bulky disease	Yes	32 (35%)	4 (18%)
IPS	0–2	68 (74%)	11 (52%)
	3–7	24 (26%)	10 (48%)
Treatment	ABVD	55 (59%)	11 (50%)
	BEACOPP	32 (34%)	6 (25%)
	MOPP/other	6 (7%)	6 (25%)

Lymph node samples from 63 HL patients were analyzed for EBV infection. *In situ* hybridization of EBERs on formalin-fixed and paraffin-embedded tissue section was carried out by following the manufacturer's instructions (Dako; Dakopatts), as previously described (23). EBV was present in the HRS cells of 20 of 63 HL cases (32%).

IL-6 and cell-free DNA plasma levels

Interleukin (IL)-6 concentration was measured in pretreatment plasma samples that had been stored at −70°C and thawed for the first time. A sandwich enzyme–linked immunoassay was used according to the manufacturer's instructions (Human IL-6 US, BioSource international, Inc.).

DNA was isolated from 400 to 800 μL plasma samples by the QIAamp UltraSens Virus Kit (QIAGEN) to facilitate the collection of fragmented DNA, eluted in 50 to 100 μL low-salt buffer, and quantified by an SYBR green-based real-time PCR assay for the β-globin gene, as previously described (24, 13).

Statistical analysis

Fisher's exact test was used to examine for differences in patient characteristics according to the presence of EBV in HRS cells or plasma. Wilcoxon-signed rank test was used for 2-sample comparisons of EBV-DNA levels according to dichotomized patient characteristics. Concentrations of viral and cellular DNA were analyzed both as continuous variable following logarithmic transformation and dichotomic variable using as cutoff point the presence of EBV-DNA or the upper normal limit of controls for cellular DNA. Correlations among the various blood parameters were calculated by Spearman rank correlation. The primary survival end point was progression-free survival (PFS) with progression during treatment, lack of complete remission at the end of first-line treatment, relapse, and death from any cause counted as adverse events. Survival curves were estimated by the Kaplan–Meier product limit method. Log-rank tests were used to analyze for differences in PFS. HRs and 95% CIs were adjusted for multiple prognostic factors by the Cox proportional hazards model. Computations were done by the Stata 10.0 software (Stata Corp.).

Results

EBV-DNA in peripheral blood compartments as a predictor for EBV-associated HL

We first compared the value of EBV-DNA detection in different peripheral blood compartments (whole blood, plasma, and mononuclear cells) to predict for EBV-associated HL, as defined by the presence of EBER in HRS cells. Determination of the viral load in plasma had the highest specificity (90%), whereas assessment of EBV in the mononuclear cell fraction was the least predictive of EBV status of HRS cells (Table 2). We therefore concentrated the following analyses on EBV-DNA concentration in plasma. The

Table 2. Diagnostic performance of EBV-DNA quantification in peripheral blood compartments predicting EBER status

	Whole Blood ($n = 43$)	Plasma ($n = 56$)	MNC fraction ($n = 55$)
Sensitivity	79% (66%–91%)	65% (52%–77%)	39% (26%–52%)
Specificity	79% (67%–91%)	90% (82%–98%)	87% (78%–95%)
Positive predictive Value	65% (50%–79%)	73% (62%–85%)	58% (45%–71%)
Negative predictive Value	89% (79%–98%)	85% (76%–95%)	74% (63%–86%)

Abbreviation: MNC, mononucleocytes.

majority of EBER-positive HL patients had a high viral DNA load in plasma, while patients with EBER-negative HL were only occasionally positive for EBV plasma DNA with a low viral load (Fig. 1).

Association of EBV in HRS cells and plasma with histological features, including the number of CD68+ macrophages

The most frequent histotype in our patient cohort was nodular sclerosis (NS), in particular the type 1 according to the BNLI (British National Lymphoma Investigation) classification (Table 1). However, cases of type 1 NS were underrepresented in EBV-associated HL, when compared with EBV-negative patients [8/20 (40%) vs. 27/40 (68%; $P = 0.06$)]. The viral load in plasma did not vary according to histotype (data not shown, $P = 0.2$).

By using the immunohistochemical scoring system proposed by Steidl and colleagues (21), we found an increased frequency (more than 5%) of tumor-infiltrating CD68+ macrophages in EBV-associated HL ($P = 0.03$; Fig. 2A–C). Increased CD68+ cell number was also associated to the presence and amount of EBV-DNA in plasma ($P = 0.02$ and $P = 0.005$, respectively; Fig. 2D).

Associations of EBV in HRS cells and plasma with patient characteristics

By looking at patients' characteristics, we found a significant association between EBV positivity and age more than 50 years (8/12, 67%) than younger patients (12/51, 24%; $P = 0.01$). In this line, plasma EBV-DNA copy number was higher in older adult EBV–associated HL than young adult EBV+ cases ($P = 0.006$, Table 3).

Furthermore, in EBV-associated HL, concentration of plasma EBV-DNA was significantly higher in advanced stage disease (stages IIB–IV; $P = 0.01$), particularly in the presence of stage IV disease ($P = 0.006$), B-symptoms ($P = 0.001$), or IPS score mote than 2 ($P = 0.007$; Table 3). In addition, there was an inverse correlation between EBV-DNA copy number and peripheral lymphocyte, but not neutrophil counts ($r = 0.61$, $P = 0.01$; Table 4).

The multivariate ANOVA analysis including older age, advanced stage, presence of B-symptoms, and the number of CD68+ cells showed that age more than 50 years ($P = 0.0006$), advanced stage ($P = 0.02$), and presence of B-symptoms ($P = 0.001$) were independently associated with higher EBV-DNA copy numbers (data not shown).

Correlations of EBV in HRS cells and plasma with other biomarkers

By considering EBV-DNA copy number as a parameter for disease activity in EBER-positive HL, we looked for

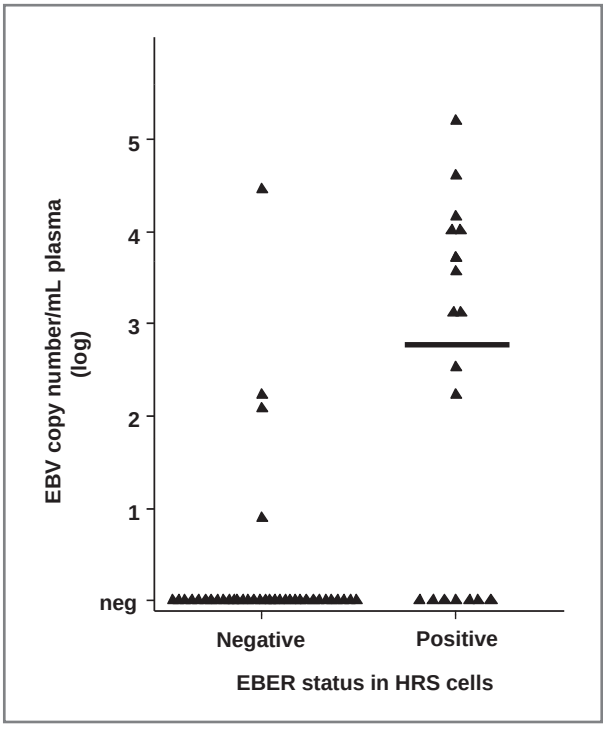

Figure 1. EBV-DNA copy number reflects EBER status in lymph nodes in patients with HL at diagnosis. Plasma EBV-DNA concentrations in EBER-positive ($n = 18$) and EBER-negative ($n = 39$) patients are shown. The horizontal line represents median value (750 copies/mL) in EBER-positive patients, while in EBER-negative patients, median EBV-DNA concentration was 0 copies/mL. The difference was statistically significant ($P < 0.0001$).

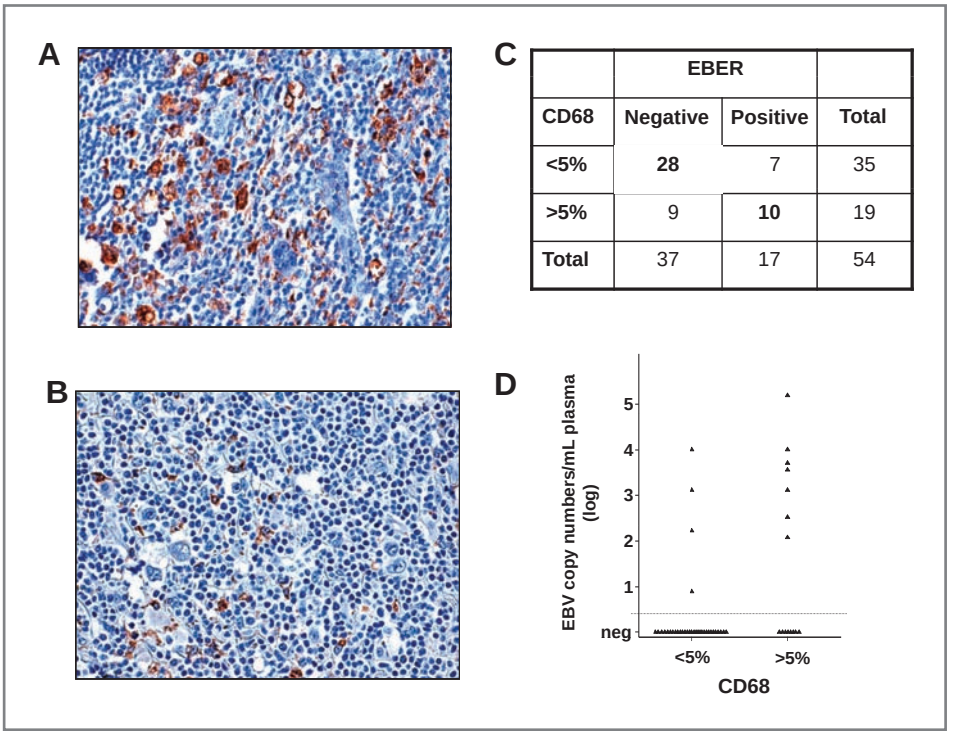

Figure 2. The viral load of EBV-DNA in plasma correlates to the frequency of CD68+ tumor–associated macrophages in lymph nodes. A, a lymph node of an EBER-positive patients with increased CD68+ macrophages, whereas (B) shows the HL tissue of an EBER-negative patient with few CD68+ macrophages. C, the results of CD68 and EBER staining (for association, $P = 0.03$). D, EBV-DNA copy number according to the number of tumor-associated CD68+ macrophages (<5%, $n = 34$; >5%, $n = 15$; $P = 0.005$).

correlations among EBV status of HRS cells, EBV-DNA load, and other biomarkers.

IL-6 levels were below detection limit in 11 of 63 (17%) patients. By limiting the analysis to patients with detectable levels, IL-6 levels were higher in patients with EBV-associated HL than EBV-negative HL (median 2.27 pg/mL; range 0.26–45.5; $n = 16$, vs. 0.89 pg/mL; range 0.03–38.3, $n = 36$, $P = 0.04$). In EBV-associated HL, IL-6 levels correlated to plasma EBV-DNA concentration ($\rho = 0.73$, $P = 0.001$; Table 4).

We recently described circulating cell-free DNA as a new biomarker in lymphoma patients (13). The median cell-free plasma DNA concentration was 26 ng/mL ($n = 62$ patients, range 1.9–656 ng/mL) with no differences according to EBV status of HRS cells [median 31 vs. 22 ng/mL for EBV positive ($n = 20$) and negative ($n = 42$) HL cases, respectively, $P = 0.2$]. EBV copy numbers significantly correlated to cell-free DNA levels only in patients with EBV-associated HL ($\rho = 0.58$, $P = 0.01$; Table 4).

Associations of EBV in HRS cells and plasma with EBV serology

A previous EBV infection was diagnosed in 94% of patients by a positive IgG antibody titer to the EBNA-1 antigen and/or to the VCA capsid antigen. Patients with EBV-associated HL often had EBNA-1 antibody titers less than 100 U/mL (8/18 cases, 44%), whereas low EBNA-1 antibody titers were only rarely observed in EBV-negative HL (5/42, 12%, $P = 0.01$). Moreover, in EBER-positive HL, EBNA-1 antibody titers inversely correlated to the viral load in plasma ($\rho = -0.62$, $P = 0.01$; Table 4). Antibody titers to VCA or against 2 other herpes family viruses, cytomegalovirus (CMV) and herpes simplex viruses (HSV)-1/2, did not differ according to the EBV status of HRS cells. In this line, there were also no correlations between VCA, CMV, and HSV-1/2 antibody titers and EBV-DNA copy number, pointing to a specific association between lower EBNA-1 antibody titers and higher EBV-DNA load in EBV-associated HL.

EBV-DNA and outcome

At a median follow-up of 25 months (range 1–81 months) from diagnosis, 14 of 91 patients developed progressive disease that translated into 84% probability of PFS (95% CI, 73%–90%). In univariate analysis, the presence of EBV-DNA in plasma was associated with a significantly shorter PFS ($P = 0.01$), as were age older than 50 years ($P = 0.005$), the number of CD68+ macrophages ($P = 0.0006$), and cell-free circulating DNA ($P = 0.0007$; Supplementary Fig. S1).

Including these parameters in a multivariate Cox proportional hazard regression analysis and adjusting the analysis for the kind of chemotherapy regimen (standard dose versus intensified regimens), only the number of CD68+ cells and the level of circulating cell-free DNA remained significant ($P = 0.03$ and 0.03, respectively), but not age or plasma EBV-DNA (Supplementary Table S1).

Discussion

We studied EBV association in HL and report several new findings: (A) the quantification of circulating EBV-DNA in

Table 3. Associations between plasma EBV-DNA concentration and clinical characteristics in EBV-associated HL

Variable	EBV-DNA Copy number/mL (median, range)	*P**
Age		
<50 y ($n = 11$)	0 (0–5,760)	0.006
>50 y ($n = 7$)	11,200 (0–135,600)	
Gender		
Female ($n = 7$)	3,600 (0–135,600)	0.6
Male ($n = 11$)	300 (0–40,000)	
Stage		
Limited ($n = 12$)	73 (0–11,000)	0.01
Advanced ($n = 6$)	12,790 (0–135,600)	
Presence of B-symptoms		
No ($n = 13$)	0 (0–11,000)	0.001
Yes ($n = 5$)	14,380 (5,760–135,600)	
Bulky disease		
No ($n = 11$)	300 (0–135,600)	0.6
Yes ($n = 7$)	1,200 (0–11,000)	
IPS		
0–2 ($n = 12$)	0 (0–11,200)	0.007
3–7 ($n = 6$)	10,070 (300–135,600)	

*P values of Wilcoxon ranksum test are given for comparison of dichotomous characteristics.

plasma improves the specificity of blood tests to identify EBV-associated HL; however, it is not a surrogate marker for EBER, as patients with a limited disease activity may not have detectable copy numbers in peripheral blood; (B) EBV-DNA copy number correlates with several other parameters of disease activity, predicting unfavorable prognosis; (C) EBV-DNA in plasma and EBV positivity of HRS cells are associated with higher frequency of CD68+ macrophages in the tumor tissue, a recently reported important prognostic marker in HL (21); (D) EBV-DNA levels inversely correlate with lymphocyte counts and EBNA-1 antibody titers, suggesting an association between EBV expansion and reduced anti-EBV immunity in the development of EBV-associated HL.

Table 4. Correlation between plasma EBV-DNA concentration and biological parameters in EBV-associated HL

Variable	Correlation coefficient (ρ)	*P**
Lymphocyte count ($n = 16$)	−0.61	0.01
Neutrophil count ($n = 16$)	−0.38	0.14
EBNA-1 titers ($n = 16$)	−0.65	0.006
VCA titers ($n = 15$)	−0.29	0.3
IL-6 concentration ($n = 18$)	0.73	0.001
Cell-free DNA ($n = 18$)	0.58	0.01

*P values are calculated by Spearman rank.

In line with previous studies, plasma was the most reliable source to predict for EBV status of HRS cells, while EBV-DNA determination in whole blood or the mononuclear cell fraction was less informative. By using a quantitative real-time PCR approach for the EBNA-1 region, we almost exclusively found high plasma levels of EBV-DNA in EBV-associated HL, while EBV-negative patients only occasionally had low levels of EBV-DNA. In 2 of 4 EBER-negative patients with positive plasma EBV-DNA, EBER was found in the surrounding tissue, raising the possibility of EBV-DNA release from bystander EBV+ B cells within the lymph node.

The sensitivity of plasma EBV-DNA as an indicator for EBV-associated HL was strongest in patients with advanced disease. All EBER-positive HLs with IPS 3 to 7 had detectable EBV-DNA in plasma, versus 42% IPS 0 to 2 (5/12; $P = 0.04$) EBER-positive HLs, pointing to the role of the quantification of EBV viral DNA in plasma as a marker of disease activity. This is further supported by the multiple correlations with other parameters of disease activity, including the levels of IL-6 and cell-free circulating DNA. We did not show serial measurements of EBV-DNA during the course of the disease, and further longitudinal studies are needed to establish whether monitoring of this biomarker will be helpful in the management of these patients.

The presence of EBV may alter the immunopathobiology of HL. Expression of some cytokines and chemokines, including IL-6, has been reported to be increased in EBV+ HL (25); however, cytokine levels and EBV tumor status did not always correlate (26, 27). In patients with EBV-associated HL, IL-6 concentration was significantly higher and directly correlated to EBV-DNA copy number in plasma. Furthermore, there was an association between EBV status of HRS cells and plasma EBV-DNA load with the frequency of tumor-infiltrating CD68+ macrophages. The number of CD68+ macrophages has recently been reported as an important prognostic marker for HL, which even outperformed the IPS in multivariate analysis (21). We confirm the importance of CD68+ cells in lymph nodes as an independent prognostic factor, being maintained in the multivariate analysis.

The number of CD68+ macrophages and the viral EBV plasma load correlated to the amount of cell-free DNA. These data are consistent with a scenario in which macrophages could be the leading contributors to the release of cell-free viral and cellular DNA, by removing apoptotic and necrotic cells in the tumor lesions (28–30). In this line, we have previously shown that circulating DNA levels are higher in HL with signs of necrosis in the tumor biopsies (13).

The role of EBV status of HRS cells as an unfavorable prognostic marker is controversial (31–35) and probably limited to older adult patients. The association of EBV status with older age, a higher proportion of tumor-infiltrating CD68+ cells in EBER-positive HL, and higher levels of circulating EBV-DNA in older patients may all point to a

different biology and disease activity in EBV-associated HL of the elderly, as factors contributing to unfavorable prognosis. These associations may also explain why EBV-DNA was not an independent prognostic factor in the multivariate analysis, which confirmed the strong prognostic impact of the number of tumor-infiltrating CD68+ macrophages and circulating DNA (13, 21).

An interesting finding of our study is the inverse correlation between EBV-DNA concentration and EBNA-1 antibody titers, pointing to a potential loss of host immunity in the development of EBV-associated HL, as proposed by other authors (6). The specificity is underlined by unchanged antibody titers to the capsid antigen VCA, 1 of the lytic antigens, and to other herpes virus antigens. Previous studies reported on elevated antibody responses to lytic antigens preceding the diagnosis of HL, suggesting viral reactivation (36), while antibody responses to the latent EBNA-1 antigen have not been well studied (10). The functional importance of low EBNA-1 antibody titers is, however, not clear, as these antibodies seem to offer little or no control over the virus in latently infected cells. EBV control is rather mediated by cytotoxic T lymphocytes (37): accordingly, in our patient series, lymphocyte counts inversely correlated to EBV-DNA. In this line, Sebelin-Wulf and colleagues observed an inverse correlation between EBV copies and lymphocyte counts, in particular CD4+ cells, in patients with EBV-associated posttransplant lymphomas (38). It will be interesting to explore whether this association reflects a specific defect in the circulating T cell populations, unable to control EBV expansion.

In conclusion, quantification of EBV-DNA copies in the peripheral blood at HL diagnosis is a specific marker for EBV association of HL, but cannot be regarded as a surrogate marker for EBER. As a biomarker, it correlates to other indicators of disease activity associated with unfavorable prognosis. Inverse correlations among EBV-DNA, EBNA-1 antibody titers, and lymphocyte counts may represent a stepping-stone to explore reduction in immunosurveillance favoring the expansion of EBV as a mechanism in the pathogenesis of EBV-associated HL.

Disclosure of Potential Conflicts of Interest

The authors have no conflicts of interest to declare.

Author Contributions

S. Hohaus was responsible for the study of design, data analysis, patients' care, and drafting the manuscript. M. Giachelia was responsible for sample preparation, circulating DNA determination, and ELISA assays. G. Massini, B. Vannata, and A. Cuccaro were responsible for data and sample collections and took part in data analysis. R. Santangelo was responsible for EBV-DNA real-time PCR. M. Martini, V. Cesarini, and T. Cenci performed immunohistochemical and EBER staining. F. D'Alo took part in patient care and establishing laboratory techniques. L.M. Larocca was responsible for histological analyses and data interpretation. M.T. Voso, R. Santangelo, G. Fadda, G. Leone, and L.M. Larocca took part in drafting the manuscript. G. Leone was responsible for patients' care and funding.

Grant Support

This work was supported by grants from Associazione Italiana per la Ricerca sul Cancro (AIRC), Ministero dell' Universita' e della Ricerca Scientifica e Tecnologica (MURST), and Fondi d'Ateneo, Linea D1, Università Cattolica del Sacro Cuore. M. Giachelia was supported by the Fondazione Roma—Progetto Cellule Staminali. G. Massini was supported by a grant from the Italian Society of Experimental Hematology (SIES).

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Received December 16, 2010; revised February 28, 2011; accepted March 7, 2011; published OnlineFirst April 8, 2011.

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