

Plasma Epstein-Barr Virus (EBV) DNA Is a Biomarker for EBV-Positive Hodgkin's Lymphoma

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Abstract Purpose: Latent Epstein-Barr virus (EBV) genomes are found in the malignant cells of approximately one-third of Hodgkin's lymphoma (HL) cases. Detection and quantitation of EBV viral DNA could potentially be used as a biomarker of disease activity.

Experimental Design: Initially, EBV-DNA viral load was prospectively monitored from peripheral blood mononuclear cells (PBMC) in patients with HL. Subsequently, we analyzed viral load in plasma from a second cohort of patients. A total of 58 patients with HL (31 newly diagnosed, 6 relapsed, and 21 in long-term remission) were tested. Using real-time PCR, 43 PBMC and 52 plasma samples were analyzed.

Results: EBV-DNA was detectable in the plasma of all EBV-positive patients with HL prior to therapy. However, viral DNA was undetectable following therapy in responding patients ($P = 0.0156$), EBV-positive HL patients in long-term remission ($P = 0.0011$), and in all patients with EBV-negative HL ($P = 0.0238$). Conversely, there was no association seen for the EBV-DNA load measured from PBMC in patients with active EBV-positive HL patients as compared with EBV-negative HL, or patients in long-term remission. EBV-DNA load in matched plasma/PBMC samples were not correlated.

Conclusions: We show that free plasma EBV-DNA has excellent sensitivity and specificity, and can be used as a noninvasive biomarker for EBV-positive HL and that serial monitoring could predict response to therapy. Additional prospective studies are required to further evaluate the use of free plasma EBV-DNA as a biomarker for monitoring response to treatment in patients with EBV-positive HL.

Latent EBV genomes are found in the malignant Hodgkin Reed-Sternberg cells of approximately one-third of Hodgkin's lymphoma (HL) cases, although the rate of involvement is as high as 100% in developing countries (1, 2). Recent studies have shown a causal relationship between infectious mononucleosis and subsequent Epstein-Barr virus (EBV)-positive HL in

young adults (3). Analysis of HLA class I polymorphisms suggest that deficient antigen presentation is associated with the risk of developing EBV-positive HL (4).

Acquisition of primary EBV infection results in life-long persistent infection in which memory B cells comprise the main reservoir with 1 to 50/10⁶ B cells infected in healthy seropositive individuals (5). Khan and colleagues showed that the frequency of EBV-infected circulating memory B cells was increased in pretreatment samples of EBV-positive HL patients compared with EBV-negative HL cases (6). They argued that their data implicates raised levels of EBV-infected B cells within peripheral blood mononuclear cells (PBMC) as a risk factor in developing EBV-positive HL. The numbers of EBV-infected cells are frequently increased in various EBV-associated diseases and that this infection burden can be monitored by measuring viral DNA load. Indeed, cellular viral load monitoring has been successfully used to identify high-risk patients who may develop EBV-associated posttransplant lymphoproliferative disease (7).

The identification of identical Hodgkin Reed-Sternberg immunoglobulin gene rearrangements in biopsy and serum samples shows that circulating Hodgkin Reed-Sternberg cells are present in the peripheral blood (8). Consistent with the notion that cell-free viral DNA may be shed from circulating apoptotic malignant cells, it has been shown that cell-free DNA is present as "naked" DNA rather than as virions (9). A number of groups have explored the value of cell-free EBV-DNA viral

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Received 9/13/05; revised 11/3/05; accepted 11/9/05.

Grant support: Queensland Cancer Fund and the British Society for Haematology. Clinical fellowships from the National Health and Medical Research Council, and Queensland State (M.K. Gandhi); R. Khanna is a Senior Research Fellow of the National Health and Medical Research Council. This study was conducted under the auspices of the Australasian Leukaemia and Lymphoma Group.

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doi:10.1158/1078-0432.CCR-05-2008

load quantification in EBV-associated malignancies. Viral load monitoring has been used in both nasopharyngeal carcinoma and natural killer cell lymphoma patients to predict the outcome of treatment (10, 11), and the likelihood of recurrence of nasopharyngeal carcinoma (12). Extension of these strategies to monitor patients with EBV-positive HL has thus far achieved limited success (9, 13, 14).

Using real-time PCR, we show that EBV-DNA can be detected and quantified in the plasma samples from all patients with EBV-positive HL prior to therapy, but then declines and becomes undetectable in chemotherapy-responsive patients. Our data suggests that free plasma EBV-DNA but not PBMC EBV-DNA viral load may be used as a noninvasive biomarker of disease response. Large prospective studies are required to further evaluate the use of free plasma EBV-DNA as a biomarker for monitoring response to treatment in patients with EBV-positive HL.

Materials and Methods

Patients. Newly diagnosed, relapsed and long-term remission (≥ 2 years from diagnosis) patients with histologically confirmed HL were eligible. Informed consent was provided as per each participating sites research ethics process. A total of 58 patients with HL (37 newly diagnosed/relapsed and 21 in long-term remission) were recruited for the study. Ninety-four samples were available for analysis collected between October 2001 and November 2004. Patients were subdivided into two cohorts. In the first cohort (October 2001 to July 2003), 43 PBMC samples were analyzed for EBV-DNA. In the second cohort (August 2003 onwards), 52 plasma samples were assessed. Paired PBMC/plasma samples were available in 12 cases (i.e., 24 samples). From both cohorts combined, blood samples were taken prior to the institution of therapy in 31 newly diagnosed and 6 relapsed patients. Serial blood samples (for plasma viral load analysis) were taken at time points following institution of therapy in four newly diagnosed and two relapsed patients. As a control, samples were taken from 21 patients in long-term remission [10 females/9 males; mean age, 29 years (range, 15-51); mean time of blood sample from diagnosis, 5 years (range, 2-33)]. Data regarding age, gender, date of diagnosis, and histology were collected in all patients. In newly diagnosed and relapsed patients,

additional prognostic information was obtained to enable the prognostic score to be calculated (15). Table 1 provides details of the patient characteristics. Irrespective of EBV tumor status, all newly diagnosed/relapsed patients were EBV-seropositive, whereas 90% of long-term remission patients were EBV-seropositive (including 87% EBV-negative HL patients). This study conformed to the tenets of the Declaration of Helsinki and informed consent was provided.

Tissue staining. All assays were carried out on sections of routinely fixed, paraffin-embedded material. LMP1 and EBER assays were done as per published guidelines (16). The distribution of HR/S cells were assessed by a morphologist on matching H&E slides prior to interpreting the LMP1 and EBER staining. Following antigen retrieval with trypsin, the anti-LMP IgG₁ or isotype control (Dako, Carpinteria, CA) was used to detect the presence of LMP1. Positive reactivity was detected as per the manufacturer's instructions incorporating 3,3'-diaminobenzidine as the chromogenic substrate (Envision kit, Dako). The EBER *in situ* hybridization assay used a commercially available hybridization kit (Dako) and custom-made probes derived from EBER-1 and EBER-2 (17). A fluoresceinated poly-dT oligonucleotide probe was used to hybridize to poly(A) mRNA in the tissue sections to serve as a control for tissue and mRNA integrity. For all stains, a known case of EBV-positive posttransplant lymphoproliferative disorder was used as a positive control.

Quantitative real-time PCR determination of the EBV-DNA load in the plasma and PBMC. DNA from plasma (400-500 μ L) and (PBMC) was extracted using a Qiagen blood kit (Valencia, CA). The purified DNA was subjected to a real-time PCR reaction. These 20 μ L reactions were done using Platinum Quantitative PCR SuperMix-UIDG (Invitrogen, Carlsbad, CA), 0.2 μ mol/L each primer and 0.1 μ mol/L fluorogenic probe. The PCR primers and probe were selected from within the *BALF5* gene encoding a subunit of EBV-DNA polymerase as described previously (18) and were synthesized by PE Applied Biosystems (Foster City, CA). Three replicate amplification reactions were set up for each sample in 0.2 mL tubes on a Rotorgene 2000 real-time PCR machine (Corbett Research, Sydney, Australia). PCR variables were 50°C for 120 seconds to activate the uracil-*N*-glycosylase enzyme, then an initial denaturation step of 95°C for 120 seconds followed by 45 cycles of 95°C for 30 seconds then 62°C for 60 seconds. The predicted PCR product was 89 bp, a size suitable for detecting the majority of the expected small fragments of DNA found in sera or plasma. Fluorescence data were acquired and a threshold cycle (C_T) value for each sample was calculated by determining the point at which the fluorescence exceeded

Table 1. Detailed patient characteristics recruited in the study

	Newly diagnosed (31) / relapsed (6)	Long-term survivors
Total (gender)	37 (14:23 F/M)	21 (10:11 F/M)
Mean age at diagnosis (y)	33 (range, 13-76)	29 (range, 15-51)
Clinical stage at entry to study (IIb/III/IV)	20 (54%)	not applicable
Prognostic score at entry to study (≥ 3)*	13 (35%)	not applicable
Time of blood sample from diagnosis	not applicable	mean, 5 y (2-33)
Histology, nodular sclerosing	76%	58%
Mixed cellularity	16%	11%
Lymphocyte-rich	3%	5%
Lymphocyte-depleted	3%	0%
Nodular lymphocyte predominant	3%	15%
Hodgkin's unclassified	0%	11%
Positive EBV serology	100%	90%
Positive EBV tissue status [†]	38%	26%

* Hasenclever/Diehl International Prognostic Score.

[†] In 2 of 58 cases, the LMP1 and EBER stains were discordant (EBER positive/LMP1 negative). Both cases were classified as EBV tissue – positive.

the threshold limit. For each analysis, a calibration curve was run in with serially diluted EBV-DNA extracted from the cell line Raji and a standard curve of the C_T values was constructed. The C_T values from test samples were then compared with the standard curve and the copy number was determined automatically. For PBMC samples, primers and probe were used as previously described to quantify the human albumin gene (19). The normalized value of the EBV-DNA load was expressed as the number of EBV copies per 10^6 PBMC calculated as the ratio EBV mean copy number / (albumin copy number / 2) $\times 10^6$. This allowed sample to sample variations to be normalized by cell count, PCR efficiency, and yield of DNA extraction. Multiple template controls were included in each assay as a negative control.

Statistics. Values were tested for significance using the Mann-Whitney, or when appropriate, the Wilcoxon matched pair test, as stated. All P values were two-tailed, with a cutoff of 0.05 for significance. Matched plasma/PBMC samples were tested for correlation using the Spearman coefficient. All statistical analyses were done using GraphPad Prism software version 4.02.

Results

Viral load from PBMC. The comprehensive data from this analysis is presented in Fig. 1A. In four of eight (50%) EBV-

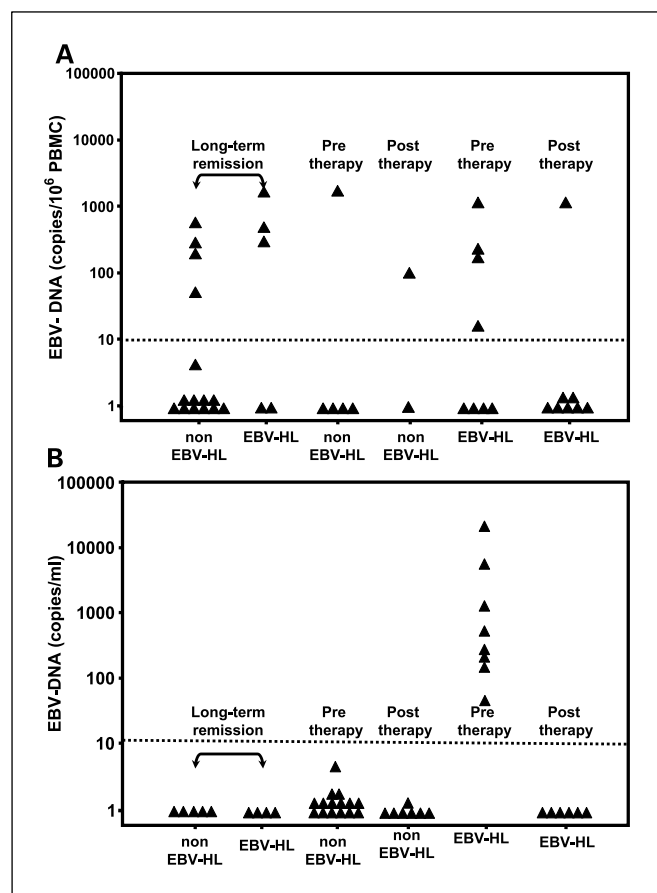


Fig. 1. Quantitative EBV viral load in HL patients. **A.** PBMC EBV-DNA in HL patients subclassified according to EBV tumor status and their relationship to therapy. The sixth column refers to posttherapy PBMC samples taken from five of the seven EBV-positive HL patients whose values are shown in column five. **B.** plasma EBV-DNA in HL patients subclassified according to EBV tumor status and their relationship to therapy. The sixth column refers to posttherapy PBMC samples taken from six of the eight EBV-positive HL patients whose values are shown in column five. In (A) and (B), an arbitrary threshold of 10 copies EBV-DNA was applied, below which, viral load was assessed as unlikely to be relevant. EBV-HL, EBV-positive HL; non – EBV-HL, EBV-negative HL.

positive HL patients prior to therapy, viral load was detectable. Following remission induction, samples were available for analysis in eight patients, and in all but one of these samples, EBV-DNA was undetectable ($P = 0.33$, Mann-Whitney test). The eight remission samples included follow-up samples from two patients whose results contributed to the eight prior to therapy samples. In these two patients, viral load was negative both at presentation and following remission induction. In EBV-negative HL patients with active disease (prior to therapy), one of five (20%) of the patients had detectable EBV-DNA (relative to EBV-positive HL, prior to therapy; $P = 0.52$, Mann-Whitney test). However, viral load was also present in 26% of EBV-negative HL patients and 57% of EBV-positive HL patients in long-term remission. The results from our small patient cohort indicate that there is no clear association between the presence of EBV-positive tumor and levels of EBV-DNA detected in PBMC.

Viral load from plasma. As opposed to PBMC, EBV-DNA was detected in all eight EBV-positive HL patients with active disease that were tested. Follow-up samples were available in six of the eight patients, and DNA became undetectable following response to therapy in all six patients ($P = 0.0156$, Wilcoxon matched pair test). Plasma EBV-DNA was also not detected in all patients with EBV-negative HL (relative to EBV-positive HL, prior to therapy; $P = 0.0238$, Mann-Whitney test) and those with long-term remission ($P = 0.0011$, Mann-Whitney test; Fig. 1B). The EBV-DNA load was highest in the EBV-positive HL patient with the highest prognostic score (IPS of 4).

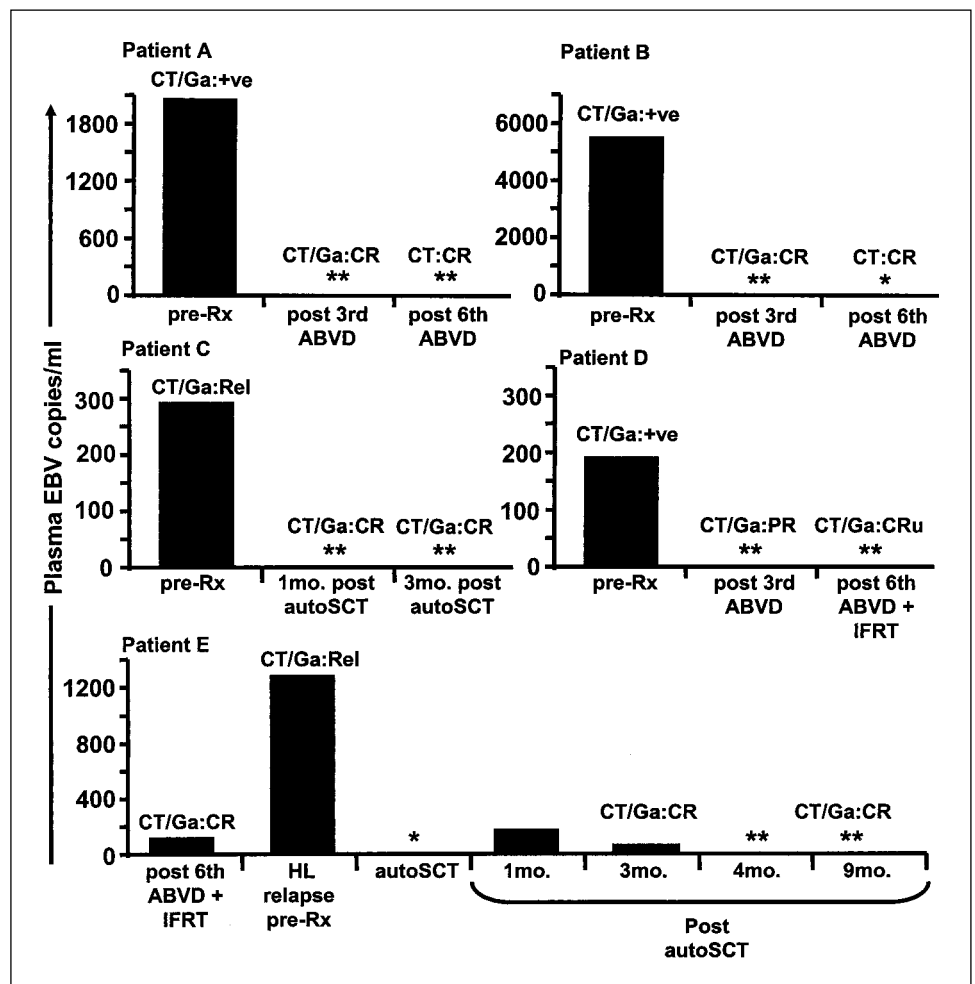
The relevance of EBV-DNA as a biomarker was further highlighted in serial analysis done in six EBV-positive HL patients who presented with active disease. In all six, response to chemotherapy was associated with decline in viral load to undetectable levels. Full details (i.e., radiographic evaluation and therapy administered) were available in five patients and are shown in Fig. 2A-E. One patient with EBV-positive HL had detectable EBV-DNA despite resolution of disease as assessed by CT scan and ⁶⁷gallium scintigraphy, and then relapsed 5 months later associated with a 10-fold increase in EBV-DNA (Fig. 2A). Consistent with previous studies, pretreatment with DNase of a serum DNA sample from this patient abrogated the amplification of PCR product (data not shown; refs. 9, 20).

Viral load in paired PBMC/plasma samples. Paired PBMC/plasma samples were available in 12 cases (i.e., 24 samples). This was comprised of two newly diagnosed EBV-positive HL patients prior to therapy (plasma 531, 31 PBMC; and 41, 2,834), one relapsed EBV-positive HL patients prior to therapy (2,771, 0), two newly diagnosed EBV-positive HL patients following remission induction (both 0, 0), one newly diagnosed EBV-negative HL patient following remission induction (0, 0), two long-term remission EBV-positive HL patients (0, 686; and 0, 3,663), and four long-term remission EBV-negative HL patients (all 0, 0). Overall, there was no significant correlation between matched pairs ($r = -0.03$; $P = 0.93$ Spearman correlation coefficient).

Discussion

Our data shows that EBV-DNA is specifically detected in the plasma of newly diagnosed and relapsed EBV-positive HL patients before treatment. This specificity is particularly highlighted by the fact that plasma samples from patients with

Fig. 2. Serial analysis of quantitative plasma EBV viral load in patients with EBV-positive HL. EBV copies per milliliter of plasma shown in relation to the timing of chemotherapy, and with respect to radiographic evaluation. Results from five patients (A-E). Patient A, 45-year-old male with newly diagnosed LR HL (stage IIIB, IPS 1); patient B, 26-year-old male with newly diagnosed NS HL (stage IIA, IPS 1); patient C, 56-year-old male with relapsed NS HL (stage IVB, IPS 3); patient D, 23-year-old female with newly diagnosed NS HL (stage IIB, IPS 2); patient E, 23-year-old female with relapsed NS HL (stage IIIA, bulky, IPS 2). NS, nodular sclerosing; LR, lymphocyte-rich; IPS, Hasenclever/Diehl International Prognostic Score; CT, computerized tomographic scan; Ga, ⁶⁷gallium scintigraphy; CR, complete remission (response criteria were defined as per published guidelines, ref. 22); CRu, complete remission unconfirmed; PR, partial remission; Rel, relapse; +ve, radiographic evidence of disease; ABVD, adriamycin, bleomycin, vinblastine, dacarbazine combination chemotherapy (23); IFRT, involved-field radiotherapy; **, not detected; *, not done.



EBV-negative HL, patients with EBV-positive HL after treatment, and patients with long-term remission (irrespective of EBV tumor status) do not show any evidence of EBV-DNA.

Of note, the DNA load was highest in the EBV-positive HL patient with the highest prognostic score, suggesting that DNA load analysis in plasma may correlate with the IPS in patients with EBV-positive HL. However, the stage and the presence of constitutional ("B") symptoms did not show any evidence of association. We acknowledge that larger prospective studies will be required to test whether there is a true correlation between IPS and plasma EBV-DNA.

Serial analysis done in EBV-positive HL patients who presented with active disease showed that response to chemotherapy was associated with decline in viral load to undetectable levels. Intriguingly, in one patient with EBV-positive HL, detectable EBV-DNA was present prior to radiographic evidence of relapsed disease. This observation is consistent with the findings by workers in the field of EBV-associated nasopharyngeal carcinoma, that persistence of viral load following primary therapy was predictive of relapse (10, 12, 21). Clearly, prospective studies of serial EBV-DNA viral load, perhaps in association with functional radiographic imaging, are required to assess the value of monitoring viral load for the early detection of relapse of EBV-positive HL. The timing of plasma viral load assessment in relation to

chemotherapy was that blood samples were measured prior to therapy, and then immediately prior to a subsequent cycle of chemotherapy as indicated (Fig. 2). We cannot rule out that therapy-induced tumor lysis may have resulted in transient elevation of viral load, which would most likely have been observed early following the initiation of therapy.

Khan and colleagues showed that cellular EBV load was increased in pretreatment samples of EBV-positive HL patients compared with EBV-negative HL cases (6). In agreement with their findings, we found that EBV-DNA was detectable in 50% of EBV-positive HL patients with active disease in contrast to only 20% of EBV-negative HL patients. Although this did not reach statistical significance, the sample size was unlikely to have been sufficiently powered and the p value should be interpreted with caution. One interpretation of the findings of Khan et al. is that a raised level of cellular EBV-DNA is a risk factor in developing EBV-positive HL. Thus, elevated PBMC EBV-DNA may be in turn due to impaired immune surveillance, resulting in increased frequency of nonneoplastic EBV-infected cells. Alternatively, the raised cellular viral load may simply reflect the presence of EBV-DNA in circulating Hodgkin Reed-Sternberg cells in patients with active EBV-positive HL. Our ability to detect viral load in patients in remission suggests that there is no clear association between the presence of EBV-positive tumor and levels of EBV-DNA detected in PBMC. Thus, PBMC EBV-DNA

cannot be recommended as a biomarker of disease activity in EBV-positive HL patients. Whereas the use of PBMC to measure EBV-DNA load in EBV-positive HL patients following EBV-specific T cell therapy will continue to have value in assessing antiviral efficacy, its role in monitoring disease response should be re-evaluated and it is possible that a different conclusion may be drawn when plasma EBV-DNA is measured.

Other workers have measured cell-free DNA by PCR in HL. Using conventional PCR, Gallagher et al. reported that EBV-DNA was detected in 91% of serum samples from patients with EBV-positive HL, whereas 23% of EBV-negative HL patients had detectable viral DNA (9). Their real-time PCR results for EBV-positive HL were similar, and only 10% of patients with EBV-negative HL had a quantifiable (low level) load, consistent with lysis of bystander EBV-positive B cells within the diseased lymph node. Using quantitative (but not real-time) PCR, Drouet and colleagues confirmed the observation that EBV-DNA was more frequently detected in serum from EBV-positive HL than EBV-negative HL, but argued that this viral DNA was probably a consequence of viremia related to increased viral replication in a nonneoplastic compartment such as the oropharynx (13). Wagner et al. detected plasma EBV-DNA by real-time PCR prior to therapy in 13 of 24 pediatric patients with EBV seropositive HL, and in none of the patients in stable remission, suggesting that viral load monitoring may be useful in disease evaluation (14). However, this study did not test tissue samples for the presence of EBV within Hodgkin Reed-

Sternberg cells and therefore was not able to stratify between EBV-positive HL and EBV-negative HL cases. The variation in sensitivity and specificity of EBV-DNA as a biomarker between these studies and ours may in part be reflected by technical differences in the assays employed. In this regard, the use of new generation reagents are likely to improve results. The sensitivity of real-time PCR varies with the efficiency and purity of DNA extraction, the segment of DNA amplified, the fluorogenic probe used, and potentially, the source of DNA (serum versus plasma). We amplified a region of *BALF5*, a subunit of the EBV-DNA polymerase gene, from plasma samples. By contrast, the study by Wagner used primers to detect the BAMH1-W region in plasma. This region is repeated a variable number of times, and therefore, their results cannot not be used to directly compare viral copy number as determined by ourselves (14). Interestingly, Gallagher and colleagues used primers to amplify the DNA polymerase region from serum, and their results are in broad agreement with our own (9). To our knowledge, no large-scale data is available comparing the relative sensitivity of EBV-DNA as assessed by real-time PCR between serum and plasma.

The data presented suggest that plasma EBV-DNA is a more sensitive and reliable biomarker than EBV-DNA from PBMC for disease evaluation of patients with EBV-positive HL. Additional prospective studies are required to further evaluate the value of free plasma EBV-DNA as a biomarker for monitoring response to treatment in patients with EBV-positive HL.

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Clin Cancer Res 2006;12:460-464.

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