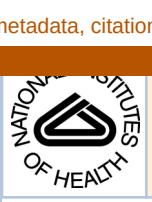

Published in final edited form as:

Diagn Mol Pathol. 2013 June ; 22(2): 91–101. doi:10.1097/PDM.0b013e318273fb43.

Atypical Epstein-Barr Viral Genomic Structure in Lymphoma Tissue and Lymphoid Cell lines

Weihua Tang, MD, PhD[1], **Hongxin Fan, MD**[2], **Jane Schroeder, DVM, PhD**[3], **Cherie H. Dunphy, MD**[1], **Ronald J. Bryant, MD**[4], **Yuri Fedoriw, MD**[1], and **Margaret L. Gulley, MD**[1,5]

[1]Department of Pathology and Laboratory Medicine, University of North Carolina, Chapel Hill, NC

[2]Department of Pathology, University of Texas Health Science Center at San Antonio, TX

[3]Department of Epidemiology, University of North Carolina, Chapel Hill, NC

[4]Department of Pathology, University of Vermont, Burlington, VT

[5]Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC

INTRODUCTION

Epstein-Barr virus (EBV) is present within the malignant lymphocytes of most endemic Burkitt lymphomas, nasal T/NK lymphomas, and lymphomas arising in patients with primary or iatrogenic immune deficiencies, and is also associated with a subset of sporadic B and T cell lymphomas.(1) The laboratory assay typically used to classify a tumor as being EBV-related is *EBV-encoded RNA in situ* hybridization, since *EBER* is expressed in latently infected cells and this assay can localize the infection to the malignant cell population.(1) Immunohistochemical stains are used to localize expressed viral proteins to tumor cells, but the spectrum of viral protein expression differs among tumor types and so there is concern for false negative test results. A tumor clone harboring a partially deleted EBV genome could go undetected by expression-based assays if the loss of genetic material results in atypical patterns of EBV gene expression.

Partially deleted or rearranged EBV DNA has been identified in some *EBER*-negative sporadic Burkitt lymphomas, which suggests that EBV DNA (or remnants of the EBV genome) may be present in a greater proportion of lymphomas than previously assumed.(2, 3) Prior molecular characterization of infected lymphomas identified two AIDS-related lymphomas with false negative tests for selected segments of the EBV genome, one of which had selective deletion of *LMP2* gene sequences, and another with selective dropout of *BZLF1* and *LMP1* amplicons.(4) Molecular characterization of infected gastric adenocarcinomas revealed an infected tumor with amplifiable *LMP1* but lacking 5 other segments of the viral genome that were queried by quantitative polymerase chain reaction (Q-PCR).(5) These examples suggest that tumors with atypical viral genomes may elude detection by commonly used laboratory assays, and raise the question of whether remnants of the viral genome are present in tumor subtypes that have not previously been associated with viral infection.

Beyond the potential for interference with laboratory tests, viral gene polymorphism could impact viral pathogenicity. Variants in the EBV genes *LMP1, EBNA2, EBERs*, and *BARF1* are reportedly more common in infected tumors than in geographically matched non-tumor

Address correspondence to: Margaret L. Gulley, MD, Dept of Pathology, University of North Carolina, 101 Manning Dr, 913 Brinkhous-Bullitt Building, Chapel Hill, NC 27599-7525, Telephone: 919-843-4595, Fax 919-966-6718, margaret_gulley@med.unc.edu.

control subjects.(6–12) Research into the clinicopathologic significance of these polymorphisms is facilitated by cataloging viral genomic variants in public databases such as the Virus Pathogen Resource at www.viprbrc.org.

In the current study we used a battery of real-time Q-PCR assays to amplify six disparate segments of the EBV genome (*LMP2*, *EBER1*, *BamH1W*, *BZLF1*, *EBNA1*, and *LMP1)* in non-Hodgkin lymphomas of varying histologic subtypes. Tumors with measurable viral loads or *EBER* localization to malignant cells by *in situ* hybridization were further studied using a panel of immunohistochemical stains to characterize LMP1 expression (associated with latent EBV infection) and BZLF1 and BMRF1 expression (associated with lytic EBV infection). Our goal was to evaluate a wide range of lymphoma subtypes for evidence of segmental viral genomic deletion, which has implications for etiologic mechanisms of viral oncogenesis and for design of laboratory tests to diagnose, monitor, and treat patients with EBV-infected tumors.

METHODS

Study Samples

Archival paraffin-embedded tumor tissue from 107 patients was retrieved from the clinical files of hospitals affiliated with the University of North Carolina and the University of Vermont. Tumors were selected to represent a spectrum of non-Hodgkin lymphoma subtypes, including lymphomas commonly associated with EBV (peripheral T cell lymphoma, diffuse large B cell lymphoma, and Burkitt lymphoma) as well as subtypes not usually associated with EBV (follicular lymphoma, marginal zone lymphoma including mucosa-associated lymphoid tumor (MALT), mantle cell lymphoma, and small lymphocytic lymphoma). Experienced hemtopathologists (CD, RB, and YF) confirmed that tumor was present in each sample and corroborated the histologic subtype using World Health Organization criteria.(13) This study was done with approval of the Institutional Review Board.

Quantitative real-time PCR

Q-PCR assays targeted and quantified six disparate regions of the EBV genome (Figure 1) in DNA extracted from two 10-micrometer thick paraffin sections cut from archival blocks, as previously described.(4, 5) All six Q-PCRs (for *BamH1W*, *EBNA1*, *LMP1*, *LMP2*, *EBER1*, and *BZLF1* segments) were previously validated on dilutions of cell line DNA and on paraffin embedded malignant tissues of known EBV status.(4, 5) One lymphoma with evidence of selective dropout of *BamH1W* amplicons was subjected to an alternate Q-PCR assay for the *BamH1W* region developed by Lo et al.(14) The alternate Q-PCR assay was validated by showing that viral loads measured using the alternate assay were equivalent to viral loads measured using our primary *BamH1W* Q-PCR assay when both assays were applied to 5 *EBER*-positive and 5 *EBER*-negative lymphomas.

For all six Q-PCRs, BLAST sequence analysis indicated no significant primer or probe homology with human DNA or other herpesvirus genomes. Cross reactivity with other herpesviruses was further examined by running each Q-PCR on purified cytomegalovirus (CMV), HHV8 (Kaposi's sarcoma associated herpesvirus, KSHV), varicella zoster virus (VZV) and herpes simplex virus (HSV1) DNA; formalin-fixed paraffin-embedded tissue from five patients diagnosed with other herpes family viruses (three with CMV, two with herpes simplex); and a paraffin-embedded pellet of the EBV-negative BL30 human lymphoma cell line.

PCR and product detection was performed on an ABI Prism 7500 Real-Time PCR instrument with Sequence Detection System software (Applied Biosystems, Foster City,

CA). Thermocycling conditions were: 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. Each 25uL reaction contained 1X TaqMan Universal Master Mix, TaqMan probe (10pmoL), and 1uL of DNA template. Assays targeting *LMP1* and *BZLF1* used 30pmoL of forward and reverse primers, while all other assays used 15pmoL of each primer. Standard curves were generated for each assay using serial 10-fold dilutions of Namalwa Burkitt lymphoma DNA (cell line from American Type Culture Collection, Rockville, MD) having two copies of the EBV genome(15) and two copies of the *APOB* human control gene per cell. Standard curves were deemed acceptable if they demonstrated analytic sensitivity to 50 or fewer copies of EBV DNA, a difference of 3.3 +/− 0.3 cycles between 10-fold dilutions, and a correlation coefficient of at least 0.99. Q-PCR of a segment of the human *APOB* gene was used to confirm the efficacy of DNA extraction and to calculate the number of cells amplified per reaction. Samples with fewer than 40 cells (as measured by Q-PCR targeting human *APOB* against a Namalwa cell line standard) were retested for *APOB* and for all six EBV DNA targets at higher or lower template volumes, and samples with consistently low *APOB* levels were excluded from further analysis.

To check for amplicon contamination, every run contained at least two “no template” controls in which nuclease-free H_2O was substituted for template. All experimental samples were run in duplicate, and mean viral loads were calculated based on the ratio of EBV to *APOB* copies in a given volume of extracted DNA, expressed as the number EBV DNA copies per 100,000 cells. Samples with no measurable EBV DNA were reported as having viral loads of zero.

Paraffin-embedded cell lines

Well described EBV-infected cell lines were fixed, embedded in paraffin, and tested by the battery of six EBV Q-PCRs. The B95-8 lymphoblastoid cell line is reported to harbor both episomal and replicative EBV genomes.(16) Chromosomal integration of the viral genome in Namalwa Burkitt lymphoma, BL30-B95-8 superinfected Burkitt lymphoma, and IB4 lymphoblastoid B cell lines affects the terminal repeat structure(17–19) which could disrupt *LMP2* reading frames, possibly interfering with intracellular signaling and lytic viral replication. Raji Burkitt lymphoma cells are reported to harbor a mixture of episomal and integrated genomes, with integration affecting the structure of the *BamH1W-Y* region.(20) The BL30-P3HR1 superinfected Burkitt line harbors the P3HR1 strain of EBV, which has multiple genomic variations (e.g. deletions, mutations) compared with the prototypic B95-8 strain.(21, 22)

Each cell line was grown in standard culture conditions, and cell pellets were fixed in 10% buffered formalin and then suspended in low-melting temperature agarose or fibrin clots prior to embedding in paraffin. Total DNA was extracted from paraffin sections, and EBV DNA was amplified by the same methods used for patient samples. The Namalwa cells that were paraffin embedded had been serially passaged for several years, whereas fresh Namalwa cell line DNA was used for assay calibration. To verify the linearity and sensitivity of each Q-PCR assay, we performed Q-PCR on serial ten-fold dilutions of template DNA extracted from paraffin-embedded Namalwa and Raji cell line samples with known EBV copy numbers (2 copies and about 55 copies of EBV DNA per cell, respectively).(15, 20, 23, 24)

EBV-encoded RNA (EBER) *in situ* hybridization

Each tumor was assayed for latent EBV infection by *EBER in situ* hybridization performed on an automated system that utilized fluorescein-labeled *EBER* probe and colorimetric detection (Ventana Benchmark, Ventana Medical Systems, Tucson AZ). A parallel

hybridization using oligo(d)T probe served as a control for RNA preservation. A molecular hematopathologist (MLG) classified tumors as *EBER*-positive if the *EBER* signal was localized to malignant appearing cells, or as *EBER*-negative if the signal was undetectable or was localized only to benign cells.

Immunohistochemistry for viral LMP1, BMRF1, and BZLF1 proteins

Immunohistochemical assays to detect expression of selected viral proteins were performed on paraffin sections of all lymphoma tissues that were *EBER*-positive or had measurable EBV DNA by any of the six Q-PCR assays. EBV LMP1 staining was done as previously described(4) using citrate retrieval and the anti-EBV LMP1-clone CS1-4 cocktail of mouse monoclonal antibodies (1:100, Dako, Carpinteria, CA). Immunohistochemical detection of the EBV replicative proteins BMRF1 and BZLF1 was performed using citrate retrieval and anti-BMRF1 clone G3-E31 (1:200 dilution, Research Diagnostics, Inc., Flanders, NJ) or anti-BZLF1 clone BZ.1 (1:25 dilution, Dako, Carpinteria, CA), with primary antibody treatment for 30 minutes at 37°C. The Super-Sensitive Non Biotin HRP Detection Kit with diaminobenzidine chromogen (Biogenex, San Ramon, CA) was used for blocking and detection. Tissues were counter-stained with hematoxylin (Dako, Glostrup, Denmark) and interpreted for signal localization to tumor cells. Positive controls were a known EBV-related Hodgkin lymphoma (for LMP1) and an oral hairy leukoplakia (for BMRF1 and BZLF1).

Statistical analyses

Means and standard deviations were compared to determine concordance of viral load measurements across the six Q-PCR assays, and to identify atypical EBV-positive cases as assessed by evidence of selective failure of one or more Q-PCR assays in a given case. We used receiver operating characteristic (ROC) analyses(25) to evaluate Q-PCR viral load threshold as a method for distinguishing between tumors with neoplastic *versus* non-neoplastic (incidental) EBV infection (as defined by the gold standard *EBER in situ* hybridization). This was accomplished by plotting the true positive rate against the false positive rate when various viral load thresholds were applied, and calculated area under the curve (AUC) was used to estimate the proportion of paired neoplastic and non-neoplastic samples that would be classified correctly for each viral load threshold. All p-values reported for statistical tests of differences in means and proportions are two-sided.

RESULTS

EBV DNA measurements by Q-PCR in infected or uninfected lymphoid cell lines

EBV copy numbers in serially diluted paraffin-embedded Namalwa and Raji cell lines demonstrated that all six Q-PCR assays were sensitive to fewer than 50 copies per PCR, and were linear over at least 3 orders of magnitude. No amplification was seen when pure viral DNA samples of CMV, HHV8, VZV or HSV1 were used as the template for Q-PCR assays (Table 1). Amplification was also negative in the *EBER*-negative BL30 human cell line and the two HSV-positive patient samples. All three CMV-related colon lesions had low levels of EBV DNA detected by at least two independent Q-PCR assays, but no amplification was observed with purified CMV DNA; therefore, we concluded that the weak EBV signal in the CMV-positive biopsies reflected true EBV DNA presence, which is not unexpected in human tissue samples given the ubiquitous prevalence of EBV infection in adults. In total, these findings suggest no cross reactivity of the six EBV Q-PCR assays with any of the other herpesvirus family members tested.

If each infected cell line contains only intact EBV genomes (without segmental deletions or gene amplifications), we would expect comparable viral load estimates across all of the Q-

PCR assays except the assay for *BamH1W,* a known repeat sequence.(4, 5) However, we observed some discrepancies among the six viral loads in most cell lines (Table 2). In four of the cell lines (Namalwa, Raji, B95-8, and BL30-B95-8) there was a >9-fold difference between the lowest and highest viral load estimate (e.g., ranging from 112 copies of *LMP2* to 1,265 copies of *LMP1* DNA in the BL30-B95-8 cell line) whereas there was less than a 5-fold difference in viral loads for the two remaining cell lines (IB4 and BL30-P3HR1, with viral loads ranging from 1 – 3 and 2 – 9 copies per cell, respectively). A noticeable trend is that *LMP2* values copy numbers were relatively low compared with copy numbers of the other EBV gene segments, which could indicate a problem with assay calibration or assay efficiency for the *LMP2* gene segment. Alternatively, it could indicate that the *LMP2* segment was consistently defective (e.g. deleted or mutated) in a way that interfered with its enumeration in each cell line, but this explanation seems less plausible because *LMP2* copy numbers were also relatively low in the naturally infected lymphomas as described below.

The Namalwa cell line is reported to have two integrated copies of the EBV genome per cell (15). We confirmed two copies (plus or minus one copy) per cell in paraffin-embedded samples of Namalwa cells based on *LMP2*, *EBER1*, *BZLF1*, *EBNA1* and *LMP1* assays (Table 2). In contrast, we measured 24 copies per cell of the *BamH1W* segment, a tandem repeat sequence with varying copy numbers across strains of EBV. Copy numbers of tandem repeat segments have been reported to fluctuate during long-term cell culture and during lytic viral replication.(26) When the *BamH1W* and *LMP2* regions are excluded from consideration, copy numbers based on the remaining four Q-PCR assays were equivalent (within about a one log range) in all six cell lines.

Q-PCR and EBER in situ hybridization results in patient samples

Nine patient tumors were excluded from further analyses because of inadequate tissue (5 MALT lymphomas), inadequate *APOB* amplification (2 diffuse large B cell lymphomas), or missing histologic subtype data (2 tissues), leaving 98 unique archival paraffin-embedded human lymphoma samples that were evaluable by Q-PCR, *EBER in situ* hybridization, and at least 2 immunohistochemical assays (Table 3).

PCR assays were negative for all six EBV segments in 50 samples and positive for at least one EBV segment in 48 samples. Mean estimated *APOB* copy numbers were comparable between PCR-negative and PCR-positive cases (PCR negative: 8,105 copies / PCR, 95% confidence interval (CI) 5,363-10,847; PCR positive: 7,223 copies / PCR, 95% CI 4,700 – 9,746; p = 0.6). All 50 PCR-negative samples were also *EBER in situ* hybridization negative.

Thirty-eight PCR-positive tumors were classified as non-neoplastic (incidental) EBV infections based on lack of localization to malignant cells by *EBER in situ* hybridization (Table 3). Only three of these tumors had amplification of all six EBV segments (Table 4). *BamH1W* was the most common target amplified from these samples (n=30/38 tissues positive), with a mean copy number of 119 EBV copies/100,000 cells (range 1 – 870). PCR-positive/*EBER*-negative samples were identified among all histologic subtypes, but were most common among the marginal zone lymphomas (10 of 15 samples, including 9 of the 14 subclassified as MALT) and mantle cell lymphomas (2 of 3 samples).

The 10 remaining PCR-positive samples were classified as neoplastic EBV infections based on *EBER* localization to malignant cells by *in situ* hybridization (4 Burkitt, 3 diffuse large B cell, and 3 T-cell lymphomas). Six of these PCR-positive/*EBER*-positive samples had high copy numbers for all EBV target segments and were classified as “typical” neoplastic infections (Table 5). The remaining 4 were classified as “atypical” infections because at least one EBV segment failed to amplify or was amplified at a relatively low level (at least

10-fold lower than the next highest value). These included one diffuse large B cell lymphoma with virtually no amplification of *BamH1W*, *BZLF1* or *EBNA1*, and three T-cell lymphomas with little or no amplification for *LMP2.* All 4 atypical samples amplified *LMP1* and *EBER1* (the latter in keeping with positive *EBER in situ* hybridization results). Mean copy numbers were lower for EBV targets amplified from atypical *versus* typical EBV-positive lymphomas, but were nonetheless 27 – 223 times higher than corresponding mean copy numbers for EBV targets amplified in PCR-positive/*EBER*-negative samples.

EBV protein expression

Immunohistochemical assays aimed at localizing lytic (BZLF1 and BMRF1) or latent (LMP1) EBV infection to neoplastic cells were negative in all 43 PCR-positive/*EBER*-negative samples tested. Five additional samples could not be assayed for LMP1 protein because tissue had been exhausted.

Among the ten *EBER*-positive tumors (Table 5), immunohistochemistry results varied. Two of six samples classified as typical infections expressed both BZLF1 (a sign of early lytic viral replication) and BMRF1 (a sign of active lytic replication), while another expressed only BZLF1, and none expressed LMP1. Two of four samples classified as atypical EBV infections expressed all three viral proteins (LMP1, BZLF1 and BMRF1). BZLF1 protein expression was negative in the atypical sample having evidence of *BZLF1* amplicon dropout, but this is not necessarily attributable to gene deletion as staining was also negative in 4 samples with high levels of *BZLF1* DNA. In all samples with visible EBV protein expression, expression was limited to a small proportion of neoplastic cells. In contrast, *EBER* was expressed in virtually all malignant cells.

Q-PCR can identify neoplastic EBV infections

ROC analyses were performed on Q-PCR data from 10 PCR-positive/*EBER*-positive and 38 PCR-positive/*EBER*-negative lymphomas to identify the viral load threshold that best distinguished neoplastic EBV infection from incidental EBV infection (Table 6). Neoplastic infection was identified with 100% sensitivity when 500 or more copies of *EBER1* or *LMP1* segments were amplified, whereas sensitivity at this copy number threshold was only 90% for *BamH1W*, *BZLF1*, and *EBNA1* segments, and only 80% for *LMP2.* These results reflect the low or negative amplification of certain EBV targets in atypical *EBER*-positive tumors (case #2681 with fewer than 500 copies of *BamH1W, EBNA1*, and *BZLF1*, and cases #2778 and #2783 with fewer than 500 copies of *LMP2*). However, the 500 copy number threshold was sufficient to identify neoplastic (*EBER*-positive) infections for all six EBV genomic targets when the 4 samples classified as atypical neoplastic infections were excluded.

False positive classification rates decreased as the copy number threshold was increased, so that all incidentally infected cases were classified correctly when a 2,000 minimum copy number threshold was applied (Table 6). All neoplastic and incidentally infected cases were correctly classified using a 2000 copy number threshold for *EBER1* or *LMP1.* Because of the atypical infection cases, only 95% of cases were correctly classified (AUC 95% CI 85 – 100%) using a 2,000 copy number threshold for *BamH1W*, *BZLF1*, or *EBNA1*, and 85% of cases were correctly classified (AUC 95% CI 70 – 100%) for *LMP2*. Overall these data suggest that use of *EBER1* or *LMP1* Q-PCR assays exceeding a threshold of 2,000 copies per 100,000 cells would be a reasonable strategy to screen lymphomas for neoplastic EBV infection.

Clinical characteristics

Age at diagnosis was available for 91 cases and gender was known for 93 cases. Age was similar for patients with PCR-positive and PCR-negative lymphomas (57 years, 95% CI 51

– 63 *versus* 52 years, 95% CI 46 – 58, p = 0.2) but was lower for patients with PCR-positive/*EBER*-positive lymphomas than PCR-positive/*EBER*-negative tumors (46 years, 95% CI 33 – 60 *versus* 59 years, 95% CI 52 – 66, p = .08). Clear gender differences were not apparent, but patients with PCR-positive lymphomas were somewhat more likely to be male than patients with PCR-negative tumors (74% *versus* 64%, p = 0.3) and patients with PCR-positive/*EBER*-positive tumors were more likely to be male than those with PCR-positive/*EBER*-negative tumors (80% *versus* 72%, p = 0.6). Pathology reports suggested that 4 cases were in immunocompromised patients, including 2 PCR-negative Burkitt lymphomas, 1 PCR-positive/*EBER*-negative diffuse large B cell lymphoma, and 1 PCR-positive/*EBER*-positive Burkitt lymphoma classified as a typical neoplastic infection. Clinical characteristics should be interpreted with caution given that our study included a small convenience sample of cases.

Case report of an atypical EBV-infected lymphoma

One atypical *EBER*-positive lymphoma (case #2681 in Table 5) had little or no amplification of *BamH1W*, *EBNA1*, and *BZLF1*, but relatively high levels *LMP2*, *EBER1*, and *LMP1* (1,745, 13,908 and 92,558 copies per 100,000 cells). These findings are consistent with deletion of the middle section of the viral genome with retention of both ends. All Q-PCR assays were repeated at least four times to confirm the viral loads for this tumor. Negative results for the *BamH1W* segment were confirmed using both primer/probe sets targeting the *BamH1W* portion of the EBV genome.

This tumor was a diffuse large B cell lymphoma arising in a 53 year old Hispanic female. The patient presented with a 3 month history of sore throat, bilateral parotid swelling, and cervical lymphadenopathy. She underwent bilateral tonsillectomy to relieve partial tracheal obstruction. Histologic examination of tonsil tissue revealed sheets of large cells with folded or cleaved nuclei and vesicular chromatin (centroblastic polymorphous appearance). Immunohistochemistry showed expression of CD45 (weak), CD20, CDw75, CD79a, BCL6, and BCL2. The large cells did not express CD10, MUM1, CD138, CD23, TdT, or CD34. CD5 and CD3 stained similar numbers of scattered small benign-appearing lymphocytes. By flow cytometry, the large cells expressed CD71 with dim CD45 and co-expression of CD20 and CD19 with no associated light chain expression or expression of CD10 or CD23. Karyotype showed t(14;18) *IGH/BCL2* in addition to complex chromosomal abnormalities: 46X,der(X)t(x;1)(p11.2;q25),t(1;3)(q?24;q27),add(2)(p21),t(14;18)(q32;q21),der(18)t(14;18)(q32;q21)[20]/ 47,idem,+21[5]. The tumor was subclassified as a malignant lymphoma, diffuse large B cell type, germinal center subtype (follicle center cell origin). HIV serology was negative. The patient responded well to six cycles of cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) plus rituximab followed by involved field radiation to the head and neck. The clinicopathologic findings in this case are not unusual for a lymphoma of this subtype, except for the atypical EBV genomic structure.

DISCUSSION

This study is the first to test for multiple segments of EBV DNA in a wide spectrum of non-Hodgkin lymphoma tissues. EBV was detected only in lymphoma subtypes that are already known to be EBV-related (Burkitt, diffuse large B cell, and T cell), and there was no evidence of EBV DNA remnants in other lymphoma subtypes (follicular, marginal zone, small lymphocytic, mantle cell). High EBV DNA copy numbers were found only in lymphomas classified as neoplastic EBV infections based on standard *EBER in situ* hybridization. Correlation between viral load and *EBER* localization supports the efficacy of quantitative (as opposed to qualitative) DNA amplification as a screening test for EBV-related malignancy. Occasional false negative Q-PCR results reinforce the utility of *EBER*

in situ hybridization as the gold standard assay for detecting tumor-associated EBV and for defining a tumor as EBV-related.

Low level EBV DNA (based on Q-PCR amplification of one or more EBV targets) was found in some lymphoma tissues despite the absence of latent (*EBER*, LMP1) or lytic (BZLF1 or BMRF1) viral gene expression, consistent with incidental infection of rare benign lymphoid cells. Interestingly, four *EBER*-positive tumors had evidence of selective drop out of portions of the EBV genome, suggesting genomic deletion or mutation involving *BamH1W, EBNA1*, *BZLF1* or *LMP2* gene segments. One of the four was a diffuse large B cell lymphoma with almost complete loss of amplicons in the central portion of the EBV genome (*BamH1W*, *EBNA1* and *BZLF1*). The remaining three cases were T cell lymphomas with marked, selective reduction of *LMP2* amplicons, implying that *LMP2* gene defects may be common in infected T cell tumors.

In prior studies, selective loss of *BZLF1*, *LMP1* or *LMP2* amplicons was identified in a fraction of AIDS lymphomas,(4) and selective loss of *LMP2* was found in a gastric adenocarcinoma.(4, 5) Another unusual gastric adenocarcinoma had loss of all but the *LMP1* amplicon, but it was not possible to determine whether this tumor contained only a small segment of the EBV genome, or if the *LMP1* amplification was false positive.(5) If the *EBER1* segment of the viral genome can be lost, then previous findings,(2–5) reinforced by data from the present study, suggest that there is not a single Q-PCR that is completely reliable for detecting tumor-associated EBV infection. This work has implications for the design of assays to identify EBV-associated malignancy. Specifically, it suggests that several disparate EBV gene segments should be assayed by Q-PCR, or that the gold standard *EBER* in *situ* hybridization assay should be used to maximize the detection of EBV in all types of malignancy.

Identifying EBV is important from a clinical standpoint since patients with EBV-infected lymphomas may be candidates for novel or alternative therapies targeting virally infected cells.(27–33) Indeed, progress has been made in treating EBV-related malignancies by infusing EBV-specific cytotoxic T cells, by inducing lytic viral replication, and/or by delivering drugs that leverage virus-driven biochemical pathways.

In clinical settings, Q-PCR of blood or plasma is frequently used to monitor tumor burden in allogeneic transplant recipients or nasopharyngeal carcinoma patients.(1, 34) The high levels of circulating EBV DNA in affected patients presumably derive from death of infected tumor cells.(35) Evidence that selected Q-PCR amplicons may be lost in primary tumor tissue implies that tumor-derived viral DNA in body fluids may also fail to amplify, potentially adversely impacting the efficacy of viral load assays in clinical settings. Consequently, it would be prudent to confirm amplification of EBV DNA in tumor tissue or in body fluid at the time of initial diagnosis before using the same assay to amplify EBV DNA in subsequent body fluid specimens as a marker of tumor burden.

Identifying and studying lymphomas with viral genomic deletion or mutation is relevant to understanding oncogenesis, since a corrupted viral coding sequence could result in abnormal viral gene expression affecting the tumorigenic potential of the viral strain. One remarkable case of diffuse large B cell lymphoma (case #2681 in this study) had evidence suggesting deletion of a large segment of the EBV genome. Assuming the deletion was contiguous across the three relevant segments, viral DNA spanning multiple latent and lytic genes as well as microRNA coding sequences would have been lost, resulting in altered transcriptional regulation of both viral and cellular genes. If true, the result implies that these EBV genome segments are not necessary to maintain EBV in lymphoma cells. EBV persistence in the absence of *EBNA1*, a gene believed to be required for propagation of

EBV DNA to daughter cells upon cell division,(36) suggests that episomal partitioning during mitosis may be obviated, which in turn suggests that the residual viral genome is integrated into host chromosomal DNA.

We used the Namalwa cell line as a standard for quantitative PCR because it has two integrated copies of the EBV genome per cell. When our battery of Q-PCR assays was used to measure viral genome copy number in a later passage of Namalwa cells and in five other well-characterized cell lines, certain parts of each viral genome had varying copy numbers that may have resulted from genetic polymorphism (mutation, deletion, gene amplification, or integration into the human genome). Altered copy number of tandem repeat sequences is a well described phenomenon, and it has been reported that numbers *BamH1W* repeat sequences can vary during long term culture, and during viral replication.(26) With the exception of the *BamH1W* repeat region, the remaining segments of the Namalwa genome varied no more than two fold (one PCR cycle) from the expected value, which supports the suitability of Namalwa as a standard for quantifying viral load. Similarly, the IB4 cell line, known to have two integrated copies of the EBV genome, had six different viral loads varying no more than two-fold (one PCR cycle) from the expected value. Given that these cell lines were fixed and paraffin embedded prior to DNA extraction, the fairly consistent viral loads measured in Namalwa and IB4 support the accuracy of EBV viral load measurements by Q-PCR in fixed tumor tissues.

The Raji Burkitt lymphoma cell line had widely varying viral loads ranging from 2 to 74 copies per cell depending on which genomic segment was targeted by our battery of six Q-PCRs. Prior work has identified both episomal and integrated EBV genomes in Raji cells, and four recurrent integration sites.(37) The relatively low *BamH1W* copy number measured in Raji cells in the present study could reflect viral genome disruption at the sites of chromosome integration previously described by Gao et al.(37)

The B95-8 cell line is a lymphoblastoid cell line that contains the prototypic wild-type EBV genome and demonstrates active lytic viral replication in a subset of infected cells. Our battery of six Q-PCRs demonstrated consistent viral loads (within one log), and an average viral load of 50 copies per cell. In the BL30-B95-8 cell line, a Burkitt lymphoma line (BL30) superinfected with EBV B95-8 virions, our battery of Q-PCRs demonstrated consistent viral loads (within one log), and an average load of 539 copies per cell. This high load (compared with an average of 5 copies per cell in the prototypic B95-8 cell line from which the virus is derived) implies that viral load is not determined by the strain of EBV exclusively. Nor is the host cell the only factor influencing viral load given that the BL30-P3HR1 line derived from the same BL30 parental line yielded much lower viral loads (average 5 copies per cell).

Our Q-PCR assays may be useful to epidemiologists seeking cost-efficient and reliable methods of detecting EBV-related tumors in large numbers of archival paraffin-embedded patient samples. Our current data suggest that a viral load of >2,000 *LMP1* or *EBER1* copies per 100,000 cells may be sufficient to identify neoplastic infections with 100% sensitivity and specificity. However, prior work has shown assay interference for *LMP1* or *EBER1* amplification by virtue of viral genomic mutation or deletion.(4, 5) Therefore two or more segments of the viral genome should be amplified to reduce the likelihood of a false negative result. When testing for low-level EBV infection (such as minimal residual disease after therapy), *BamH1W* appears to be the most sensitive of the Q-PCR assays,(1, 4, 5) probably because it targets a reiterated sequence. The presence of at least 2,000 copies of any EBV DNA segment is a reasonable surrogate for infected neoplasia, but confirmatory testing by *EBER in situ* hybridization is necessary to demonstrate EBV localization to the lesion of interest. Histochemical assays for BZLF1, BMRF1 or LMP1 protein expression do

not appear to add value as part of a screening strategy for neoplastic infections, but they may be useful for cancer diagnosis and classification.(1)

In conclusion, we have shown that a battery of Q-PCR assays is sensitive and specific for detecting tumor-associated EBV in archival paraffin-embedded patient samples. Our findings suggest that a threshold of 2,000 EBV copies per 100,000 cells can be used to distinguish tumors with neoplastic *versus* incidental EBV infection. Evidence of viral genome variation (such as point mutation, deletion, or chromosomal integration) in a subset of cases is relevant to laboratory medicine practice and has implications for disease pathogenesis. Atypical viral genome structure could interfere with certain assays aimed at viral detection, emphasizing the need to target more than one viral genome segment in order to maximize identification of infected tumors. Importantly, we did not identify any viral genomic alteration that would have rendered a tumor-specific infection undetectable by *EBER in situ* hybridization, which reinforces the role of *EBER in situ* hybridization as the gold standard assay for defining EBV-related malignancy. Our study showed that among *EBER*-negative tumors, there is scant evidence to support a hit-and-run hypothesis whereby the virus was present during lymphomagenesis but then was partially eliminated, thus retaining only remnants of the viral genome in malignant cells. Further work is needed to precisely characterize viral genomic variants as facilitated by emerging next generation sequencing strategies. Results should be correlated with clinicopathologic findings to shed light on the oncogenic impact of atypical viral genomes.

Acknowledgments

The authors thank Sandra H. Elmore and Mohammed Siddiqui for technical support.

References

1. Gulley ML, Tang W. Laboratory assays for Epstein-Barr virus-related disease. J Mol Diagn. 2008; 10:279–292. [PubMed: 18556771]
2. Razzouk BI, Srinivas S, Sample CE, et al. Epstein-Barr Virus DNA recombination and loss in sporadic Burkitt's lymphoma. J Infect Dis. 1996; 173:529–535. [PubMed: 8627013]
3. Gan YJ, Razzouk BI, Su T, et al. A defective, rearranged Epstein-Barr virus genome in EBER-negative and EBER-positive Hodgkin's disease. Am J Pathol. 2002; 160:781–786. [PubMed: 11891176]
4. Ryan JL, Fan H, Glaser SL, et al. Epstein-Barr virus quantitation by real-time PCR targeting multiple gene segments: a novel approach to screen for the virus in paraffin-embedded tissue and plasma. J Mol Diagn. 2004; 6:378–385. [PubMed: 15507678]
5. Ryan JL, Morgan DR, Dominguez RL, et al. High levels of Epstein-Barr virus DNA in latently infected gastric adenocarcinoma. Lab Invest. 2009; 89:80–90. [PubMed: 19002111]
6. Speck SH, Jia Y, Wang Y, et al. EBV framed in Burkitt lymphoma Sequence analysis of the Epstein-Barr virus (EBV) BRLF1 gene in nasopharyngeal and gastric carcinomas New variations of Epstein-Barr virus-encoded small RNA genes in nasopharyngeal carcinomas, gastric carcinomas, and healthy donors in northern China Unique variations of Epstein-Barr virus-encoded BARF1 gene in nasopharyngeal carcinoma biopsies Widespread sequence variation in the Epstein-Barr virus latent membrane protein 2A gene among northern Chinese isolates Sequence analysis of Epstein-Barr virus EBNA-2 gene coding amino acid 148–487 in nasopharyngeal and gastric carcinomas LMP1 strain variants: biological and molecular properties Novel mechanisms of EBV-induced oncogenesis. Nat Med. 2002; 8:1086–1087. [PubMed: 12357244]
7. Raab-Traub N. Novel mechanisms of EBV-induced oncogenesis. Curr Opin Virol. 2012
8. Mainou BA, Raab-Traub N. LMP1 strain variants: biological and molecular properties. J Virol. 2006; 80:6458–6468. [PubMed: 16775333]
9. Jia Y, Wang Y, Chao Y, et al. Sequence analysis of the Epstein-Barr virus (EBV) BRLF1 gene in nasopharyngeal and gastric carcinomas. Virol J. 2010; 7:341. [PubMed: 21106109]

10. Wang Y, Zhang X, Chao Y, et al. New variations of Epstein-Barr virus-encoded small RNA genes in nasopharyngeal carcinomas, gastric carcinomas, and healthy donors in northern China. J Med Virol. 2010; 82:829–836. [PubMed: 20336720]
11. Wang X, Wang Y, Wu G, et al. Sequence analysis of Epstein-Barr virus EBNA-2 gene coding amino acid 148–487 in nasopharyngeal and gastric carcinomas. Virol J. 2012; 9:49. [PubMed: 22348267]
12. Wang Y, Wang XF, Sun ZF, et al. Unique variations of Epstein-Barr virus-encoded BARF1 gene in nasopharyngeal carcinoma biopsies. Virus Res. 2012; 166:23–30. [PubMed: 22406129]
13. Swerdlow, SH.; Campo, E.; Jaffe, ES., et al. WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues. Lyon: IARC; 2008.
14. Lo YM, Chan LY, Lo KW, et al. Quantitative analysis of cell-free Epstein-Barr virus DNA in plasma of patients with nasopharyngeal carcinoma. Cancer Res. 1999; 59:1188–1191. [PubMed: 10096545]
15. Lawrence JB, Villnave CA, Singer RH. Sensitive, high-resolution chromatin and chromosome mapping in situ: presence and orientation of two closely integrated copies of EBV in a lymphoma line. Cell. 1988; 52:51–61. [PubMed: 2830981]
16. Pan YR, Fang CY, Chang YS, et al. Analysis of Epstein-Barr virus gene expression upon phorbol ester and hydroxyurea treatment by real-time quantitative PCR. Arch Virol. 2005; 150:755–770. [PubMed: 15785969]
17. Gulley ML, Raphael M, Lutz CT, et al. Epstein-Barr virus integration in human lymphomas and lymphoid cell lines. Cancer. 1992; 70:185–191. [PubMed: 1318776]
18. Takakuwa T, Luo WJ, Ham MF, et al. Identification of Epstein-Barr virus integrated sites in lymphoblastoid cell line (IB4). Virus Res. 2005; 108:133–138. [PubMed: 15681063]
19. Hurley EA, Klaman LD, Agger S, et al. The prototypical Epstein-Barr virus-transformed lymphoblastoid cell line IB4 is an unusual variant containing integrated but no episomal viral DNA. J Virol. 1991; 65:3958–3963. [PubMed: 1645805]
20. Takakuwa T, Luo WJ, Ham MF, et al. Integration of Epstein-Barr virus into chromosome 6q15 of Burkitt lymphoma cell line (Raji) induces loss of BACH2 expression. Am J Pathol. 2004; 164:967–974. [PubMed: 14982850]
21. Murray RJ, Young LS, Calender A, et al. Different patterns of Epstein-Barr virus gene expression and of cytotoxic T-cell recognition in B-cell lines infected with transforming (B95.8) or nontransforming (P3HR1) virus strains. J Virol. 1988; 62:894–901. [PubMed: 2828684]
22. Jansson A, Johansson P, Li S, et al. Activity of the LMP1 gene promoter in Epstein-Barr virus-transformed cell lines is modulated by sequence variations in the promoter-proximal CRE site. J Gen Virol. 2007; 88:1887–1894. [PubMed: 17554019]
23. Anvret M, Karlsson A, Bjursell G. Evidence for integrated EBV genomes in Raji cellular DNA. Nucleic Acids Res. 1984; 12:1149–1161. [PubMed: 6320116]
24. Brandsma J, Miller G. Nucleic acid spot hybridization: rapid quantitative screening of lymphoid cell lines for Epstein-Barr viral DNA. Proc Natl Acad Sci U S A. 1980; 77:6851–6855. [PubMed: 6256764]
25. Altman DG, Bland JM. Diagnostic tests 3: receiver operating characteristic plots. BMJ. 1994; 309:188. [PubMed: 8044101]
26. Allan GJ, Rowe DT. Size and stability of the Epstein-Barr virus major internal repeat (IR-1) in Burkitt's lymphoma and lymphoblastoid cell lines. Virology. 1989; 173:489–498. [PubMed: 2556840]
27. Kenney S. Theodore E. Woodward Award: Development of Novel, EBV-Targeted Therapies for EBV-Positive Tumors. Trans Am Clin Climatol Assoc. 2006; 117:55–74. [PubMed: 18528464]
28. Merlo A, Turrini R, Dolcetti R, et al. Immunotherapy for EBV-associated malignancies. Int J Hematol. 2011; 93:281–293. [PubMed: 21336546]
29. Destro F, Sforza F, Sicurella M, et al. Proteasome inhibitors induce the presentation of an Epstein-Barr virus nuclear antigen 1-derived cytotoxic T lymphocyte epitope in Burkitt's lymphoma cells. Immunology. 2011; 133:105–114. [PubMed: 21342184]
30. Shirley CM, Chen J, Shamay M, et al. Bortezomib induction of C/EBPbeta mediates Epstein-Barr virus lytic activation in Burkitt lymphoma. Blood. 2011; 117:6297–6303. [PubMed: 21447826]

31. Feng WH, Kenney SC. Valproic acid enhances the efficacy of chemotherapy in EBV-positive tumors by increasing lytic viral gene expression. Cancer Res. 2006; 66:8762–8769. [PubMed: 16951192]

32. Jones K, Nourse J, Corbett G, et al. Sodium valproate in combination with ganciclovir induces lysis of EBV-infected lymphoma cells without impairing EBV-specific T-cell immunity. Int J Lab Hematol. 2010; 32:e169–174. [PubMed: 19196381]

33. Meng Q, Hagemeier SR, Fingeroth JD, et al. The Epstein-Barr virus (EBV)-encoded protein kinase, EBV-PK, but not the thymidine kinase (EBV-TK), is required for ganciclovir and acyclovir inhibition of lytic viral production. J Virol. 2010; 84:4534–4542. [PubMed: 20181711]

34. Gulley ML, Tang W. Using Epstein-Barr viral load assays to diagnose, monitor, and prevent posttransplant lymphoproliferative disorder. Clin Microbiol Rev. 2010; 23:350–366. [PubMed: 20375356]

35. Ryan JL, Fan H, Swinnen LJ, et al. Epstein-Barr Virus (EBV) DNA in plasma is not encapsidated in patients with EBV-related malignancies. Diagn Mol Pathol. 2004; 13:61–68. [PubMed: 15167006]

36. Frappier L. Role of EBNA1 in NPC tumourigenesis. Semin Cancer Biol. 2012; 22:154–161. [PubMed: 22206863]

37. Gao J, Luo X, Tang K, et al. Epstein-Barr virus integrates frequently into chromosome 4q, 2q, 1q and 7q of Burkitt's lymphoma cell line (Raji). J Virol Methods. 2006; 136:193–199. [PubMed: 16806502]

Figure 1.
EBV genome map. BamH1 restriction enzyme cut sites shown above the map result in fragments named from largest to smallest in alphabetical order, with lower case letters following Z. Below the map are the locations of the six gene segments evaluated in this study.

Table 1

Specificity of Epstein-Barr Viral Load Assays

Sample*	Human *APOB***	EBV target segment, copy number per PCR					
		LMP2	***EBER1***	***BamH1W***	***BZLF1***	***EBNA1***	***LMP1***
CMV DNA, purified, Towne strain	0	0	0	0	0	0	0
HHV8 (KSHV) DNA, purified (ABI)	3	0	0	0	0	0	0
VZV DNA, purified (ABI)	0	0	0	0	0	0	0
HSV-1 DNA, purified (ABI)	0	0	0	0	0	0	0
Colon biopsy, CMV colitis	145	0	7	<1	4	0	0
Colon biopsy, CMV colitis	2,033	1	3	7	9	6	<1
Colon biopsy, CMV colitis	4,962	<1	6	3	11	5	0
Esophageal biopsy, HSV infection	191	0	0	0	0	0	0
Esophageal biopsy, HSV infection	584	0	0	0	0	0	0
BL30 Cell Line, *EBER*-negative	690	0	0	0	0	0	0

*CMV= cytomegalovirus; KSHV= Kaposi's sarcoma associated herpesvirus (HHV8); HSV= herpes simplex virus; ABI, Advanced Biotechnologies, Inc., Columbia, MD

**Human *APOB* copy number reflects the number of cells represented in the reaction. Human *APOB* detected in purified HHV8 (KSHV) DNA (3 copies) probably emanated from the human cell line used to prepare the viral DNA, while purified VZV, HSV-1 and CMV DNA preparations had no evidence of human DNA contamination.

Table 2

Copy number of EBV target segments in paraffin embedded, Epstein-Barr virus infected cell lines

Cell Line	EBV segment copy number, per cell						Previously reported EBV copy number per cell (citation)	Previously reported viral genome structure (citation)
	LMP2	*EBER1*	*BamH1W*	*BZLF1*	*EBNA1*	*LMP1*		
Namalwa	1	2	24	3	2	1	2 (15)	Integrated via terminal repeats (17)
IB4	1	1	1	2	3	2	2 (19)	One copy integrated via BamH1C, another via EcoR1I (18, 19)
Raji	6	24	2	20	13	74	55 (15, 23, 24)	Episomal and integrated via BamH1W and BamH1D (20)
B95-8	8	67	53	48	50	74	18 (16)	Episomal with active replication
BL30-B95-8	112	375	607	357	516	1,265	22 (19)	Integrated via terminal repeats (17)
BL30- P3HR1	2	6	4	5	4	9	35 (19)	Episomal (17, 19)

Table 3

EBV infection by lymphoma subtype, with evidence of intact (typical) *versus* selective amplicon drop out (atypical) EBV genomic sequence*

			EBV Q-PCR Positive.		
Lymphoma subtype	**Number of cases**	**EBV Q-PCR Negative**	**Non- neoplastic**	**Neoplastic, Typical**	**Neoplastic, Atypical**
Burkitt	19	11	4	4	0
Diffuse large B cell	19	8	8	2	1
T cell	18	9	6	0	3
Follicular	13	7	6	0	0
Marginal zone/MALT	15	5	10	0	0
Small lymphocytic	11	9	2	0	0
Mantle cell	3	1	2	0	0
Total	98	50	38	6	4

*EBV Q-PCR negative: samples with no amplification of any of the six EBV target segments.

Non-neoplastic: amplification of one or more EBV targets, but negative for EBV-encoded RNA (*EBER*) in neoplastic cells based on *in situ* hybridization.

Neoplastic, Typical: *EBER*-positive tumor with amplification of all six EBV targets at comparable levels.

Neoplastic, Atypical: EBER-positive tumor with evidence of low EBV genome copy number (<10% of the next highest copy number) for at least one EBV target segment.

Table 4

Clinicopathologic characteristics of 38 *EBER*-negative lymphomas having at least one amplifiable EBV target*

						Q-PCR EBV copies/100,000 cells					
Case ID#	**Lymphoma Subtype[b]**	**Anatomic Site**	**Age**	**Sex**	***APOB*, copies**	***LMP2***	***EBER1***	***BamH1W***	***BZLF1***	***EBNA1***	***LMP1***
2618[a]	Burkitt	nodal	8	M	8,652	0	0	0	1	0	0
2746	Burkitt	ovary	72	F	25,550	0	0	1	0	0	0
2624	Burkitt	omentum	12	M	1,495	0	0	4	0	0	0
2747	Burkitt	testicle	50	M	9,339	0	2	0	0	0	0
2629	DLBL	nodal	38	M	10,918	0	106	153	45	112	42
2630[c]	DLBL	gastric	58	M	350	68	592	222	47	878	849
2631	DLBL	nodal	56	M	2,782	0	90	302	24	135	0
2634	DLBL	nodal	15	M	1,753	0	0	0	5	0	0
2680	DLBL	gallbladder	56	M	657	0	303	13	172	93	0
2635	DLBL	nodal	53	M	6,089	3	0	0	0	0	0
2695	DLBL	lung	50	F	4,519	0	0	3	0	0	0
2640	DLBL	nodal	59	F	1,673	0	0	3	0	0	0
2636	Follicular	skin	49	F	1,097	0	0	9	0	0	0
2639	Follicular	nodal	41	M	333	0	0	35	0	0	0
2683	Follicular	nodal	44	M	4,616	0	17	0	0	0	0
2689	Follicular	nodal	85	M	28,253	0	1	2	3	1	0
2694	Follicular	nodal	44	M	4,342	0	0	11	38	58	0
2684[a]	Follicular	nodal	65	M	19,673	0	1	0	2	0	0
2645	Mantle	nodal	74	M	886	0	0	6	0	0	0
2647	Mantle	nodal	62	M	5,789	0	16	8	1	4	0
2708[a]	MALT	gastric	62	M	5,847	0	0	10	5	40	0
2709	MALT	gastric	76	F	7,325	21	77	191	322	504	882
2711[a]	MALT	gastric	88	M	156	0	0	25	0	0	0
2713	MALT	gastric	78	M	892	0	0	23	0	0	0
2716	MALT	ocular	85	F	5,247	0	0	9	5	0	0
2719	MALT	orbit	81	F	19,557	0	14	83	39	66	89
2720	MALT	orbit	69	F	4,412	22	572	676	620	1,199	1,512

						Q-PCR EBV copies/100,000 cells					
Case ID#	**Lymphoma Subtype[b]**	**Anatomic Site**	**Age**	**Sex**	***APOB*, copies**	***LMP2***	***EBER1***	***BamH1W***	***BZLF1***	***EBNA1***	***LMP1***
2722	MALT	lung	63	M	130	0	0	68	239	0	0
2725	MALT	duodenum	73	M	275	209	411	870	1,128	593	0
2614[a]	MZL	submandib.	77	M	4,680	0	0	3	0	0	0
2756	SLL	nodal	80	F	22,852	0	7	0	0	2	0
2758	SLL	brain	74	M	1,028	0	0	18	100	0	0
2765	PTCL	nasopharynx	64	M	170	0	725	713	607	334	0
2769	PTCL	tonsil	-	-	30,262	0	72	40	42	35	53
2770	PTCL	nodal	-	-	30,303	4	167	30	166	97	0
2782	PTCL	epiglottis	-	M	8,193	0	0	1	0	14	0
2785	T cell, AILT	nodal	48	M	2,100	0	0	23	0	0	0
2775	T cell, AILT	nodal	67	F	22,785	0	2	0	3	0	0
Summary Statistics											
Overall Mean					8,026	9	846	94	95	110	90
Overall Median					4,568	0	0	10	3	0	0
% of samples PCR-positive					100%	16%	47%	79%	58%	45%	16%
Mean result in PCR-positive samples					8,026	55	176	119	164	245	571

[*] *EBER in situ* hybridization was negative or localized to non-neoplastic cells only. All tested samples were negative for BZLF1, BMRF1 and LMP1 protein expression in neoplastic cells unless noted.

[a] inadequate tissue forLMP1 immunohistochemistry.

[b] DLBL: diffuse large B-cell; MALT: mucosa associated lymphoid tissue, a subtype of marginal zone lymphoma (MZL); SLL: small lymphocytic lymphoma; PTCL: peripheral T-cell lymphoma, not otherwise specified (NOS); T cell, AILT: angioimmunoblastic T cell lymphoma.

[c] In setting of immune dysfunction.

Table 5

Clinicopathologic characteristics and Q-PCR results for 10 *EBER*-positive lymphomas*

ID#	Lymphoma subtype[a]	Anatomic Location	Age	Sex	*APOB* copies per PCR	Q-PCR result, EBV copies/100,000 cells						Immunohistochemistry [b]		
						LMP2	*EBER1*	*BamH1W*	*BZLF1*	*EBNA1*	*LMP1*	BZLF1	BMRF1	LMP1
Typical neoplastic infections														
2616	Burkitt	axillary	34	M	90	286,347	808,719	351,659	2,382,038	2,291,069	1,490,334	1%	Neg.	Neg.
2619[c]	Burkitt	penis	43	M	6,769	16,781	480,006	56,126	620,772	527,596	1,434,316	10%	10%	Neg.
2745	Burkitt	abdomen	44	M	3,415	2,901,271	7,121,827	3,239,437	3,261,607	3,332,537	37,478,296	Neg.	Neg.	Neg.
2621	Burkitt	nodal	29	M	1,566	86,405	361,821	488,799	640,499	333,168	2,592,924	Neg.	Neg.	Neg.
2632	DLBL	pelvis	42	M	2,682	103,677	594,112	52,928	352,170	342,645	919,562	10%	5%	Neg.
2633	DLBL	gastric	71	M	4,026	54,581	463,578	255,935	749,374	572,410	4,881,438	Neg.	Neg.	Neg.
Mean					3,091	574,844	1,638,344	740,814	1,334,410	1,233,238	8,132,812			
Median					3,049	95,041	537,059	303,797	694,937	550,003	2,041,629			
Atypical neoplastic infections														
2681	DLBL	tonsil	53	F	12,870	1,745	13,908	**2**	**0**	**0**	92,558	Neg.	Neg.	Neg.
2778	PTCL	tongue	75	F	5,885	**0**	10,385	16,428	18,880	23,109	11,140	<1%	<1%	<1%
2780	T cell, AILT	lung	27	M	3,318	**3,794**	108,629	67,645	57,723	64,178	103,789	10%	5%	3%
2783	NK/T cell	nasal	-	M	1,126	**331**	29,756	21,882	36,424	8,314	40,272	Neg.	Neg.	Neg.
Mean					5,800	1,468	40,670	26,489	28,257	23,900	61,940			
Median					4,602	1,038	21,832	19,155	27,652	15,712	66,415			

*All samples classified as neoplastic EBV infections based on localization of EBV to the malignant cells by *EBER in situ* hybridization.

Typical neoplastic infections: Amplification of all EBV target segments at comparable levels.

Atypical neoplastic infections: Evidence of low EBV genome copy number (<10% of the next highest copy number) for at least one EBV target segment, and marked in **BOLD.**

[a]DLBL: Diffuse large B-cell; PTCL: peripheral T-cell lymphoma, not otherwise specified; T cell, AILT: angioimmunoblastic T cell lymphoma; NK/T cell: Extranodal NK/T cell lymphoma.

[b]Proportion of neoplastic cells expressing each protein. Neg.: no visible protein expression.

[c]In setting of immune dysfunction.

Table 6

Receiver operator characteristic (ROC) by various Q-PCR copy number thresholds as used to classify EBV infection as neoplastic or non-neoplastic (incidental) in paraffin-embedded lymphomas *

	500 copy/100,000 cell threshold				1000 copy/100,000 cell threshold				2000 copy/100,000 threshold			
EBV Target	**Sensitivity**	**Specificity**	**AUC**	**95% CI**	**Sensitivity**	**Specificity**	**AUC**	**95% CI**	**Sensitivity**	**Specificity**	**AUC**	**95% CI**
LMP2	0.80	1.0	0.90	0.77 – 1.0	0.80	1.0	0.90	0.77 – 1.0	0.70	1.0	0.85	0.70 – 1.0
EBER1	1.0	0.92	0.96	0.92 – 1.0	1.0	1.0	1.0	1.0 – 1.0	1.0	1.0	1.0	1.0 – 1.0
BamH1W	0.90	0.92	0.91	0.80 – 1.0	0.90	1.0	0.95	0.85 – 1.0	0.90	1.0	0.95	0.85 – 1.0
BZLF1	0.90	0.92	0.91	0.80 – 1.0	0.90	0.97	0.94	0.83 – 1.0	0.90	1.0	0.95	0.85 – 1.0
EBNA1	0.90	0.89	0.90	0.79 – 1.0	0.90	0.97	0.94	0.84 – 1.0	0.90	1.0	0.95	0.85 – 1.0
LMP1	1.0	0.92	0.96	0.92 – 1.0	1.0	0.97	0.99	0.96 – 1.0	1.0	1.0	1.0	1.0 – 1.0
Any EBV target **	1.0	0.87	0.93	0.88 – 0.99	1.0	0.95	0.99	0.94 – 1.0	1.0	1.0	1.0	1.0 – 1.0

* Analyses based on data from 48 archival paraffin embedded lymphoma samples with amplification of at least one of six EBV target segments, and classified as a neoplastic (N = 10) or non-neoplastic (N = 38) infection based on *EBER in situ* hybridization.

AUC: area under the curve, which represents the probability that the threshold specified will correctly distinguish a random pair of *EBER*-positive and *EBER*-negative cases as neoplastic and non-neoplastic, respectively. 95% CI: 95% confidence interval for the AUC.

** Any one of the six EBV target segments above the threshold.