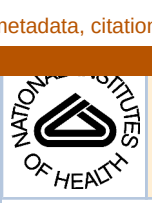

Published in final edited form as:

Clin Cancer Res. 2010 April 1; 16(7): 2055–2064. doi:10.1158/1078-0432.CCR-09-2424.

Viral Response to Chemotherapy in Endemic Burkitt Lymphoma

Weihua Tang[1], **Paula Harmon**[2], **Margaret L. Gulley**[1,3], **Charles Mwansambo**[4], **Peter N. Kazembe**[5], **Francis Martinson**[6], **Clifford Wokocha**[4], **Shannon C. Kenney**[7], **Irving Hoffman**[6], **Carlie Sigel**[1], **Susan Maygarden**[1], **Mariah Hoffman**[8], and **Carol Shores**[2,3]

[1] Department of Pathology and Laboratory Medicine, University of North Carolina at Chapel Hill (UNC-CH)

[2] Department of Otolaryngology/Head & Neck Surgery, UNC-CH

[3] Lineberger Comprehensive Cancer Center, UNC-CH

[4] Department of Pediatrics, Kamuzu Central Hospital, Lilongwe, Malawi

[5] Baylor College of Medicine Children's Foundation, Lilongwe, Malawi

[6] Department of Medicine, Division of Infectious Disease, UNC-CH

[7] Departments of Medicine and Oncology, University of Wisconsin, Madison

[8] UNC Project- Malawi, Lilongwe, Malawi

Abstract

Purpose—Some Epstein-Barr virus (EBV)-directed therapies are predicted to be effective only when lytic viral replication occurs. We studied whether cyclophosphamide chemotherapy induces EBV to switch from latent to lytic phases of infection in a series of EBV-associated Burkitt lymphomas.

Experimental Design—Children with first presentation of an expanding, solid maxillary or mandibular mass consistent with Burkitt lymphoma underwent fine needle aspiration just prior to initiation of cyclophosphamide and again 1 to 5 days later. Aspirated cells were examined for latent and lytic EBV infection using *in situ* hybridization to EBV-encoded RNA (*EBER*), immunohistochemical analysis of the lytic EBV proteins BZLF1 and BMRF1, reverse transcription PCR targeting BZLF1 transcripts, and EBV viral load measurement by quantitative PCR.

Results—Among 21 lymphomas expressing *EBER* prior to chemotherapy, 9/10 still expressed *EBER* on day 1 after therapy while only 2/11 (18%) specimens still expressed *EBER* at days 3 to 5, implying that chemotherapy was fairly effective at eliminating latently infected cells. Neither of the lytic products, BZLF1 or BMRF1, was significantly upregulated at the post-therapy time-points examined. However, EBV genomic copy number increased in 5/10 samples 1 day after treatment began, suggesting that viral replication occurs within the first 24 hours.

Conclusion—Cyclophosphamide may induce the lytic phase of EBV infection and is fairly effective in diminishing *EBER*-expressing tumor cells within 5 days. These findings provide the

Corresponding author and reprint requests: Margaret L. Gulley, MD, Dept of Pathology and Laboratory Medicine, University of North Carolina, 101 Manning Dr, 913 Brinkhous-Bullitt Building, Chapel Hill, NC 27599-7525, margaret_gulley@med.unc.edu.

Translational Relevance: The presence of Epstein-Barr virus (EBV) genome within malignant cells of endemic Burkitt lymphoma makes EBV an appealing target for therapy. Mechanistic data in cell lines and mouse models suggests that ganciclovir enhances tumor cell killing by chemotherapy *only* if the chemotherapy induces lytic viral replication. Our prospective study of naturally infected African Burkitt lymphoma patients found evidence of viral replication within the first 24 hours after starting cyclophosphamide. This result supports design of a phase two trial of cyclophosphamide and ganciclovir in endemic Burkitt lymphoma.

rationale for a trial testing synergistic tumor cell killing using cyclophosphamide with a drug like ganciclovir targeting lytically infected cells.

Keywords

Epstein-Barr Virus; Burkitt lymphoma; lytic infection; cyclophosphamide; ganciclovir

Introduction

Epstein–Barr virus (EBV) is a human gamma herpes virus that establishes a persistent infection in more than 90% of the world's population. EBV DNA has been detected in numerous tumors including Hodgkin and non-Hodgkin lymphomas, undifferentiated nasopharyngeal carcinoma, gastric carcinoma, and primary brain lymphomas in AIDS patients. EBV is closely associated with endemic Burkitt lymphoma in sub-Saharan Africa.

Like other herpes viruses, EBV exists inside cells in two alternative modes: latent infection and lytic replication (1). In the latent form of infection, EBV persists largely within memory B lymphocytes where only a small fraction of viral genes are expressed including EBV-encoded RNA (*EBER*). Upon cell division, replication of the viral genome is mediated by host cell DNA polymerase (1). The lytic form of infection is required for packaging of virions permitting transmission from host to host. In the lytic phase, most viral genes are expressed in a carefully orchestrated sequential fashion mediating viral genomic replication, encapsidation, and release of infectious virions accompanied by lysis and death of the host cell (2).

Induction of EBV replication can be achieved using radiation therapy, chemotherapy, or other manipulations inducing the lytic phase (3–6). Lytic phase infection promotes destruction of EBV-positive tumor cells, at least in cell culture models and in transgenic mouse models (7–12). It is timely and important to translate successful laboratory models into clinical practice. Latently infected malignancies such as Burkitt lymphoma may respond to lytic induction with associated host cell death. In fact, lytic cell death may be even more effective *in vivo* than *in vitro* when immune recognition of foreign viral antigens contributes to cell destruction.

A clinical trial by Chan and colleagues explored feasibility of lytic induction by azacytidine in ten patients with various EBV-related malignancies (13). Azacytidine is a DNA methyltransferase inhibitor that reactivates transcription of genes that were silenced by CpG island methylation. When administered to patients, there was significant demethylation of CpG islands in promoters of latent and lytic EBV genes encoding immunogenic proteins. By immunohistochemistry, only one patient had detectable lytic protein expression, suggesting that the effect was minimal, transient, or promptly progressed to cell death (13).

Histone deacetylase (HDAC) inhibitors such as sodium butyrate are well known inducers of lytic replication in Burkitt lymphoma cell lines (6,14,15). A clinical trial of the HDAC inhibitor, romidepsin, in T cell lymphoma patients resulted in secondary EBV-related lymphoproliferations in two of 120 treated patients (16). While these lymphoproliferations are likely to be multifactorial, it is feasible that HDAC inhibitors and other chemotherapeutic agents initiating lytic replication (like methotrexate (17)) could function to enhance killing of EBV-related neoplasia at a cost of increasing risk of secondary EBV-related lymphoproliferations. Interestingly, withdrawl of methotrexate therapy usually leads to regression of the associated EBV-driven lymphoproliferation, presumably by relieving the drug's immunosuppressive effect (18).

If lytic cycle inducers could be rendered even more potent, perhaps they might be capable of eliminating every malignant cell of an infected tumor. Intriguing studies show that some lytic

phase proteins (e.g. EBV thymidine kinase and BGLF4) can phosphorylate nucleoside analog drugs like ganciclovir to a toxic form, enhancing killing of the host cell and also of surrounding cells (e.g. latently infected tumor cells) (3,10,19,20). Therefore, addition of nucleoside analog drugs may achieve outcomes beyond what could be achieved with cytotoxic therapy alone. In a clinical trial of arginine butyrate plus ganciclovir in 15 patients with EBV-related lymphoma, four patients had complete response and 6 others had partial response (21). Most responses occurred within the first week of therapy, and tumor lysis was unexpectedly rapid in several patients (21).

Whether there is synergy between traditional chemotherapy and nucleoside analog therapy in Burkitt lymphoma patients remains to be tested. Burkitt lymphoma is the most common lethal malignancy of children between the ages of 3 to 15 years in the tropical regions of Africa, and this cancer is nearly always EBV-associated (22–24). In nearly all cases, the EBV gene expression pattern is very restricted. LMP1 and LMP2 are *not* expressed and, due to Qp promoter usage for EBNA1 transcription, only EBNA1 but not the other EBNAs are expressed (25,26). In resource-poor settings in Africa, Burkitt lymphoma is often treated using single agent cyclophosphamide with survival rates of 64% among children with maxillary or mandibular tumors compared to only 33% for those having more disseminated disease (25, 27,28).

Despite the potential for application to human disease, it has yet to be proven that chemotherapy induces lytic EBV gene expression *in vivo* in Burkitt lymphoma patients. Demonstrating lytic EBV expression in tumor tissue following treatment with chemotherapy may justify a new combined therapeutic approach to treating EBV-related malignancies. In the current study, we examined the extent to which lytic replication is induced by single agent cyclophosphamide in African children with Burkitt lymphoma. Fine needle aspirates of tumor were collected before and again 1 to 5 days after initiation of cyclophosphamide therapy, and the specimens were examined for evidence of latent and lytic EBV infection using histochemical and molecular methods. Our findings lay the groundwork for a possible clinical trial testing the addition of ganciclovir to initial chemotherapy as a way to enhance cancer-specific cytotoxicity.

Materials and Methods

Subjects

This research was approved by the UNC Medical Institutional Review Board and the Malawi National Health Sciences Research Committee. Consent was obtained from each patient's guardian. Children were admitted to the pediatric Burkitt Lymphoma ward of Kamuzu Central Hospital in Lilongwe, Malawi from August 2007 to June 2008. Patients age 3 to 15 years were recruited if they had a rapidly expanding mass involving the maxilla or mandible that was superficial and amenable to safe biopsy. All patients were studied at initial presentation of disease, and none had a previous history of cancer or cancer treatment. All patients were scheduled to receive cyclophosphamide therapy. Patients were excluded if they had hemoglobin <8g/dL, platelets <50,000/uL, or known intolerance or allergy to lidocaine. One study-specific pediatrician (CW) performed all fine needle aspirate (FNA) procedures. Treatment plans did not change as a result of enrollment. Patients were treated with cyclophosphamide 40mg/kg IV, with a plan to repeat every 2 weeks for at least 6 cycles.

Prior to the first round of cyclophosphamide, the skin overlying the mandibular or maxillary mass was anesthetized with topical 2.5% lidocaine and 2.5% prilocaine (EMLA cream, AstraZeneca) and an FNA obtained using a 23-gauge needle. The aspirated material was promptly transferred into a vial containing 20 mL of PreservCyt solution (Hologic, Inc., Bedford, MA), pumping the syringe several times to rinse. In the first half of the study, the

aspiration procedure was repeated 3 to 5 days after initiation of the first round of cyclophosphamide therapy. After interim analysis of laboratory data, subsequent patients had their second aspiration on day 1 rather than on day 3 to 5 to test whether viral replication occurred earlier in the time course following initiation of treatment. Specimens were refrigerated for up to six weeks before shipment in batches to University of North Carolina at Chapel Hill where 10 mL of the aspirate was used to make a paraffin- embedded cell block and the remaining 10 mL was used for nucleic acid extraction.

In situ hybridization

To localize latently infected cells, *in situ* hybridization was performed using an *EBER* probe, with an oligo-dT probe run in parallel as an RNA preservation control, on a BenchMark XT system (Ventana Medical System, AZ) according to manufacturer instructions. Results were interpreted by two pathologists (MLG and WT) who characterized each specimen as having abundant, few or no detectable *EBER*-expressing cells. Nuclear *EBER* signal was considered legitimate evidence of EBV infection. The proportion of *EBER*-expressing cells was calculated based on counts of *EBER*-positive cells and total nucleated cells in three 40x microscopic fields.

Real-time polymerase chain reaction (PCR) to measure EBV viral load

To measure EBV genomic copy number, quantitative PCR targeting the BamH1W segment was performed on DNA extracted from scrolls of each paraffin embedded cell block using the QIAamp DNA Mini Kit (QIAGEN, Inc). In 4 subjects there was insufficient DNA in the paraffin embedded sample so total nucleic acid was extracted from PreservCyt-suspended cells using the the QIAamp DNA Mini Kit. Viral load was measured as previously described (29) on an ABI 7500 real time PCR instrument using primers and a TaqMan probe targeting the EBV *BamH1W* segment in duplex with an assay targeting the human *APOB* gene that serves as a normalizer for the number of cells represented in the reaction. DNA from the Namalwa Burkitt lymphoma cell line was used as a standard by which to quantitate both EBV and *APOB* (29). EBV genomic copy number was reported as EBV copies per 100,000 cells.

Quantitative-rtPCR to detect EBV lytic gene expression

EBV lytic gene expression was detected by quantitative reverse transcription PCR (Q-rtPCR) targeting *BZLF1* mRNA. First, RNA was extracted from 10 mL of FNA preservative using the RNeasy Mini Kit (Qiagen) and DNA was removed by on-column deoxyribonuclease digestion for 15 min at room temperature using the RNase-Free DNase Set (Qiagen). Total RNA was eluted into 30 μL of ribonuclease-free water and cDNA was synthesized immediately using random hexamers and the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). RNA preservation and cDNA quality was confirmed using Q-rtPCR targeting human *ABL1* exons 10 and 11. Primers and TaqMan probes listed in Table 1 were chosen to span introns so that any residual genomic DNA would be unlikely to amplify (30). Positive controls included RNA from the B95.8 and Namalwa cell lines. No template controls verified absence of amplicon contamination.

Immunohistochemistry to detect EBV lytic gene expression

To detect EBV lytic protein expression in cell blocks, immunohistochemistry was performed using antibodies targeting the immediate-early lytic protein BZLF1 and the early lytic protein BMRF1. Briefly, unstained sections were deparaffinized, rehydrated and then incubated overnight at 4°C in monoclonal antibody targeting either BZLF1 (ZEBRA, M7005, DAKO Corporation, Carpinteria) or BMRF1 (Clone#:G3-E31, Fitzgerald, MA) using the blocking and detection protocols in the Super-Sensitive Non Biotin HRP Detection Kit (Biogenex). Oral hairy leukoplakia was used as a positive control for both lytic proteins. A control stain of the B cell marker CD20 (Chemicon international, Temecula, CA) was run in parallel to verify

protein detectability in each specimen. Results were interpreted by two pathologists (MLG and WT) who characterized each specimen as having abundant, few or no detectable BZLF1 or BMRF1-expressing cells.

Statistical analysis

Association of *EBER* positivity between 1 day and 3–5 day treatment groups was assessed using a chi-square test. Exact Wilcoxon rank-sum tests were used to compare differences in median viral loads and median *EBER*-expressing cell proportions, respectively, between specimens collected on treatment day 1 *versus* specimens collected on days 3–5 (SAS 9.2, SAS Institute Inc, Cary, NC).

Results

Clinical and cytological features

Thirty-three patients ranging from 4 to 14 years old with 20 males and 13 females were enrolled. Only those patients confirmed to have EBV-related Burkitt lymphoma based on clinical and cytologic features were further studied.

The first 16 enrolled patients had a second FNA collected 3–5 days after starting the first round of cyclophosphamide. Five of the 16 patients were excluded because initial hemoglobin was <8g/dL (n=1), the subject left the hospital before the second FNA was done (n=1), or FNA was *EBER*-negative at both time-points (n=3). Among the latter three subjects with *EBER*-negative aspirates, the first had cytologic features of an *EBER*-negative diffuse large cell lymphoma, the second had rare atypical large cells in a background of acute and chronic inflammation, and the third had reactive-appearing mixtures of inflammatory cells. Further evaluation would be required to distinguish reactive or infectious processes from Hodgkin lymphoma, non-Hodgkin lymphoma, or other tumor types.

The next 17 enrolled patients had a second FNA collected only one day after starting the first round of cyclophosphamide. Seven of the 17 were excluded because initial hemoglobin was <8g/dL (n=1), an FNA sample was lost (n=1), or the initial FNA was *EBER*-negative (n=5). Among the latter five patients with *EBER*-negative aspirates, the first had multinucleated giant cells and abundant eosinophils, the second had a few CD20-negative large cells and many macrophages, and the next three patients had peripheral blood although the post-treatment aspirate of one patient contained scattered *EBER*-positive large cells suggesting that the needle had missed an *EBER*-expressing tumor in the pre-treatment sampling. Overall, a total of 21 patients had adequate FNAs before and after chemotherapy. There were no complications.

The 21 patients confirmed to have EBV-related Burkitt lymphoma based on clinical and cytologic features combined with *EBER*-positivity ranged from 5 to 14 years old with 13 males and 8 females. Typical cytologic features included enlarged lymphoid cells, fine granular blast-like chromatin, and tingible body-laden macrophages, all of which are characteristic of Burkitt lymphoma. CD20 was expressed in larger atypical lymphoid cells in all cases (Figure 1).

EBER expression is diminished or lost after therapy

Latent EBV infection was identified by *EBER in situ* hybridization in all 21 specimens collected before therapy was initiated. As shown in Table 2, *EBER* was still expressed in nearly all specimens collected 1 day after treatment. By 3–5 days after treatment, *EBER* was undetectable in the majority of patients (Figure 1). The difference is statistically significant ($p = 0.0019$).

Total nucleated cell number was visibly decreased in all post-therapy specimens compared to pre-treatment levels. There was often a lower proportion of atypical lymphoid cells and, in

some cases, no cytomorphologic evidence of malignancy in the post-therapy specimens. *EBER*-expressing cells were only rarely visible by microscopy in patients sampled 3–5 days after therapy. In contrast, *EBER*-expressing cells were easily identified in all but one patient (#15) sampled on day 1 after therapy. (See Table 3.) These findings suggest that cyclophosphamide has a rapid effect in diminishing latent infection to nearly undetectable levels in the majority of patients within 5 days after initiation of treatment.

EBV DNA levels implicate viral replication after chemotherapy

Human *APOB* gene copy number was measured by real-time PCR as a surrogate for the number of nucleated cells represented in each DNA amplification reaction. *APOB* results helped adjust for fact that the number of cells aspirated differed from specimen to specimen. *APOB* levels typically went down after therapy (average 2-fold decrease), which correlates with the cytologic observation of fewer nucleated cells after treatment.

EBV viral load in tumor aspirates was measured by real-time PCR, and the results were rendered quantitative by virtue of normalization to the number of nucleated cells as represented by the human *APOB* gene results. EBV viral load was high in all 21 lymphoma specimens tested at the time of initial diagnosis before starting therapy. As shown in figure 2, EBV load fell precipitously in the 11 samples that were aspirated 3–5 days after treatment (average 9-fold loss of EBV). However, in specimens collected just one day after therapy, EBV viral load often did not decrease substantially compared with diagnostic specimens, and in fact it *increased* in half of the specimens (5 of 10 cases; Table 4.) The average increase in EBV load was 30-fold in the ten tumors sampled at day 1 compared to pretreatment levels. Even after excluding the outlier subject (case #16) with an exceptionally high rise in EBV load, the average viral load still rose 8-fold among tumors sampled at day 1 compared to pretreatment levels. An increase in EBV load suggests that the EBV genome has replicated during the first day after initiation of chemotherapy. This rise in viral load, in the face of diminishing numbers of cells, is consistent with lytic viral replication.

EBV lytic gene BZLF1 expression is detected in few Burkitt lymphoma tissues

To test whether lytic infection was the explanation for increased EBV viral load immediately after treatment, viral lytic gene expression was examined by Q-rtPCR targeting the EBV lytic transcript, *BZLF1*. BZLF1 is normally expressed very early in lytic phase and is considered to be the master switch controlling exit from latency to lytic viral replication.

Extracted RNA was successfully amplified in all specimens using PCR with a TaqMan probe targeting an endogenous control transcript (human *ABL1* mRNA). BZLF1 transcripts were expressed in only four samples, 3 collected before initiation of therapy (from patient #4, #11, and #19) and only one collected after therapy. The latter, from patient #12, was aspirated on day 1 after therapy and expressed low-level BZLF1. Overall, these data suggest no significant up-regulation of this lytic viral factor at the post-therapy time-points examined.

All three of the tumors having evidence of lytic infection prior to therapy had dramatic drops in EBV viral load after chemotherapy.

EBV lytic proteins were not expressed by immunohistochemistry

To further characterize any lytic infection, immunohistochemistry was performed using monoclonal antibodies against BZLF1 and BMRF1 which represent markers of lytic EBV infection. Microscopic examination revealed no detectable expression of either protein in any of the pre- or post-therapy aspirates. Control staining showed successful detection of both proteins in oral hairy leukoplakia tissue. Furthermore, each aspirate in this study was successfully stained for a protein (CD20) that is ubiquitously expressed in normal and

malignant B lymphocytes, diminishing the likelihood that technical failure is responsible for the negative viral lytic expression results in these aspirates. These data suggest that our immunohistochemical method is not sensitive enough to detect these lytic proteins, or that the timing of specimen collection was not optimal for detecting transient expression of these factors. It should be noted that BZLF1 is an immediate early lytic factor and BMRF1 is an early factor, meaning that both are expressed at an early time-point during the sequential process of viral replication and cell lysis.

Discussion

This study found EBV DNA levels, as measured by Q-PCR, frequently rise within one day of the start of cyclophosphamide therapy for Burkitt lymphoma. By day 5, the viral load and the tumor burden are already greatly diminished. The initial rise in EBV levels in some day 1 tumors could reflect viral DNA replication. The mechanism of EBV DNA replication is hypothesized to be lytic infection, although evidence for expression of the early viral genes BZLF1 and BMRF1 was lacking at the time-points that were examined.

The lytic phase of EBV infection is normally marked by sequential expression of a cascade of proteins that co-opt cellular functions leading to replication of the viral genome followed by packaging of viral DNA and export of infectious virions (1). The earliest marker of lytic viral infection is expression of the immediate-early protein, BZLF1, followed by other early markers such as BMRF1 (11,31,32). These lytic genes are usually silent or are expressed only focally at low level in Burkitt lymphoma tissues (4,26).

The current study confirmed that BZLF1 and BMRF1 were not expressed in Burkitt lymphoma by immunohistochemistry, although the more sensitive rtPCR method revealed BZLF1 in 3/21 pretreatment tumors. Our study is the first to examine expression of these lytic factors in the first few days after initiation of chemotherapy. We showed low-level expression of BZLF1, detectable only by a sensitive rtPCR assay, in only one post-therapy aspirate. Despite a general lack of evidence for replicative gene expression, EBV viral load increased above pre-treatment levels in 50% of day-one aspirates, implying that viral replication had already occurred within the first 24 hours of treatment. Viral load then fell but remained detectable at days 3 to 5. It is uncertain how much of the remaining viral DNA is intracellular *versus* packaged in virions or existing as naked viral genomes from dying tumor cells.

The time-course of events implies that replicative infection could be initiated within hours of exposure to cyclophosphamide. In cell line models, Countryman et al showed that the kinetics of lytic induction depends on the host cell type and the dose and timing of the stimulus (33). In the Akata Burkitt lymphoma cell line stimulated by crosslinking surface immunoglobulin receptors, Wen et al showed that BZLF1 mRNA was expressed as early as 1.5 hours and disappeared in 24 hours, while the encoded protein was expressed from 6 to 12 hours after induction (5). In humans, we found it difficult to track the kinetics of replicative infection. After enrolling our first cohort of Burkitt lymphoma patients and finding no replicative gene expression at time-points of 3 to 5 days, we proceeded to enroll additional patients for evaluation just one day after initiation of therapy and we still seem to have missed the putative window of replicative viral gene upregulation, although we were able to demonstrate viral load increases implicating lytic phase of infection. Re-biopsy at multiple time-points is difficult, so a detailed timeline of tumor-based profiles is probably not feasible in humans.

We hypothesize that transient expression of lytic proteins permits viral DNA synthesis while simultaneously triggering immune recognition of foreign immunogenic peptides leading to engulfment by macrophages, which contributes to the “starry sky” histology that is characteristic of Burkitt lymphoma. Lytic infection is notoriously difficult to document, even

in oropharyngeal tissues where replicative infection occurs routinely as evidenced by periodic shedding of virions into saliva (34).

In the current study, the proportion of *EBER* expressing cells was similar to baseline at days 1 and 3, although by days 4 to 5 all specimens had undetectable *EBER* in concert with the disappearance of malignant-appearing cells. In future studies, one could explore whether the initial response to chemotherapy, in terms of serially measured viral loads or infected cell counts, predicts long term outcome. Because blood sampling is less invasive than tumor aspiration, circulating markers of latent and replicative viral infection should be examined (35,36).

The observed decline in *EBER*-expressing cells could result from elimination of infected tumor cells or from *EBER* downregulation in tumor cells. Lytic infection is associated with diminished *EBER* expression, at least in some lesions and cell models (37,38). Although transcriptional regulation of *EBER* genes is poorly understood, recent evidence shows that MYC can bind to and activate the *EBER1* gene promoter (39–41). Promoter methylation is a proposed mechanism for silencing *EBER1* and *EBER2* transcription (37,42). Altered availability or enhanced degradation of *EBERs* must also be considered as potential cause of diminished *EBER* expression (43). Relief from EBV's tumorigenic and antiapoptotic effects might explain, at least in part, the efficacy of chemotherapy.

The anti-tumor effect of cyclophosphamide therapy is dramatic, and its introduction to clinical medicine beginning over 50 years ago represents one of the great historic breakthroughs in cancer management (44). Nevertheless, a significant fraction of treated patients eventually relapse and die. Clearly more effective therapies are needed for Burkitt lymphoma and for other EBV-related malignancies that, in aggregate, are estimated to affect about 1% of the world's population. EBV is an appealing therapeutic target because it is likely to be involved in pathogenesis of the neoplasm and, when present, it is generally found within every malignant cell of a given infected malignancy (4,10,45–47). Considerable work has been done to explore the possibility of destroying EBV-infected cells by means of inducing lytic infection (7–10, 13,46,48–50). Infection terminating in cell lysis or immune destruction is an appealing endpoint for cancer therapy.

In conclusion, we found evidence supporting EBV lytic induction in Burkitt lymphoma patients almost immediately after the first dose of cyclophosphamide, a drug that is very commonly used in Africa as single agent therapy. Evidence of lytic replication lays the groundwork for a clinical trial testing the efficacy of adding a nucleoside analog to enhance cancer-specific cytotoxicity of cyclophosphamide.

Acknowledgments

Funding: This study was supported by an American Academy of Otolaryngology-Head and Neck Surgery CORE Resident Research Grant, the UNC Medical Endowment fund, and NIH supplement grant R01-CA66519 (SCK).

We thank Gregory Sturtz, Mervi Eeva, Nana Feinberg, Bruce Israel, and Rosalie Dominik of the University of North Carolina at Chapel Hill for their administrative and technical expertise. We also thank Debbie Kamwendo MS and the staff of the UNC Project in Lilongwe. Finally, we thank the study subjects and their parents for brave and altruistic participation in this investigation.

References

1. Kieff, E.; Rickinson, A. Epstein-Barr Virus and Its replication. In: Knipe, D.; Howley, P., editors. Fields Virology. 4. Philadelphia, PA: Lippincott William & Wilkins; 2001. p. 2511-73.
2. Kieff, E.; Rickinson, A. Epstein-Barr Virus and its replication. In: Knipe, D.; Howley, P., editors. Fields Virology. 4. Philadelphia: Lippincott William & Wilkins; 2001. p. 2511-73.

3. Westphal EM, Blackstock W, Feng W, Israel B, Kenney SC. Activation of lytic Epstein-Barr virus (EBV) infection by radiation and sodium butyrate in vitro and in vivo: a potential method for treating EBV-positive malignancies. Cancer Res 2000;60:5781–8. [PubMed: 11059774]
4. Labrecque LG, Xue SA, Kazembe P, Phillips J, Lampert I, Wedderburn N, et al. Expression of Epstein-Barr virus lytically related genes in African Burkitt's lymphoma: correlation with patient response to therapy. Int J Cancer 1999;81:6–11. [PubMed: 10077144]
5. Wen W, Iwakiri D, Yamamoto K, Maruo S, Kanda T, Takada K. Epstein-Barr virus BZLF1 gene, a switch from latency to lytic infection, is expressed as an immediate-early gene after primary infection of B lymphocytes. J Virol 2007;81:1037–42. [PubMed: 17079287]
6. Miller G, El-Guindy A, Countryman J, Ye J, Gradoville L. Lytic cycle switches of oncogenic human gammaherpesviruses. Adv Cancer Res 2007;97:81–109. [PubMed: 17419942]
7. Feng WH, Hong G, Delecluse HJ, Kenney SC. Lytic induction therapy for Epstein-Barr virus-positive B-cell lymphomas. J Virol 2004;78:1893–902. [PubMed: 14747554]
8. Feng WH, Israel B, Raab-Traub N, Busson P, Kenney SC. Chemotherapy induces lytic EBV replication and confers ganciclovir susceptibility to EBV-positive epithelial cell tumors. Cancer Res 2002;62:1920–6. [PubMed: 11912175]
9. Westphal EM, Mauser A, Swenson J, Davis MG, Talarico CL, Kenney SC. Induction of lytic Epstein-Barr virus (EBV) infection in EBV-associated malignancies using adenovirus vectors in vitro and in vivo. Cancer Res 1999;59:1485–91. [PubMed: 10197618]
10. Kenney S, Ge JQ, Westphal EM, Olsen J. Gene therapy strategies for treating Epstein-Barr virus-associated lymphomas: comparison of two different Epstein-Barr virus-based vectors. Hum Gene Ther 1998;9:1131–41. [PubMed: 9625252]
11. Rooney CM, Rowe DT, Ragot T, Farrell PJ. The spliced BZLF1 gene of Epstein-Barr virus (EBV) transactivates an early EBV promoter and induces the virus productive cycle. J Virol 1989;63:3109–16. [PubMed: 2542618]
12. Ragoczy T, Heston L, Miller G. The Epstein-Barr virus Rta protein activates lytic cycle genes and can disrupt latency in B lymphocytes. J Virol 1998;72:7978–84. [PubMed: 9733836]
13. Chan AT, Tao Q, Robertson KD, Flinn IW, Mann RB, Klencke B, et al. Azacitidine induces demethylation of the Epstein-Barr virus genome in tumors. J Clin Oncol 2004;22:1373–81. [PubMed: 15007085]
14. Countryman JK, Gradoville L, Miller G. Histone hyperacetylation occurs on promoters of lytic cycle regulatory genes in Epstein-Barr virus-infected cell lines which are refractory to disruption of latency by histone deacetylase inhibitors. J Virol 2008;82:4706–19. [PubMed: 18337569]
15. Sculley TB, Buck M, Gabrielli B, Parsons PG, Krauer KG. A histone deacetylase inhibitor, azelaic bishydroxamic acid, shows cytotoxicity on Epstein-Barr virus transformed B-cell lines: a potential therapy for posttransplant lymphoproliferative disease. Transplantation 2002;73:271–9. [PubMed: 11821743]
16. Ritchie D, Piekarz RL, Blombery P, Karai LJ, Pittaluga S, Jaffe ES, et al. Reactivation of DNA viruses in association with histone deacetylase inhibitor (HDI) therapy: a case series report. Haematologica. 2009
17. Feng WH, Cohen JI, Fischer S, Li L, Sneller M, Goldbach-Mansky R, et al. Reactivation of latent Epstein-Barr virus by methotrexate: a potential contributor to methotrexate-associated lymphomas. J Natl Cancer Inst 2004;96:1691–702. [PubMed: 15547182]
18. Rizzi R, Curci P, Delia M, Rinaldi E, Chiefa A, Specchia G, et al. Spontaneous remission of "methotrexate-associated lymphoproliferative disorders" after discontinuation of immunosuppressive treatment for autoimmune disease. Review of the literature. Med Oncol 2009;26:1–9. [PubMed: 18461290]
19. Moore SM, Cannon JS, Tanhehco YC, Hamzeh FM, Ambinder RF. Induction of Epstein-Barr virus kinases to sensitize tumor cells to nucleoside analogues. Antimicrob Agents Chemother 2001;45:2082–91. [PubMed: 11408227]
20. Roychowdhury S, Peng R, Baiocchi RA, Bhatt D, Vourganti S, Grecula J, et al. Experimental treatment of Epstein-Barr virus-associated primary central nervous system lymphoma. Cancer Res 2003;63:965–71. [PubMed: 12615710]

21. Perrine SP, Hermine O, Small T, Suarez F, O'Reilly R, Boulad F, et al. A phase 1/2 trial of arginine butyrate and ganciclovir in patients with Epstein-Barr virus-associated lymphoid malignancies. Blood 2007;109:2571–8. [PubMed: 17119113]
22. Magrath I, Jain V, Bhatia K. Epstein-Barr virus and Burkitt's lymphoma. Seminars in Cancer Biology 1992;3:285–95. [PubMed: 1335792]
23. Burkitt D, O'Conor GT. Malignant lymphoma in African children. I. A clinical syndrome. Cancer 1961;14:258–69. [PubMed: 14448554]
24. Schaefer BC, Woisetschlaeger M, Strominger JL, Speck SH. Exclusive expression of Epstein-Barr virus nuclear antigen 1 in Burkitt lymphoma arises from a third promoter, distinct from the promoters used in latently infected lymphocytes. Proc Natl Acad Sci U S A 1991;88:6550–4. [PubMed: 1713681]
25. Niedobitek G, Agathanggelou A, Rowe M, Jones EL, Jones DB, Turyaguma P, et al. Heterogeneous expression of Epstein-Barr virus latent proteins in endemic Burkitt's lymphoma. Blood 1995;86:659–65. [PubMed: 7605996]
26. Rowe DT, Rowe M, Evan GI, Wallace LE, Farrell PJ, Rickinson AB. Restricted expression of EBV latent genes and T-lymphocyte-detected membrane antigen in Burkitt's lymphoma cells. Embo J 1986;5:2599–607. [PubMed: 3023050]
27. Kazembe P, Hesseling PB, Griffin BE, Lampert I, Wessels G. Long term survival of children with Burkitt lymphoma in Malawi after cyclophosphamide monotherapy. Med Pediatr Oncol 2003;40:23–5. [PubMed: 12426682]
28. Orem J, Mbidde EK, Weiderpass E. Current investigations and treatment of Burkitt's lymphoma in Africa. Trop Doct 2008;38:7–11. [PubMed: 18302850]
29. Ryan JL, Fan H, Glaser SL, Schichman SA, Raab-Traub N, Gulley ML. 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;6:378–85. [PubMed: 15507678]
30. Kubota N, Wada K, Ito Y, Shimoyama Y, Nakamura S, Nishiyama Y, et al. One-step multiplex real-time PCR assay to analyse the latency patterns of Epstein-Barr virus infection. J Virol Methods 2008;147:26–36. [PubMed: 17870188]
31. Zalani S, Holley-Guthrie E, Kenney S. Epstein-Barr viral latency is disrupted by the immediate-early BRLF1 protein through a cell-specific mechanism. Proc Natl Acad Sci U S A 1996;93:9194–9. [PubMed: 8799177]
32. Kenney S, Kamine J, Holley-Guthrie E, Lin JC, Mar EC, Pagano J. The Epstein-Barr virus (EBV) BZLF1 immediate-early gene product differentially affects latent versus productive EBV promoters. J Virol 1989;63:1729–36. [PubMed: 2538653]
33. Countryman J, Gradoville L, Bhaduri-McIntosh S, Ye J, Heston L, Himmelfarb S, et al. Stimulus duration and response time independently influence the kinetics of lytic cycle reactivation of Epstein-Barr virus. J Virol 2009;83:10694–709. [PubMed: 19656890]
34. Hudnall SD, Ge Y, Wei L, Yang NP, Wang HQ, Chen T. Distribution and phenotype of Epstein-Barr virus-infected cells in human pharyngeal tonsils. Mod Pathol 2005;18:519–27. [PubMed: 15696119]
35. Gulley ML, Tang W. Laboratory assays for Epstein-Barr virus-related disease. J Mol Diagn 2008;10:279–92. [PubMed: 18556771]
36. Ryan JL, Fan H, Swinnen LJ, Schichman SA, Raab-Traub N, Covington M, et al. Epstein-Barr Virus (EBV) DNA in plasma is not encapsidated in patients with EBV-related malignancies. Diagn Mol Pathol 2004;13:61–8. [PubMed: 15167006]
37. Greifenegger N, Jager M, Kunz-Schughart LA, Wolf H, Schwarzmann F. Epstein-Barr virus small RNA (EBER) genes: differential regulation during lytic viral replication. J Virol 1998;72:9323–8. [PubMed: 9765483]
38. Middeldorp JM, Brink AA, van den Brule AJ, Meijer CJ. Pathogenic roles for Epstein-Barr virus (EBV) gene products in EBV-associated proliferative disorders. Crit Rev Oncol Hematol 2003;45:1–36. [PubMed: 12482570]
39. Niller HH, Salamon D, Ilg K, Koroknai A, Banati F, Bauml G, et al. The in vivo binding site for oncoprotein c-Myc in the promoter for Epstein-Barr virus (EBV) encoding RNA (EBER) 1 suggests a specific role for EBV in lymphomagenesis. Med Sci Monit 2003;9:HY1–9. [PubMed: 12552250]

40. Ruf IK, Rhyne PW, Yang C, Cleveland JL, Sample JT. Epstein-Barr virus small RNAs potentiate tumorigenicity of Burkitt lymphoma cells independently of an effect on apoptosis. J Virol 2000;74:10223–8. [PubMed: 11024153]
41. Felton-Edkins ZA, Kondrashov A, Karali D, Fairley JA, Dawson CW, Arrand JR, et al. Epstein-Barr virus induces cellular transcription factors to allow active expression of EBER genes by RNA polymerase III. J Biol Chem 2006;281:33871–80. [PubMed: 16956891]
42. Banati F, Koroknai A, Salamon D, Takacs M, Minarovits-Kormuta S, Wolf H, et al. CpG-methylation silences the activity of the RNA polymerase III transcribed EBER-1 promoter of Epstein-Barr virus. FEBS Lett 2008;582:705–9. [PubMed: 18258198]
43. Elia A, Vyas J, Laing KG, Clemens MJ. Ribosomal protein L22 inhibits regulation of cellular activities by the Epstein-Barr virus small RNA EBER-1. Eur J Biochem 2004;271:1895–905. [PubMed: 15128299]
44. Brock N. Oxazaphosphorine cytostatics: past-present-future. Seventh Cain Memorial Award lecture. Cancer Res 1989;49:1–7. [PubMed: 2491747]
45. Kelley GL, Rickinson A. Burkitt lymphoma: revisiting the pathogenesis of a virus-associated malignancy. Hematology Am Soc Hematol Educ Program 2007:277–84. [PubMed: 18024641]
46. Ambinder RF, Robertson KD, Moore SM, Yang J. Epstein-Barr virus as a therapeutic target in Hodgkin's disease and nasopharyngeal carcinoma. Semin Cancer Biol 1996;7:217–26. [PubMed: 8946606]
47. Gutierrez MI, Judde JG, Magrath IT, Bhatia KG. Switching viral latency to viral lysis: a novel therapeutic approach for Epstein-Barr virus-associated neoplasia. Cancer Res 1996;56:969–72. [PubMed: 8640787]
48. Chan AT, Ma BB, Lo YM, Leung SF, Kwan WH, Hui EP, et al. Phase II study of neoadjuvant carboplatin and paclitaxel followed by radiotherapy and concurrent cisplatin in patients with locoregionally advanced nasopharyngeal carcinoma: therapeutic monitoring with plasma Epstein-Barr virus DNA. J Clin Oncol 2004;22:3053–60. [PubMed: 15284255]
49. Ambinder RF. Viruses as potential targets for therapy in HIV-associated malignancies. Hematol Oncol Clin North Am 2003;17:697–702. v–vi. [PubMed: 12852651]
50. Chen C, Li D, Guo N. Regulation of cellular and viral protein expression by the Epstein-Barr virus transcriptional regulator Zta: implications for therapy of EBV associated tumors. Cancer Biol Ther 2009;8:987–95. [PubMed: 19448399]

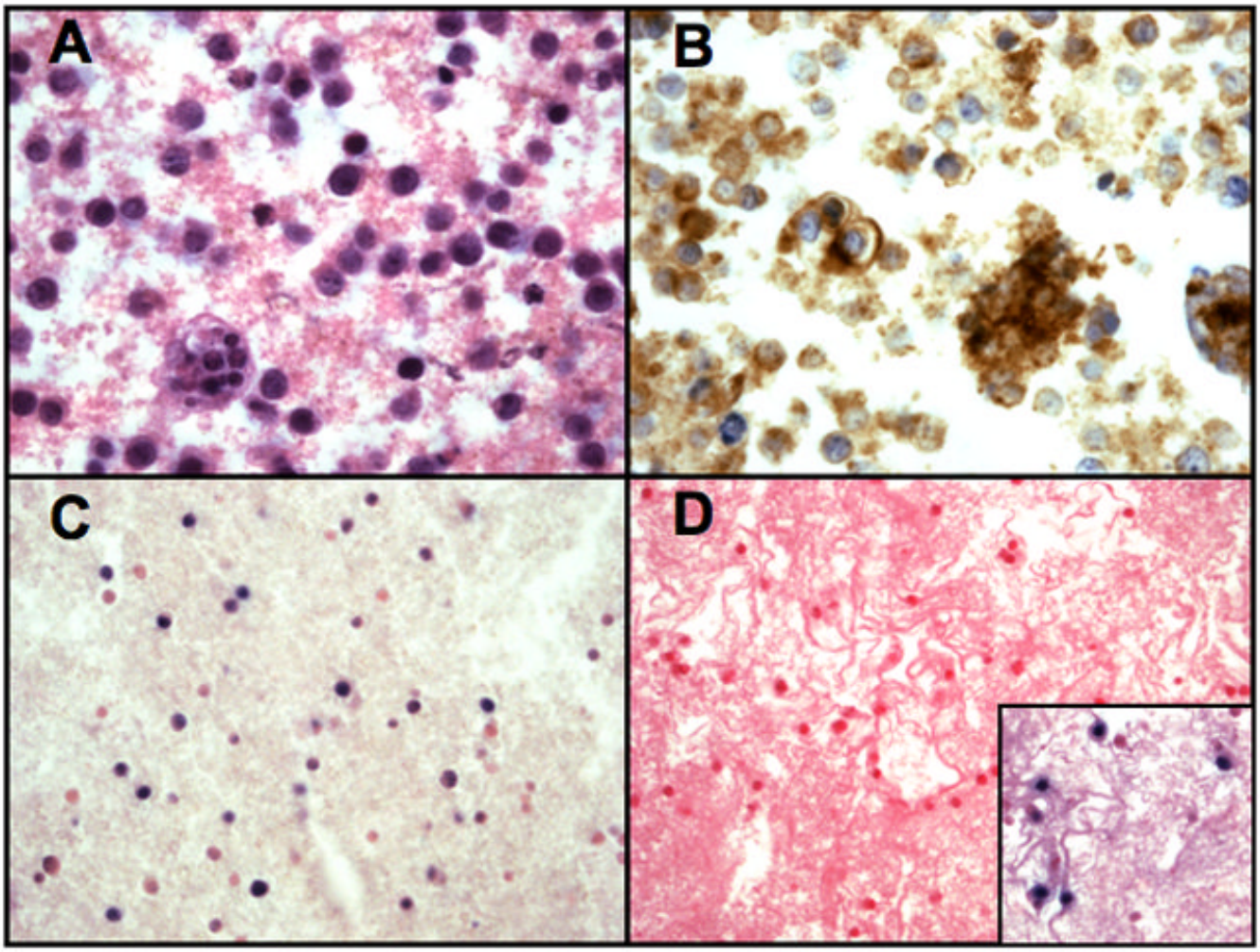

Figure 1. Cytologic and histochemical features demonstrate loss of *EBER*-expressing Burkitt lymphoma cells after cyclophosphamide therapy

(A) In a newly diagnosed patient, hematoxylin and eosin stain reveals features of Burkitt lymphoma including atypical lymphoid cells with fine, blast-like chromatin, and a large cell-laden macrophage in the lower left. (B) Immunohistochemistry reveals CD20 localized to cytoplasm and membrane of atypical cells. (C) *EBER* is expressed by *in situ* hybridization in many nuclei, whereas smaller lymphoid cells stain only with the eosin counterstain; (D) In an aspirate from the same patient 4 days after start of chemotherapy, *EBER* is not expressed while a control hybridization targeting oligo-dT (inset) shows RNA is preserved in most cells.

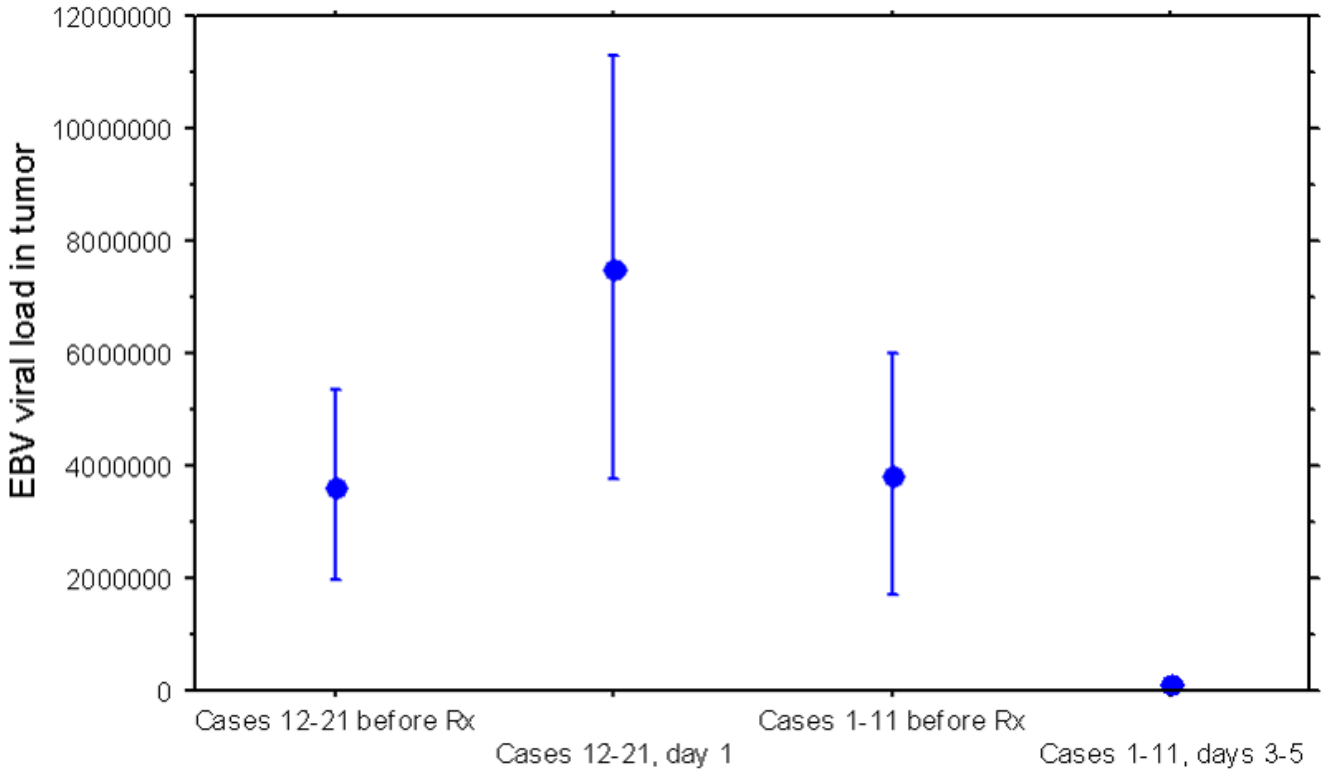

Figure 2. EBV viral load may increase initially but then falls consistently in post-therapy tumor specimens

EBV viral load, expressed in copies per 100,000 cells, is displayed as mean with standard error bars in each clinical subgroup of Burkitt lymphomas. Both pre-treatment groups have similar distributions of viral burden. Mean viral load is even higher among tumors sampled on day one, but has declined by days 3 to 5 after start of chemotherapy.

Table 1

Primer and probe sequences used in Q-rtPCR

Target gene	Sequence	
ABL1		
Forward primer	5′-agagctgcagagcacagagaca-3′	
Reverse primer	5′-gctcttttcgagggagcaatg-3′	
TaqMan probe	5′-FAM-atggtccagaggatcgctctctccct-TAMRA-3′	
BZLF1		Position*
Forward primer	5′-acgcacaccgaaaccacaa-3′	90,397-90,379
Reverse primer	5′-cttaaacttggcccggcatt-3′	90,162–90,181
TaqMan probe spanning spliced mRNA	FAM-aatcgcattcctccagcgattctgg-TAMRA	90,227–90,242; 90,367–90,375

* Position in the EBV genome relates to NCBI Accession number AJ507799

Table 2

Frequency of latent EBV detection in tumors before and after start of cyclophosphamide therapy

		After Therapy*	
	Before Therapy	**Day 1 (n=10)**	**Days 3–5 (n=11)**
Positive *EBER* stain	21	9	2
Negative *EBER* stain	0	1	9

* Results are significantly different in day 1 versus day 3–5 collections, p value = 0.0019

Table 3

Proportion of *EBER*-expressing cells declines in most tumors by 4–5 days after start of cyclophosphamide therapy, as assessed by microscopy

		EBER-expressing cells as a proportion of total nucleated cells			
Case number	**Day collected post-therapy**	**Before therapy**	**After therapy***	**Fold-change****	**Fold-change in total cell number**
1	5	0.06	0	0	0.57
2	5	0.23	0	0	0.23
3	4	0.36	0	0	0.15
4	4	0.66	0	0	0.01
5	4	0.27	0	0	0.38
6	4	0.28	0	0	0.22
7	4	0.73	0	0	0.09
8	4	0.70	0	0	0.02
9	3	0.18	0.71	4.05	0.03
10	3	0.21	0.54	2.57	0.39
11	3	0.69	0	0	0.03
Mean	**4**	**0.40**	**0.11**	**0.60**	**0.19**
Median	**4**	**0.28**	**0**	**0**	**0.15**
12	1	0.15	0.23	1.59	0.61
13	1	0.44	0.50	1.12	0.26
14	1	0.23	0.13	0.57	0.16
15	1	0.39	0.00	0.00	0.32
16	1	0.61	0.22	0.36	0.00
17	1	0.39	0.24	0.62	0.68
18	1	0.27	0.50	1.82	0.53
19	1	0.21	0.33	1.60	0.05
20	1	0.15	0.67	4.58	0.37
21	1	0.42	0.23	0.55	0.60

Clin Cancer Res. Author manuscript; available in PMC 2011 April 1.

		EBER-expressing cells as a proportion of total nucleated cells			
Case number	**Day collected post-therapy**	**Before therapy**	**After therapy***	**Fold-change****	**Fold-change in total cell number**
Mean	**1**	**0.33**	**0.31**	**1.28**	**0.36**
Median	**1**	**0.33**	**0.24**	**0.87**	**0.35**

* Median *EBER*-positive cell proportion was lower in tumors sampled on days 3–5 compared to day 1 ($p = 0.017$).

** Median fold-change in *EBER*-positive cell count was lower in tumors sampled on days 3–5 compared to day1 ($p < 0.014$).

Table 4

EBV viral load results as measured by real-time PCR

			EBV copies/100,000 cells*		
Case number	**Day collected post-therapy**	**Fold-change in cell number (*APOB* gene)**	**Before therapy**	**After therapy**	**Fold-change**
1	5	0.89	684877	312755	0.460
2	5	0.01	209501	31429	0.150
3	4	0.42	990627	7872	0.010
4	4	0.08	232444	5555	0.020
5	4	0.22	538058	1015	0.002
6	4	1.40	24399000	579509	0.020
7	4	0.07	3488468	17949	0.005
8	4	1.04	7169565	33333	0.005
9	3	0.17	2201256	134909	0.060
10	3	1.10	330844	154949	0.470
11	3	0.02	1968976	8000	0.004
Mean	**4**	**0.49**	**3837602**	**117025**	**0.110**
Median	**4**	**0.22**	**990627**	**31429**	**0.020**
12	1	2.65	10556522	3824590	0.360
13	1	0.13	4119544	**12380000**	**3.010**
14	1	0.02	470307	**758015**	**1.610**
15	1	0.01	133907	23529	0.180
16	1	0.03	170152	**39058491**	**229.550**
17	1	0.77	487755	364000	0.750
18	1	0.89	2334907	**7215556**	**3.090**
19	1	0.26	15911481	242857	0.020
20	1	0.02	2072262	1736190	0.840
21	1	0.87	152632	**9533333**	**62.460**

			EBV copies/100,000 cells*		
Case number	Day collected post-therapy	Fold-change in cell number (*APOB* gene)	Before therapy	After therapy	Fold-change
Mean	1	0.56	3640947	7513656	30.187
Median	1	0.20	1280008	2780390	1.225

*Median viral load was higher in tumors sampled on day 1 compared to days 3–5 ($p < 0.001$). Median fold change in viral load was also higher in tumors sampled on day 1 compared to days 3–5 ($p < 0.001$).