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Downregulation of the Polyamine Regulator Spermidine/Spermine N¹-Acetyltransferase by Epstein-Barr Virus in a Burkitt's Lymphoma Cell Line

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Abstract

Transition of Akata Burkitt's lymphoma (BL) from a malignant to nonmalignant phenotype upon loss of Epstein-Barr virus (EBV) is evidence for a viral contribution to tumorigenesis despite the tight restriction of EBV gene expression in BL. Examination of global cellular gene expression in Akata subclones that retained or lost EBV identified spermidine/spermine N¹-acetyltransferase (SAT1), an inducible enzyme whose catabolism of polyamines affects both apoptosis and cell growth, as one of a limited number of cellular genes downregulated upon EBV loss. Re-infection of the EBV-negative Akata clone reduced *SAT1* mRNA to a level comparable with the parental EBV-positive Akata. EBV-positive Akata cells demonstrated decreased SAT1 enzyme activity concomitant with altered intracellular polyamine constituents. Reduction of SAT1 in EBV-positive BL was a transcriptional effect. Forced expression of the viral BCL2 homologue, BHRF1, in an EBV-negative Akata clone reduced SAT1 mRNA. Whereas dysregulated *c-myc* may enhance expression of ornithine decarboxylase elevating polyamine synthesis, EBV repression of polyamine catabolism becomes a complementary alteration favorable to BL lymphomagenesis.

Keywords

EBV; spermidine/spermine N¹-acetyltransferase; polyamines; Burkitt's lymphoma; c-myc; Akata

1. Introduction

Epstein-Barr virus (EBV) is a prevalent gammaherpesvirus associated with a diverse array of lymphoid and epithelial cell malignancies, one being endemic Burkitt's lymphoma (BL). Common to all BL tumors is a characteristic chromosomal translocation that places c-MYC into the immunoglobulin gene locus (Dalla-Favera et al., 1982; Taub et al., 1982), with the constitutive overexpression of c-MYC being sufficient for oncogenesis. Transgenic mice carrying the c-MYC translocation develop BL-like lymphomas in the absence of EBV (Kovalchuk et al., 2000; Zhu et al., 2005). Similarly, sporadic and AIDS-associated BL are frequently EBV-negative, unlike the endemic form of disease. The virus' contribution to BL

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pathogenesis has been further obscured by the restricted viral gene expression program (latency I) found in EBV-positive tumors, wherein the EBV-encoded growth promoting genes are downregulated and only EBV nuclear antigen 1 (EBNA1), the non-coding EBV encoded RNAs (EBERs) and the BamHI A Rightward Transcripts (BARTs) are expressed (Rowe et al., 1987).

The possibility that EBV may act as cofactor to counter the proapoptotic effects of high c-MYC expression first became apparent from studies of a BL-derived cell line, Akata, initially positive for EBV, but after long-term culture developed subclones that had lost virus (Shimizu et al., 1994). Notably, presence of EBV was necessary to maintain the Akata malignant phenotype, as measured by growth in low serum, colony formation in soft agar, and tumor formation in SCID mice (Chodosh et al., 1998; Komano et al., 1998; Shimizu et al., 1994). EBV-negative subclones were more prone to apoptosis than the parental cells. Re-infection of EBV-negative Akata subclones restored the tumor phenotype, providing further corroboration of a complementary role for EBV in BL development (Komano et al., 1998; Ruf et al., 1999).

Attempts to define the survival advantage that EBV confers on Akata have indicated an upregulation of the anti-apoptotic BCL-2 protein and growth signals through interleukin-10 production (IL-10) (Kitagawa et al., 2000; Ruf et al., 1999). Forced expression of the EBV-encoded RNAs (EBERs), two RNA polymerase III transcripts abundantly expressed in BL, partially restored the tumor phenotype. EBV-negative Akata clones expressing EBERs formed tumors in SCID mice with enhanced IL-10 expression, albeit time to tumor development was longer than in the infected EBV-positive Akata (Komano et al., 1999; Ruf et al., 2000). EBERs have been shown to protect against some apoptotic stimuli such as interferon- α , glucocorticoid, cycloheximide, and hypoxia and may upregulate BCL-2 under some conditions (Komano et al., 1999; Nanbo et al., 2002; Ruf et al., 2000). However, EBERs were unable to restore survival under serum starvation, suggesting that other viral genes may contribute to the EBV-dependent malignant phenotype (Ruf et al., 2000).

In the study presented here we sought to further clarify cellular pathways modulated by the EBV latency I gene expression pattern that contribute to the BL tumor phenotype. We used global transcriptional profiling comparing EBV-positive Akata clones to their EBV-negative counterparts generated not after extensive passage but by treatment with hydroxyurea, an inhibitor of ribonucleotide reductase that forced rapid loss of EBV episomes (Chodosh et al., 1998). We identified an EBV-dependent downregulation of spermidine/spermine N¹-acetyltransferase (SAT1), a rate-limiting enzyme involved in the catabolism of polyamines (Casero and Pegg, 1993). These aliphatic cations, essential for regulation of cell growth and apoptosis, are elevated in a number of cancers including BL (Gerner and Meyskens, 2004). Such a downregulation of the catabolic pathway by EBV identifies a complementary alteration to c-MYC induction of polyamines biosynthesis, highlighting the importance of polyamine metabolism in EBV-associated BL lymphomagenesis.

2. Materials and Methods

2.1. Cell culture and reagents

EBV-negative Akata and MutuI cell clones were generated by hydroxyurea treatment as previously described (Chodosh et al., 1998). BL2 is an EBV-negative BL cell line, and an EBV-positive counterpart was generated by infection with the B958 EBV strain. Akata EBV-positive clones (1B4, 1B6, and 4C4) and -negative counterparts (2A8, 3F2, and 2E4) including the BL2 and Mutu derived cell lines and clones were grown in RPMI 1640 media supplemented with 10% fetal bovine serum (Hyclone), 2 mM L-glutamine, 100 IU/ml penicillin and 100 μ g/ml streptomycin. Low serum media contained 0.1% fetal bovine

serum unless otherwise indicated. EBV(-) 2A8 was reinfected with recombinant Akata virus (EBVneo^r, gift of L. Hutt-Fletcher) (Borza and Hutt-Fletcher, 1998), and selected on G418 (MediaTech). For induction of lytic replication, 5×10^5 cells/ml were incubated with 10 μ g/ml anti-human IgG (Cappel). Western blot analysis was performed as described previously (Moody et al., 2005). Protein lysates were prepared by lysis of equal cell numbers in 4 $\times$ laemmli buffer. After transfer onto nylon membranes, membranes were hybridized with phosphorylated ERK and pan ERK antibodies (Santa Cruz) at a 1:1000 dilution. For detection of BHRF1, immunoblots were probed with a 1:500 dilution of the BHRF1 antibody (Millipore, 5B11). A secondary HRP conjugated antibody was used for detection by standard methods.

2.2. Microarray expression profiling

Logarithmically growing EBV(-) 2A8 and EBV(+) 1B6 cell clones were seeded at 2.5×10^5 cells/ml and grown overnight in complete media. Since the EBV-induced tumor phenotype is observed in low serum (Chodosh et al., 1998; Komano et al., 1998; Shimizu et al., 1994), cells were exposed to 1% serum for 4 h. RNA was harvested using STAT-60 (Teltest) as per manufacturer's protocol and integrity was monitored on an Agilent 2100 Bioanalyzer. Double-stranded cDNA was synthesized from 10 μ g total RNA using a Superscript cDNA Synthesis Kit (Invitrogen) in combination with a T7-(dT)₂₄ primer. Biotinylated cRNA was transcribed in vitro using the BioArray High Yield RNA Transcript Labeling Kit (ENZO Biochem), purified using the GeneChip Sample Cleanup Module (Affymetrix), and fragmented by incubation in fragmentation buffer (200 mM Tris-acetate, pH 8.1, 500 mM potassium acetate, 150 mM magnesium acetate) at 94°C for 35 min. Fifteen micrograms of fragmented biotin-labeled cRNA was hybridized to the Human Genome U95Av2 Array (Affymetrix), interrogating a total of 12,625 human genes. Arrays were incubated, washed, and stained with 10 μ g/ml streptavidin-R phycoerythrin (Vector Laboratories) followed by 3 μ g/ml biotinylated goat anti-streptavidin antibody (Vector Laboratories) as per manufacturer's protocol, then read with a GeneChip Scanner 3000. Expression signals were analyzed using Microarray Analysis Suite 5 (Affymetrix). Arrays were globally scaled to a target intensity value of 2500 in order to compare individual experiments. The absolute call, direction of change, and fold change of gene expressions between samples were calculated with Data Mining Tool 3.0 (Affymetrix). Significant genes were identified by filtering data using Spotfire software according to these criteria: 1) signal values had to be statistically significant ($p < 0.05$) as calculated by Spotfire's T-test/Anova algorithm; 2) detection calls had to be present in at least 1 out of 6 samples; 3) the average signal log ratio of the 3 experiments was set at an absolute value ≥ 0.9 . Microarray data has been deposited in Gene Expression Omnibus (Accession#GSE19761).

2.3. Reverse-transcription PCR

RNA was harvested using STAT-60. DNase-treated RNA was used for first-strand cDNA synthesis (Wagner et al., 2004). For real-time quantitative (RQ) RT-PCR of SAT1 message, primers and TaqMan probe were designed using PrimerExpress and amplification was measured by the ABI Prism 7000 Sequence Detection System (Applied Biosystems). One hundred nanograms of cDNA were amplified in TaqMan Universal PCR Master mix (Applied Biosystems) with 300 nM of primers and 200 nM of probe. Negative controls included RT-negative and template-negative samples. Serially diluted cDNA was used to validate the RQ-PCR assay relative to endogenous human GAPDH (Applied Biosystems p/n 4310884E). GAPDH served as a normalization control and the $\Delta\Delta$ CT method was used for quantitation (User Bulletin #2, Applied Biosystems). Standard cycling conditions were used setting 60°C as the annealing temperature for SAT1 and GAPDH. Statistical analysis was performed using single factor Anova analysis. RT-PCR detection of SAT1 spliced variant and EBV BHRF1 and BZLF1 was performed using primers as described in Table 1. One

hundred nanograms of cDNA was amplified using 500 nM of primers using 1 unit of Go Taq polymerase (Promega). Thermocycling condition following a 94°C 5minute denaturation consisted of 35 cycles at 94°C for 30 sec, annealing temperature in Table 1 for 30 sec, 72°C for 30 sec and ending with 7 minute extension time. PCR products were visualized after agarose gel electrophoresis by ethidium bromide staining, or by hybridization with ³²P-labeled oligonucleotides probes (Table 1) following Southern blotting onto nylon membranes (Southern, 1975).

2.4. Spermidine acetylation assay

Cells seeded at 2×10^5 per ml were grown for 24 hrs followed by treatment for 24 hrs with 10 μ M N¹, N¹¹-di(ethyl)norspermine (DENSPM; Tocris Cookson Inc.). DENSPM-treated cells were resuspended at 2×10^7 per ml in 5 mM HEPES pH 7.2 containing 1 mM DTT, and sonicated for 5 min at 20% power (W-380 sonicator, Heat Systems Ultrasonic, Inc.). Protein extracts were cleared by high speed microfuge centrifugation at 4°C. SAT1 activity in each cell clone was measured by acetylation of spermidine as previously described (Matsui and Pegg, 1981). Briefly, in a 100 μ l reaction, 70 μ l of protein extract was incubated with 3 mM spermidine (Sigma-Aldrich), 100 mM Tris-HCL (pH 7.8), and 10 μ l [¹⁴C] acetyl-CoA (4 μ Ci/ml, MP Biochemicals) for 10 min at 37°C. Reactions were stopped by addition of 1M NH₂OH-HCL, and boiled for 3 min. Insoluble proteins were removed by high speed centrifugation. Fifty microliters of reaction mix were spotted onto cellulose phosphate paper disks (Whatmann P81), washed $\times 5$ with distilled water, followed by 3 washes with absolute ethanol. Disks were air-dried and scintillation counted. Statistical analysis was performed using the Student's t-Test.

2.5. Analysis of intracellular polyamine pools

EBV-positive and -negative cell clones were seeded at 2×10^5 cells/ml in 10% or 0.1% serum growth media and harvested at 72 or 24 hrs, respectively. Intracellular polyamines using a modified dansylation detection method (Kabra et al., 1986). One hundred microliters of 0.2 M perchloric acid containing 125 μ M of 1,6 diamino hexamine was added to every 1×10^6 cells as an internal standard. Cells were sonicated on ice for 2 hrs in a Bransonic 1210 ultrasonic cleaner, and insoluble material was cleared by centrifugation. To each 100 μ l of cell extract (1×10^6 cell equivalents), 350 μ l of 5 mg/ml dansyl chloride and 50 μ l of 1.8 mg/ml sodium carbonate were added and incubated at 55°C for 2 hrs. Twenty microliters of 100 mg/ml proline was added and vortexed until clear. Dansylated polyamines were extracted with 1 ml toluene, dried under nitrogen, and reconstituted with 30 μ l of acetonitrile. Separation of the dansylated polyamines was performed by reverse-phase HPLC with fluorescence detector (Agilent Technologies) using a C18 column (Agilent hypersil ODS). Columns were eluted with the following 4 stepped linear gradients: 1) 6 min in 20% acetonitrile with 80% of 1% acetic acid/0.03% triethylamine (HAcTEA), 2) 35 min with 62% acetonitrile and 38% HAcTEA, 3) 40 min with 65% acetonitrile and 35% HAcTEA, 4) 45 min with 95% acetonitrile and 5% HAcTEA. Fluorescent signal emission was detected at 525 nm (excitation at 340 nm). Chromatogram peaks were quantified with Chemstation for liquid chromatography version 9 (Agilent Technologies). For each run, standard curves for putrescine, spermidine, spermine and their acetylated counterparts were performed. Sample values were normalized to the internal standard and reported as pmol per 10^6 cells. Six independent experiments were performed and statistical analysis was performed using single factor Anova.

2.6. Transfections

A BHRF1 expression construct was created by amplifying the BHRF1 open reading frame from EBV-strain B958 using the Expand Long Template PCR system (Boehringer Mannheim) (forward primer, CGCCGAATTCATCTTGTAGAGCAAGATGGC and reverse

primer, GCGCCTCGAGCAATGACCCTGATTCATATA). The PCR product was cloned into the *EcoRI* and *XhoI* multiple cloning site of the pLXSN vector and verified by sequencing. The EBV(-)clone 2A8 was transfected using the Amaxa Nucleoporation system (program N-16). Briefly, cells were seeded at 2×10^5 cells/ml 2 days prior transfection. 1×10^6 logarithmically growing cells were resuspended in Amaxa V solution and transfected with 2 μ g of endotoxin free plasmid DNA. Stably transfected cells were selected with 350 μ g/ml G418.

3. Results

3.1. Transcriptional profiling to identify altered cellular gene expression in the Akata EBV-dependent tumor phenotype

Using Affymetrix U95A GeneChips, we examined variations in global gene expression between the EBV-positive (1B6) and EBV-negative (2A8) Akata clones derived from hydroxyurea treatment. Because growth under conditions of serum starvation provides one measure of the Akata tumor phenotype (Chodosh et al., 1998; Ruf et al., 1999; Shimizu et al., 1994), cell clones were serum starved for 4 hours prior to RNA harvest. The short incubation time was intended to minimize cell death and other potential downstream effects of low serum treatment, while at the same time initiating expression changes contributing to the tumorigenic phenotype. Twenty-three genes showed statistically significant ($p < 0.05$) differences of 1.9-fold or greater in the EBV-positive Akata clone compared to its EBV-negative counterpart in three independent experiments (Table 2). The genes were equally split between the upregulated (11 genes) and downregulated (12 genes) groups. The small number of gene expression changes (approximately 0.2% of the some 12,000 queried) attests to both the relatedness of clones rapidly “cured” of EBV by hydroxyurea (Chodosh et al., 1998) to their EBV-positive counterparts. Two independent probesets identified DNA damage-inducible transcript 3 (DDIT3) and spermidine/spermine N1-acetyltransferase (SAT1) being differentially expressed in EBV-positive and -negative Akata BL. We focused our attention on SAT1, as a second alteration in polyamine metabolism dependent on EBV in BL: the first being the c-myc translocation characteristic of BL.

C-myc and its family members are transcription factors that promote cell growth and transformation by regulating the transcription of target genes required for proliferation (Baudino and Cleveland, 2001). Among the c-myc target genes for c-myc is ornithine decarboxylase (ODC), a key regulatory enzyme driving polyamine biosynthesis (Bello-Fernandez et al., 1993) (Fig.1). Elevated ODC activity with resultant higher polyamine levels are detected in rapidly proliferating cells and in various tumors including BL (Bello-Fernandez et al., 1993), implicating the synthetic pathway in tumorigenesis. Polyamines are aliphatic cations (putrescine, spermidine, and spermine) involved in multiple functions that support cell survival and growth. Irreversible inhibitors of ODC, such as difluoromethylornithine (DFMO), act as cytostatic agents that arrest the cell cycle and cell growth (Meyskens and Gerner, 1999). On the other hand, SAT1 is a rate-limiting enzyme that catalyzes the acetylation of spermidine and spermine and with polyamine oxidase (PAO) results in polyamine degradation. Thus, we investigated how EBV manipulated SAT1 expression in the Akata BL cell line.

3.2 EBV-dependent repression of SAT1 expression

To define the conditions required for downregulation of SAT1 mRNA in the EBV-positive clone 1B6, real-time quantitative RT-PCR (RQ-RT-PCR) was used to measure the levels of SAT1 mRNA in three EBV-positive and EBV-negative Akata clones under growth conditions of low (0.1%) or normal (10%) serum (Fig. 2A). Using the $\Delta\Delta$ CT method, the relative amount of SAT1 transcripts was normalized to GAPDH and compared to the EBV-

positive Akata 1B4 clone (10% serum) as the calibrator. After 48 hours of serum starvation, three EBV-positive Akata clones showed a comparable decrease in SAT1 mRNA relative to EBV-negative counterparts (average of 4.2 ± 0.3 SAT1 transcripts/100ng RNA versus 8.0 ± 1.1 SAT1 transcripts/100ng RNA, respectively; $p < 0.05$) as was shown by microarray. In 10% serum, a reduction in SAT1 mRNA levels was still apparent in EBV-positive clones showing a two-fold reduction in SAT1 mRNA relative to EBV-negative clones (average of 1.2 ± 0.2 SAT1 transcripts/100ng RNA versus 2.8 ± 0.2 SAT1 transcripts/100ng RNA, respectively; $p < 0.01$) (Fig. 2A).

To determine if the downregulation of SAT1 mRNA observed was dependent on EBV, we quantified SAT1 mRNA in the EBV-negative Akata cell clone 2A8 re-infected with a recombinant Akata EBV strain (EBVneo^r). Re-infection of EBV-negative Akata cells has previously been shown to restore the tumor phenotype (Komano et al., 1998; Ruf et al., 1999). Indeed, the reinfected 2A8 clone reduced SAT1 mRNA levels by 2.5-fold, comparable to that found in the parental EBV-positive Akata BL cell clones (Fig. 2B). To expand our observations of EBV repression of SAT1 to other EBV-positive and -negative BL cell lines, we quantified SAT1 mRNA levels in paired EBV-negative and -positive BL cell lines (MutuI and BL2). The MutuI EBV-negative clone was cured of EBV episomes by hydroxyurea similar to the EBV-negative Akata clones used in this study. The EBV-negative MutuI clone and BL2 cell lines displayed already reduced SAT1 mRNA levels that were similar or below that measured for the EBV-positive Akata clones. In Mutu and BL2 cells, EBV did not further repress SAT1 mRNA levels (Fig. 2C). One difference between these BL cell lines is that, in contrast to the Akata-derived EBV-negative BL clones, the Mutu-derived EBV-negative clone and the BL2 cell line retain their malignant phenotype despite loss of the viral episome (Chodosh et al., 1998). These results suggest that repression of SAT1 occurs in the BL malignant phenotype.

3.3. EBV maintains reduced SAT1 mRNA levels in conditions known to induce SAT1 expression

To determine if experimental activation of SAT1 is capable of overcoming its repression in EBV-positive cells relative to their EBV-negative counterparts, we first examined the effect of B-cell receptor (BCR) crosslinking known to induce SAT1 expression in B cells (Nitta et al., 2001). To ensure that EBV-positive and -negative clones were capable of being similarly activated by IgG crosslinking, phosphorylation of ERK was measured after 30 minutes of stimulation as the downstream readout of BCR activation. As shown in Fig 3A, treatment with IgG resulted in upregulation of phosphorylated ERK 1 and 2 in both the EBV-positive and -negative Akata BL clones. Densitometric analysis of phospho-ERK to pan ERK showed a slightly increased activation in the EBV-positive Akata BL of ~1.8 fold compared to the EBV-negative Akata clones.

Using the EBV-negative (2A8) and EBV-positive (1B6) clones, SAT1 mRNA levels were measured after treatment with anti-human IgG for 12 and 24 hours. BCR crosslinking produced a time-dependent increase in SAT1 mRNA in the EBV-negative clone 2A8 whereas levels remained largely repressed in the EBV-positive clone (1B6) (Fig. 3B). The initial 2.5 fold difference in mock treated samples increased to a 4-fold difference by 24 hours (Fig. 3B). The two treatments shown to induce SAT1 expression, BCR crosslinking (Fig. 3B) and serum starvation (Fig. 2A), are commonly used to activate EBV lytic replication (Takada et al., 1991). Indeed, expression of lytic BHRF1 was observed in the EBV-positive clone (1B6) at 12 and 24 hours post-IgG stimulation (Fig 3C). Expression of the immediate early gene, BZLF1, mRNA confirmed activation of lytic replication at 12 hours following IgG stimulation. Thus, EBV-dependent repression of SAT1 under both treatment conditions potentially is maintained not only during the restrictive type I latency characteristic of BL cells but during the lytic phase of the EBV life cycle as well.

To more directly manipulate polyamine metabolism in paired Akata cell clones, we used N1, N11 diethylnorspermine (DENSPM), a synthetic polyamine analog that downregulates polyamine biosynthesis and potently induces SAT1 mRNA and activity (Bernacki et al., 1992; Fogel-Petrovic et al., 1993; Fogel-Petrovic et al., 1996). DENSPM induction of SAT1 activity involves increased SAT1 mRNA stability, translation efficiency, and protein stability (Fogel-Petrovic et al., 1993; Fogel-Petrovic et al., 1996; Libby et al., 1989; Parry et al., 1995; Xiao and Casero, 1996). Treatment with DENSPM mimics high polyamine concentrations, activating feedback loops that turn off the biosynthetic pathways and turn on the catabolic pathway regulated by SAT1. The DENSPM polyamine analog does not substitute for the growth stimulating effects of polyamines; instead, DENSPM results in depletion of polyamine pools, growth inhibition, and in some cases apoptosis. Three EBV-positive and EBV-negative Akata BL clones were treated with DENSPM, and the amount of SAT1 mRNA was quantified by RQ-RT-PCR. DENSPM treatment increased SAT1 mRNA levels in all clones regardless of EBV status, but the approximately 2 fold reduction in SAT1 found in untreated EBV-positive clones was maintained (Fig. 3D).

3.4 SAT1 mRNA levels reflect enzymatic catabolic activity

To address whether mRNA levels reflected the biological activity of SAT1, we took advantage of DENSPM's ability to increase SAT1 enzyme activity several orders of magnitude above the mRNA increase (Fogel-Petrovic et al., 1996). Basal SAT1 activity is typically below the limit of detection in acetylation assays, and this proved to be the case with our Akata BL cell clones. Extracts from DENSPM-treated cells were incubated with spermidine and ^{14}C radiolabeled acetylCoA and the amount of ^{14}C labeled spermidine was quantified by scintillation counting as a measure of SAT1 activity (Fig. 3E) (Matsui and Pegg, 1981). Decreased spermidine acetylation was detected in the three EBV-positive as compared to EBV-negative Akata cell clones (mean of 470 ± 62 CPM/ 10^6 cells versus 1103 ± 188 CPM/ 10^6 cells, respectively; $p < 0.05$). Decreased SAT1 activity in the EBV-positive Akata clones (2.3 fold) correlated with the fold reduction in mRNA level (Fig. 3D) suggesting that EBV regulation of SAT1 primarily occurs at the RNA level.

3.5. EBV reduction of SAT1 affects polyamine levels in Akata BL

Having established a correlation between reduction in SAT1 mRNA and SAT1 enzymatic activity, we next examined the impact on intracellular polyamine levels. SAT1 regulates levels of the long chained polyamines, spermidine and spermine, through acetylation of these substrates, which are either cleaved by polyamine oxidase to generate smaller chained polyamines or excreted from the cell, resulting in polyamine depletion (Fig. 1). To determine if reduced SAT1 mRNA and enzyme activity in the EBV-positive Akata cells resulted in altered intracellular polyamine levels, we performed reverse phase HPLC analysis using extracts from EBV-positive and -negative Akata clones.

In 10% serum, the EBV-positive Akata clones had a 1.8-fold decrease ($p < 0.05$) in acetylspermidine as compared to the EBV-negative clones (Table 3). Because acetylspermidine is a direct acetylation product of SAT1, decreased acetylspermidine levels demonstrated a functionally relevant change consequent to reduced catabolism of polyamines in EBV-positive clones. There were no significant differences in the levels of intracellular putrescine, spermidine and spermine, possibly reflecting the comparable growth of both sets of clones under serum-rich conditions and/or compensatory mechanisms that modulate polyamine levels. Lack of alterations in polyamine pools have been previously reported even when basal SAT1 was reduced by 80% using siRNA in human melanoma cells, reflecting coordinate regulation of the biosynthetic/catabolic and import/export pathways that maintain polyamine homeostasis (Chen et al., 2003).

After 24 hours in 0.1% serum where EBV-positive Akata clones demonstrate a growth advantage over EBV-negative Akata clones (Chodosh et al., 1998), there was a statistically significant 2-fold increase in spermine levels in the EBV-positive Akata clones compared to their EBV-negative counterparts (Table 3). Spermine has been shown to function as negative regulator of BCR-mediated apoptosis (Nitta et al., 2001) and as a free radical scavenger (Ha et al., 1998). Spermidine and putrescine were slightly elevated in the EBV-positive Akata clones, suggesting an overall increase in the natural polyamine level in EBV-positive Akata cells. Together, these results confirm that the reduced SAT1 mRNA levels in EBV-positive Akata clones functionally affect polyamine pools, the elevation of which may contribute to the EBV-induced tumor phenotype in Akata BL cells.

3.6. Transcriptional regulation of SAT1 in EBV-positive Akata

Arboviral and adenoviral infections have previously been shown to induce alternatively spliced, non-functional variants of SAT1, which also occur in response to changes in polyamine concentration, irradiation, and hypoxia (Hyvonen et al., 2006; Nikiforova et al., 2002). One alternatively spliced product, termed SAT1-X, includes an additional 110bp exon X between exons 3 and 4 that results in premature translation termination of SAT1 protein and nonsense-mediated mRNA decay (Hyvonen et al., 2006; Nikiforova et al., 2002). A second splice variant deleting exon 4 has also been observed upon infection with the Semliki Forest arbovirus (Nikiforova et al., 2002) (Hyvonen et al., 2006). Since primers for our RQ-RT-PCR assays (Table 1) framed the exon 3:4 junction of SAT1, we examined if increased alternative splicing that interfered with formation of the exon 3:4 junction to decrease in SAT1 mRNA observed in the presence of EBV. First, we examined if alternative spliced variants of SAT1 were generated in Akata clones using primers to SAT1 exon 3 (RQ-SAT1-F) and exon 6 (P6) (Table 1) that would detect all three transcripts. Only two amplicons were detected by Southern blotting, that of standard SAT1 and a larger variant consistent with SAT1-X (data not shown). We confirmed that the larger splice variant was indeed SAT1-X, using RT-PCR primers to exon 1 (P1) coupled with a primer specific for exon X (P4) (Fig. 4A, top panel). The SAT1-X splice variant was detected in both EBV-positive and EBV-negative Akata clones; however, the EBV-positive clones showed decreased SAT1-X comparable to the previously observed reduction in SAT1 mRNA (Fig. 2). To quantify the alternative spliced SAT1-X relative to SAT1, we used the earlier RQ-RT-PCR primers directed to exons 3 and 4 to amplify both SAT1-X and SAT1, determining their relative abundance by Southern hybridization (Fig. 4A, bottom panel). Densitometric analysis of three independent biological replicates showed that the ratio of SAT1-X isoform to SAT1 was slightly lower in EBV-positive clones compared to the EBV-negative clones, excluding the role of alternative splicing in the EBV-dependent reduction in SAT1.

With evidence of two SAT1 transcripts being reduced by EBV, we examined if EBV affected SAT1 at the level of transcription. Using RQ-RT-PCR primers and probes designed to quantify unspliced SAT1 (exon2 and intron 2), EBV-positive clones averaged 1 ± 0.02 SAT1 pre-mRNA transcripts/100ng RNA as compared to 2.3 ± 0.09 SAT1 pre-mRNA transcripts/100ng RNA in their EBV-negative counterparts (Fig. 4B). This 2.3 fold reduction in SAT1 pre-mRNA in EBV-positive Akata BL clones was similar to the reduction observed in spliced transcripts, suggesting regulation by EBV at the level of transcription initiation or pre-mRNA transcript stability.

3.7. BHRF1 as a negative modulator of SAT1 expression

To identify the EBV viral gene product responsible for downregulation of SAT1 mRNA levels, EBV-negative Akata cells were transfected with expression vectors carrying EBERs or EBNA1, previously reported to restore the tumorigenic phenotype and enhance survival

of the BL cells (Kennedy et al., 2003; Komano et al., 1999; Ruf et al., 2000). In both transient and stable expression of the EBERs or EBNA1 in EBV-negative Akata cells, SAT1 mRNA levels were not altered relative to cells transfected with a vector control (data not shown). Following a hint provided by earlier studies using representation difference analysis of paired Akata clones, BHRF1 mRNA, a viral homolog of the anti-apoptotic factor BCL-2, was detected (unpublished observations, Y. Gan and J. W. Sixbey). Although BHRF1 which could have been produced from a small fraction of Akata BL cells undergoing lytic replication, we examined if a latent form of BHRF1 was expressed in our EBV-positive Akata clones. Latent forms of heterogeneous BHRF1 transcripts that contained spliced leader exons have been reported in a variety of latently-infected B cell lines (Austin et al., 1988; Bodescot and Perricaudet, 1986; Pfitzner et al., 1987). Recently, a Wp promoter linked W/BHRF1-spliced transcript was described that is constitutively expressed as a latent protein in growth-transformed cells and is capable of providing protection against apoptotic stimuli in BL cells (Kelly et al., 2009) (Fig 5A).

To determine if a latent form of BHRF1 was expressed in our EBV-positive Akata clones, we used RT-PCR to amplify W2-BHRF1 or Y2-BHRF1 spliced transcripts in our latently infected EBV-positive Akata clones using previously described primers (Kelly et al., 2009; Oudejans et al., 1995; Speck et al., 1999). As shown in Figure 5B (top and middle panels), W2-BHRF1 spliced transcripts were detected in all three clones, and Y2-BHRF1 spliced transcripts were detected in two of three clones. Spliced forms with and without the Y2 exon have been previously reported (Kelly et al., 2009). An additional primer set that amplified only the BHRF1 open reading frame (primers C/D) corroborated presence of the BHRF1 transcript (Fig. 5B, bottom panel). As support for expression of latent forms of BHRF1, the BZLF1 mRNA expression was absent in the 1B6 clone (Fig. 3C). Furthermore, H2/BHRF1 primers (Lear et al., 1992) that detect transcripts initiating from the lytic promoter located immediately upstream in the H2 exon did not yield a product in the 1B6 EBV-positive clones corroborating expression of a latent form of BHRF1 in the EBV-positive Akata BL clones (Fig 3C). We have been unable to detect latent BHRF1 protein in our EBV-positive Akata clones by immunoblot analysis likely due to low levels of expression. In contrast, we are able to detect BHRF1 protein after IgG stimulation of lytic replication (Fig. 5D).

To determine if BHRF1 was responsible for repression of SAT1 expression, we cloned the BHRF1 coding region into the pLXSN expression vector, excluding the 3' untranslated region of the transcript to exclude potential contributions from encoded viral BHRF microRNAs. The EBV-negative Akata clone, 2A8, was transfected with the BHRF1-expressing vector or the corresponding vector control. As shown in Figure 5C and D, BHRF1 transfected cells expressed the transgene as detected at the mRNA and protein level. Analysis of two independent stable transfectant expressing BHRF1 demonstrated a 40% reduction in SAT1 mRNA as compared to the vector control (Fig. 5E), implicating BHRF1 as a negative modulator of SAT1 mRNA expression in Akata BL.

4. Discussion

In this study, we demonstrated an EBV-dependent reduction in SAT1 mRNA levels in BL, which impacts SAT1 enzyme activity and polyamine levels. Evidence from a reinfected EBV-negative clone and single expression of the BHRF1 viral gene product supported EBV in reducing SAT1 mRNA levels. This EBV-dependent reduction in SAT1 becomes a complementary alteration to the c-myc translocation in BL. Downregulation of the catabolic polyamine metabolism by EBV negates the natural compensatory response triggered by c-MYC induction of polyamine biosynthesis, likely preserving c-MYC enhanced polyamine levels required for cell proliferation while reducing polyamine depletion-induced programmed cell death (Ha et al., 1997). The observation of low levels of SAT1 mRNA in

two additional BL cell lines that suggested that reduction of SAT1 mRNA levels was favorable event in tumorigenic BL cell lines in the absence of EBV.

In defining the relationship between the presence of EBV and reduction in SAT1 expression, a consistent repression of SAT1 mRNA levels was observed in infected Akata cells under growth and stimulatory conditions known to increase SAT1 expression (Fig. 2 and 3). In a prior study by Yuan, et al. that also examined gene expression changes between EBV-positive and -negative Akata after BCR crosslinking using microarray analysis, SAT1 was not identified as a candidate gene among nearly 200 changes in gene expression (Yuan et al., 2006). The reason for failed detection of SAT1 in the prior study is unknown; however, in our Akata BL cell lines reduction of SAT1 and its consequence was validated by various methods. Although we were unable to directly measure protein levels, reduction in SAT1 mRNA correlated with decreased enzyme activity (Fig. 3C). Furthermore, the observation of a 2-fold reduction in acetyl-spermidine in the EBV-positive Akata BL clones compared to their negative counterparts provided a functional consequence for the EBV-dependent reduction in SAT1 mRNA levels (Table 3). While no significant changes in putrescine, spermidine or spermine were measured when grown in serum rich conditions, the decrease in acetyl-spermidine suggested a slower metabolic turnover of polyamines.

Homeostatic mechanisms likely maintain polyamine levels in the EBV-positive clones such that the growth of the EBV-positive and -negative clones did not differ when cultured in serum rich conditions (Persson, 2009). In contrast, under serum starvation conditions where EBV-positive Akata BL clones demonstrated increased survival compared to their EBV-negative counterparts (Chodosh et al., 1998; Komano et al., 1998; Ruf et al., 1999), EBV-positive clones showed a two-fold increase in spermine levels correlating to the ~2-fold decrease in SAT1 mRNA levels in these cells. Since exogenous addition of spermine can protect against glucocorticoid and B-cell receptor-mediated apoptosis (Hegardt et al., 2001; Nitta et al., 2001), an increase in spermine levels in the EBV-positive Akata cells may confer the resistance to apoptosis reported for the EBV-positive Akata BL cell line in response to serum deprivation (Ruf et al., 1999). Surprisingly, spermine levels in both EBV-positive and -negative Akata BL cells were extremely low. In most cultured mammalian cell systems, spermine and spermidine constituted the majority of the polyamine pool, and their levels are roughly equivalent. To exclude any biases in our HPLC assay, we examined intracellular polyamine pools in two additional EBV-negative BL cell lines (Ramos and BL2). In Ramos BL the ratio of spermidine/spermine was nearly equivalent; in the BL2 cell line, spermine was only 6 times lower than spermidine (data not shown). Since these BL cell lines did not show the ~350 fold discrepancy in spermidine/spermine ratio seen in Akata BL, such low spermine levels may be characteristic of the Akata BL cell line.

Polyamines play a vital role in the lifecycle of a number of viruses particularly in viral replication. Polyamines (spermidine and spermine) are found within virion preparations from a widespread group of viruses, including influenza (Bachrach et al., 1974), vaccinia (Lanzer and Holowczak, 1975), adenovirus (Shortridge and Stevens, 1973), and herpes simplex (HSV-1) (Gibson and Roizman, 1971). Inhibition of polyamine biosynthesis using the ODC inhibitor, DFMO, diminishes HCMV, vaccinia, and HSV-1 progeny production (Tuomi et al., 1980; Tys and Williamson, 1982; Williamson, 1985) emphasizing a requirement for polyamines during viral replication. Whereas some viruses enhance polyamine biosynthesis to meet that requirement (Williamson, 1985), a few are known to inhibit polyamine catabolism. Arbovirus and adenovirus family members, for example, induce an alternatively spliced, nonfunctional form of SAT1 mRNA (Hyvonen et al., 2006; Nikiforova et al., 2002). EBV did not induce alternatively spliced variants of SAT1, but repression of SAT1 mRNA was noted at the pre-mRNA level. The mechanism behind this repression is under investigation.

EBV's suppression of SAT1 in face of BCR crosslinking (Fig. 3B) suggested viral regulation of SAT1 as a survival response during EBV lytic replication. Antigenic stimulation is posited as one means for EBV reactivation from latency in vivo. From the standpoint of normal B lymphocyte physiology, signaling through the BCR initiates outcomes that vary from survival, proliferation or apoptosis, depending on activation of co-stimulatory molecules, the maturation state of the B cell, and the antigenic stimulus. BCR-mediated apoptosis involves not only c-MYC, known to be transiently upregulated prior to the onset of apoptosis, but also polyamines, which are reduced after BCR crosslinking (Leider and Melamed, 2003; Nitta et al., 2001). Suppression of SAT1 by EBV after BCR crosslinking would counter this effect to maintain polyamine levels during viral replication. Furthermore, the early lytic gene, BHRF1, and viral BCL2 homolog was identified as a viral mediator potentiating this effect.

BHRF1 is known to enhance survival of BL cells under conditions that otherwise induce cell death by apoptosis (Henderson et al., 1993; Komano and Takada, 2001). Although the mechanism was not addressed here, BHRF1 may act in a similar fashion to BCL-2, where BCL2-overexpression was shown to reduce SAT1 activity to avert polyamine depletion-induced cell death (Holst et al., 2008). Although the authors did not measure the effect of BCL2 overexpression on SAT1 transcripts, they speculated that expression of BCL2 might sequester K-RAS to mitochondria, limiting its participation with PPAR-gamma to transcriptionally induce SAT1. Whether BHRF1 expression altered K-RAS or PPAR-gamma function is unknown.

Detection of latent forms of BHRF1 in our EBV-positive suggested a role repressing SAT1 not only during lytic replication but also during latency. Although we did not expect to find BHRF1 in latently infected Akata BL cells, the existence of this message in Akata cells was somewhat predicted by detection of BHRF1-derived miRNAs (Pratt et al., 2009). Latent forms of BHRF1 have now been demonstrated to be constitutively expressed in growth transformed lymphocytes where it confers resistance to apoptotic stimuli (Kelly et al., 2009; Kelly et al., 2006). In BL tumors containing an EBNA2-deletion mutant virus, the latent BHRF1 protein was shown to counter the apoptotic effects of c-MYC overexpression and serve as cofactor in BL pathogenesis (Kelly et al., 2009).

In the broader context of how a B-cell transforming human tumor virus complements the cellular genetic alteration represented by translocation of *c-myc* into an immunoglobulin locus, our results shed light on the significance of perturbations in polyamine metabolism initiated by c-MYC enhancement of ODC. Disruptions of polyamine homeostasis are considered remarkably difficult to achieve, the overexpression of ODC alone typically fails to increase levels of the higher polyamines spermidine and spermine (Janne et al., 2004). What EBV seems to have accomplished by repression of the catabolic activity of SAT1 in the context of ODC overexpression is accumulation of the higher polyamines, as reflected by our detection of elevated levels of spermine in serum deprived Akata cells. Such synchronous alteration of biosynthetic and catabolic arms of polyamine metabolism may be responsible for both the enhanced cell proliferation and the increased protection from apoptosis characteristic of EBV-positive BL.

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Highlights

- EBV-positive Akata BL demonstrated reduced SAT1 mRNA levels.
- EBV-positive Akata BL showed altered SAT1 enzyme activity and polyamine levels.
- Reduction of SAT1 in EBV-positive Akata BL occurred at the level of transcription.
- Forced expression of the EBV BCL2 homolog, BHRF1 reduced SAT1 mRNA levels.

Figure 1.

Schematic of polyamine synthetic and catabolic pathways. Polyamines are synthesized by the decarboxylation of two amino acids (L-ornithine and L-methionine). Decarboxylation of L-ornithine is catalyzed by ornithine decarboxylase (ODC) to produce the diamine, putrescine. The second aspect of biosynthesis begins with the decarboxylation of L-methionine catalyzed by S-adenosylmethionine decarboxylase resulting in aminopropyl groups that are attached to putrescine or spermidine by spermidine synthase and spermine synthase, respectively, to form the longer chained polyamines, spermidine and spermine. Catabolism of polyamines is regulated by SAT1, which can acetylate both spermidine and spermine. The acetylated polyamines are in turn cleaved by polyamine oxidase to generate the shorter chained polyamines. In addition, acetylated polyamines are rapidly removed from the cell to aid in the depletion of polyamines. Thus, the combined action of biosynthesis and catabolism determine the polyamine levels within a cell (adapted from (Gerner and Meyskens, 2004)).

Figure 2.

EBV-dependent repression of SAT1 mRNA levels. A) SAT1 mRNA levels in EBV-positive BL clones compared to BL clones “cured” of virus under 0.1% serum starvation (striped bars) and 10% serum (solid bars). The mean and standard error of the mean (SEM) from five independent experiments is shown. Values are reported relative to the EBV-positive 1B4 clone grown in 10% serum. B) SAT1 repression upon re-infection of EBV-negative Akata BL clone. SAT1 mRNA levels in the EBV-negative Akata clone (2A8) infected with a recombinant EBV-Akata strain (BDLF3⁻, striped bar) was compared to the uninfected EBV-negative 2A8 clone (grey bar) and the EBV-positive 1B6 clone (black bar). The mean and SEM from two independent experiments is shown relative to the re-infected clone. C) Comparison of SAT1 mRNA levels in paired EBV-positive and -negative BL cell lines derived from Akata, MutuI, and BL2. The mean and SEM from 2 independent experiments is shown. Grey boxes denote the EBV-negative BL cell line while black shaded bars denote the EBV-positive BL cell lines.

Figure 3.

Maintenance of EBV-dependent repression of SAT1 levels with inducers of SAT1 expression. A) ERK activation following BCR crosslinking. Western blot analysis was performed to detect phosphorylated ERK (pERK) and pan-ERK after 30 minutes of BCR crosslinking. B) Effect of BCR crosslinking on SAT1 mRNA levels. RNA was harvested from cells treated with 10ug/ml anti-human IgG at 12 and 24 hours. Mock samples were harvested at 24 hours. Values are reported relative to the mock-treated, EBV-positive 1B6 clone. The mean and SEM from two independent experiments is shown. C) Top panel: BHRF1 expression after 12 and 24 hours following BCR crosslinking. Shown is a Southern hybridization to RT-PCR amplicons using primers H2/D specific for the lytic form of BHRF1. Bottom panel: BZLF1 expression at 12 hours following BCR crosslinking. Shown is an image from an ethidium bromide stained agarose gel of RT-PCR amplicons using BZLF1 specific primers. D) Effect of polyamine analog treatment on SAT1 mRNA levels in EBV-positive and -negative BL clones. Cells were treated for 24 hours with 10uM diethylnorspermidine (DENSPM) or mock-treated with vehicle control. The average and SEM from four independent experiments is shown relative to the value from the 1B4 clone. E) Comparison of SAT1 activity in DENSPM-treated, EBV-positive and negative BL clones. SAT1 activity was measured using an in vitro spermidine acetylation enzyme assay. Shown is the mean and SEM from 5 independent experiments.

Figure 4.

Mechanism of SAT1 transcriptional repression in EBV-positive and -negative Akata clones. A) Analysis of SAT1 splice variants in EBV-positive and -negative Akata clones. Top panel is RT-PCR using primers P1 and P4 which are specific for exon 1 and exon X amplifying the SAT1-X splice variant. Shown is a Southern hybridization using a random-labeled SAT1 cDNA probe. Middle panel is the ethidium bromide stained gel showing the corresponding loading control showing RT-PCR amplification with primers specific to GAPDH. Bottom panel is a RT-PCR amplification using the real-time primers to SAT1 exon 3 and exon 4. Shown is a Southern hybridization using a random-labeled SAT1 cDNA probe. The ratio of SAT1-X to SAT1 is shown determined from densitometric analysis. B) Analysis of SAT1 pre-mRNA levels in EBV-positive and -negative Akata clones. The levels for SAT1 pre-mRNA was measured by RQ-RT-PCR using primers specific to SAT1 intron 2 and exon 2 and a TaqMan probe. The data was normalized to GAPDH and relative expression was determined using the $\Delta\Delta C_t$ method. The mean and SEM from three independent experiments are shown. Relative SAT1 mRNA levels were compared to the value from the 1B4 clone arbitrarily set to 1.

Figure 5.

Identification of a negative viral modulator of SAT1 mRNA expression. A) Schematic of alternatively spliced latent BHRF1 transcripts (Kelly et al., 2009). B) Latent BHRF1 expression in EBV-positive Akata BL. BHRF1 expression in 3 EBV-positive clones was determined by RT-PCR using primers specific for W2, Y2, or BHRF1 exons. Shown are Southern hybridizations to RT-PCR products using the various primer pairs. Images were cropped to remove extraneous lanes. Top Panel represents W2/BHRF1 transcripts. Middle panel represents Y2/BHRF1 transcripts. Bottom panel shows BHRF1 transcripts carrying the coding region. B958 was used as a positive control, and the EBV-negative 2A8 clone was used as a negative control. RT-positive and RT-negative lanes are indicated. C) BHRF1 mRNA expression in stably transfected EBV-negative Akata cell lines. The EBV-negative clone, 2A8, was transfected with a BHRF1 expression plasmid or vector alone as a control, and stable cell lines were generated. Shown is a Southern blot of RT-PCR amplicons using primers to the BHRF1 coding region. D) Detection of BHRF1 protein in transfected EBV-negative 2A8 Akata BL clone. Shown is an immunoblot using a BHRF1 specific antibody. Lanes are 1: vector control; 2: IgG induced EBV+Akata BL; 3 and 4: 2A8+BHRF1; 5: B958 (latency III) as positive control. E) Reduction of SAT1 mRNA in cells stably expressing BHRF1. SAT1 was determined using RQ-RT-PCR as previously described. Vector control was set arbitrarily to 1. Shown is the mean and SEM determined from duplicate seedings of two independently generated stably expressing cell lines.

Table 1

Oligonucleotide primers and probes used in this study.

Name	Sequence (5'-3')	Genomic position/ Accession#	Tm (°C)	Ref.
RQ-SSAT-F (exon 3)	GGT TGC AGA AGT GCC GAA A	330-348 / NM_002970	60	
RQ-SSAT-R (exon 4)	CCA ATC CAC GGG TCA TAG GT	422-403 / NM_002970	60	
TaqMan SSAT probe (exon 3/4)	FAM-CAC TGG ACT CCG GAA GGA CAC AGC A-TAMRA	352-376 / NM_002970	---	
RQ-SSATUN-F (exon 2)	TGG CTA AAT ATG AAT ACA TGG AAG AAC A	1587-1614 / HSSSPN1AG	60	
RQ-SSATUN-R (intron 2)	AGG CAC CGA ACG CTT GTC	1677-1660 / HSSSPN1AG	60	
RQ-SSATUN-Probe	FAM-AAA AAG GTA ATT CAA CAG TGG CGG GAC G-TAMRA	1629-1656 / HSSSPN1AG	---	
P1 (SSAT exon 1)	AGC CAC TGC CTC TGA CTG	7-25 / NM_002970	50	
P4 (SSAT exon X)	GAG ACT GTA ACC TTC CGG AG	214-195 / AY841998	50	
P6 (SSAT exon 6)	CTC TTC ACT GGA CAG ATC AG	621-601 / NM_002970	50	
GAPDH-F	GAA GGT GAA GGT CGG AGT	108-125 / NM_002046	50	
GAPDH-R	GAA GAT GGT GAT GGG ATT TC	333-314 / NM_002046	50	
BHRF1-C (CDS)	GTG CAT GGA AAT GGT A	42142 – 42157/ AJ507799	50	(a)
BHRF1- D (CDS)	AAG GCT TGG GTC TCC	42381 – 42367/ AJ407799	50	(a)
BHRF1 W2	TGG TAA GCG GTT CAC CTT CAG	33242- 33262/ AJ507799	50	(b)
BHRF1 Y2 (Cp / Wp initiated)	TAC GCA TTA GAG ACC ACT TTG AGC C	35609- 35633/ AJ507799	50	(b)
BHRF1 probe	TGC ATC CTG TGT TGG AGC TAG CAG CA	42161-42186/ AJ507799	---	
BHRF1 H2 (Hp initiated)	GTC AAG GTT TCG TCT GTG TG	41542 -41561/ AJ507799	50	(c)
BZLF1-F	CTTGGCCCGGCATTTTCT	90168-90185/ AJ50799	60	
BZLF1-R	ACGACGCACACGGAACCC	90400-90383/ AJ507799		

^a(Speck et al., 1999),^b(Kelly et al., 2009),^c(Oudejans et al., 1995)

Table 2

Global transcription profiling of EBV-positive and –negative Akata BL clones.

ProbeSet ID	Gene Symbol	Description	Fold Δ *	P value
Downregulated				
808_at	RAB27B	RAB27B, member RAS oncogene family	-14.6	0.0054
36261_at	SYT17	synaptotagmin XVII	-4.2	0.0003
41027_at	FOXC1	forkhead box C1	-3.2	0.0098
39298_at	ST3GAL6	ST3 beta-galactoside alpha-2,3-sialyltransferase 6	-2.3	0.0003
34107_at	PFKFB2	6-phosphofructo-2-kinase	-2.7	0.0147
756_at	ITPR2	inositol 1,4,5-triphosphate receptor, type 2	-2.7	0.0224
35459_at	RGS13	regulator of G-protein signaling 13	-2.5	0.0036
1173_g_at	SAT1	spermidine/spermine N1-acetyltransferase 1	-2.4	0.0002
38228_g_at	MITF	microphthalmia-associated transcription factor	-2.3	0.0089
34304_s_at	SAT1	spermidine/spermine N1-acetyltransferase 1	-2.3	0.0178
32916_at	PTPRE	protein tyrosine phosphatase, receptor type, E	-2.1	0.0417
34342_s_at	SPP1	secreted phosphoprotein 1	-2.0	0.0159
40024_at	STAC	SH3 and cysteine rich domain	-1.9	0.0033
Upregulated				
39420_at	DDIT3	DNA-damage inducible transcript 3	1.9	0.0273
39827_at	DDIT4	DNA-damage-inducible transcript 4	2.0	0.0048
35842_at	IL6ST	interleukin 6 signal transducer (gp130, oncostatin M receptor)	2.0	0.0409
35799_at	DNAJB9	DnaJ (Hsp40) homolog, subfamily B, member 9	2.0	0.0077
34777_at	ADM	adrenomedullin	2.1	0.0232
37044_at	PDIA5	protein disulfide isomerase family A, member 5	2.2	0.0195
442_at	HSP90B1	heat shock protein 90kDa beta (Grp94), member 1	2.4	0.0006
41371_at	GTF2H4	general transcription factor IIH, polypeptide 4, 52kDa	2.6	0.0461
39037_at	AFF1	AF4/FMR2 family, member	2.6	0.0332
40143_at	FAM53B	family with sequence similarity 53, member B	2.8	0.0072
40201_at	DDC	dopa decarboxylase	3.3	0.0192
1842_at	DDIT3	DNA-damage-inducible transcript 3	4.4	0.0098

* Fold Difference was calculated as $2^{\text{signal log ratio}}$, where the signal log ratio is the intensity value from the EBV-positive Akata clone (1B6) compared to the EBV-negative Akata clone (2A8).

Table 3

Intracellular polyamine pools in EBV-negative and -positive BL clones.

Sample	Putrescine pmol/10 ⁶ cells	Acetyl spermidine pmol/10 ⁶ cells	Spermidine pmol/10 ⁶ cells	Acetyl spermine pmol/10 ⁶ cells	Spermine pmol/10 ⁶ cells
EBV-negative BL clones (10% FBS)	2.14±0.25	3.98±0.70	170.70±7.59	nd.	0.50±0.06
EBV-positive BL clones (10% FBS)	1.70±0.15	2.16±0.27*	175.39±7.41	nd.	0.58±0.06
EBV-negative BL clones (0.1% FBS)	0.65±0.10	1.49±0.47	177.45±16.82	nd	0.47±0.05
EBV-positive BL clones (0.1% FBS)	0.79±0.14	1.09±0.28	212.17±17.33	nd	0.88±0.15**

Polyamine amounts are reported as the mean of four clones ± standard error of the mean, nd=none detected

* $p < 0.05$,

** $p < 0.015$