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The role of promoter methylation in Epstein-Barr virus (EBV) microRNA expression in EBV-infected B cell lines

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Abbreviations: 5-aza-Cd, 5-aza-2'-deoxycytidine; BARTs, *Bam*HI A rightward transcripts; Cp, *Bam*HI C promoter; CSTs, complementary strand transcripts; EBERs, EBV-encoded RNAs; EBNA, EBV nuclear antigen; EBV, Epstein-Barr virus; LCL, lymphoblastoid cell lines; miRNA, microRNAs; NPC, nasopharyngeal carcinoma; Qp, BamHI Q promoter; TSA, trichostatin A; Wp, *Bam*HI W promoter

Abstract

Epstein-Barr virus (EBV) microRNAs (miRNAs) are expressed in EBV-associated tumors and cell lines, but the regulation mechanism of their expression is unclear yet. We investigated whether the expression of EBV miRNAs is epigenetically regulated in EBV-infected B cell lines. The expression of BART miRNAs was inversely related with the methylation level of the BART promoter at both steady-state and following 5-aza-2'-deoxycytidine treatment of the cells. The expression of BHRF1 miRNAs also became detectable with the demethylation of Cp/Wp in latency I EBV-infected cell lines. Furthermore, *in vitro* methylation of the BART and Cp promoters reduced the promoter-driven transactivation. In contrast, tricostatin A had little effect on the expression of EBV miRNA expression as well as on the BART and Cp/Wp promoters. Our results suggest that promoter methylation, but not histone acetylation, plays a role in regulation of the EBV miRNA expression in EBV-infected B cell lines.

Keywords: decitabine; DNA methylation; Herpesvirus 4, human; microRNAs; promoter regions, genetic

Introduction

Epstein-Barr virus (EBV) is a ubiquitous human herpesvirus implicated in several lymphomas and carcinomas (Young and Rickinson, 2004). Three different latency patterns have been demonstrated depending on the use of distinct latency promoters. In latency III, long (up to 120 kb) primary transcripts synthesized from activated *Bam*HI C promoter (Cp) or *Bam*HI W promoter (Wp) are alternatively spliced to produce mature transcripts encoding six different EBV nuclear antigen (EBNA) proteins (EBNA-1, -2, -3A, -3B, -3C, and -LP) (Abbot *et al.*, 1990). In latency I and II, *Bam*HI Q promoter (Qp) is activated to produce EBNA1, while both Wp and Cp become silent due to DNA methylation (Li and Minarovits, 2003; Tao and Robertson, 2003). In addition, EBV-encoded RNAs (EBERs) and *Bam*HI A rightward transcripts (BARTs) are expressed in all latently-infected cells (Middeldorp *et al.*, 2003; van Beek *et al.*, 2003). BART transcripts were originally identified in the nude mouse-passaged nasopharyngeal carcinoma (NPC) cell line C15 (Hitt *et al.*, 1989; Gilligan *et al.*, 1990) and also known as the complementary strand transcripts (CSTs) or BARF0 RNAs (Gilligan *et al.*, 1990; Smith, 2001). BART transcripts are expressed at particularly high levels in NPC but also expressed in EBV infected peripheral blood B cells (Chen *et al.*, 1992; Brooks *et al.*, 1993).

EBV encodes two groups of viral microRNAs (miRNAs): BHRF1 miRNAs and BART miRNAs. Expression of the EBV miRNAs has been observed not only in EBV-infected cell lines (Pfeffer *et al.*, 2004; Cai *et al.*, 2006; Grundhoff *et al.*, 2006) but also in EBV-associated tumors (Cai *et al.*, 2006; Kim *et al.*, 2007; Jun *et al.*, 2008). The expression pattern of BHRF1 miRNAs in certain cells is related to the EBV latencies in those cells (Pfeffer *et al.*, 2004; Cai *et al.*, 2006; Grundhoff *et al.*, 2006). BHRF1 miRNAs sequences are located within introns of the BHRF1 gene that lies downstream of Cp/Wp and upstream of Qp (Pfeffer *et al.*, 2004; Grundhoff *et al.*, 2006) and transcribed as part of the long EBNA transcripts during latency III (Cai *et al.*, 2006). In latency I and II, the long transcripts encompassing EBNAs and BHRF1 miRNAs cannot

be produced as Cp/Wp are silenced due to DNA methylation (Li and Minarovits, 2003; Tao and Robertson, 2003).

Unlike the latency III restricted BHRF1 miRNAs expression, BART miRNAs are expressed in all three latencies (Pfeffer *et al.*, 2004; Cai *et al.*, 2006). The expression level of BART miRNAs correlates with the expression of BART transcripts in EBV-infected cell lines (Cai *et al.*, 2006; Edwards *et al.*, 2008; Amoroso *et al.*, 2011), supporting the notion that BART miRNAs are processed from the BART transcripts. Recently, Pratt *et al.* (Pratt *et al.*, 2009) reported that BART miRNAs are expressed at 25-50-fold different levels in various EBV-infected cell lines. They found that the steady-state levels of BART miRNAs in EBV-infected cell lines did not correlate with the number of viral templates per cell and were not elevated by lytic induction (Pratt *et al.*, 2009). Thus far, it is unclear why the level of BART miRNAs expression varies greatly in different cells.

miRNA expression can be regulated transcriptionally and post-transcriptionally. Several transcription factors have been shown to activate or repress the transcription of primary miRNAs (Chen *et al.*, 2005; O'Donnell *et al.*, 2005; Rao *et al.*, 2006; He *et al.*, 2007). miRNA processing can be modulated by RNA-binding proteins. For example, Lin-28 and Lin-28B inhibit let-7 maturation by inhibiting primary miRNA or precursor miRNA cleavage (Heo *et al.*, 2008; Viswanathan *et al.*, 2008), while HnRNP A1 enhances processing of miR-18a (Guil and Cáceres, 2007; Michlewski *et al.*, 2008). Expression of miRNAs is also known to be regulated by epigenetic mechanisms, such as promoter methylation (Bueno *et al.*, 2008; Toyota *et al.*, 2008) or histone acetylation (Scott *et al.*, 2006; Grady *et al.*, 2008; Saito *et al.*, 2009).

In this study, we investigated if the expression of BART miRNAs is epigenetically regulated in EBV-positive B cell lines.

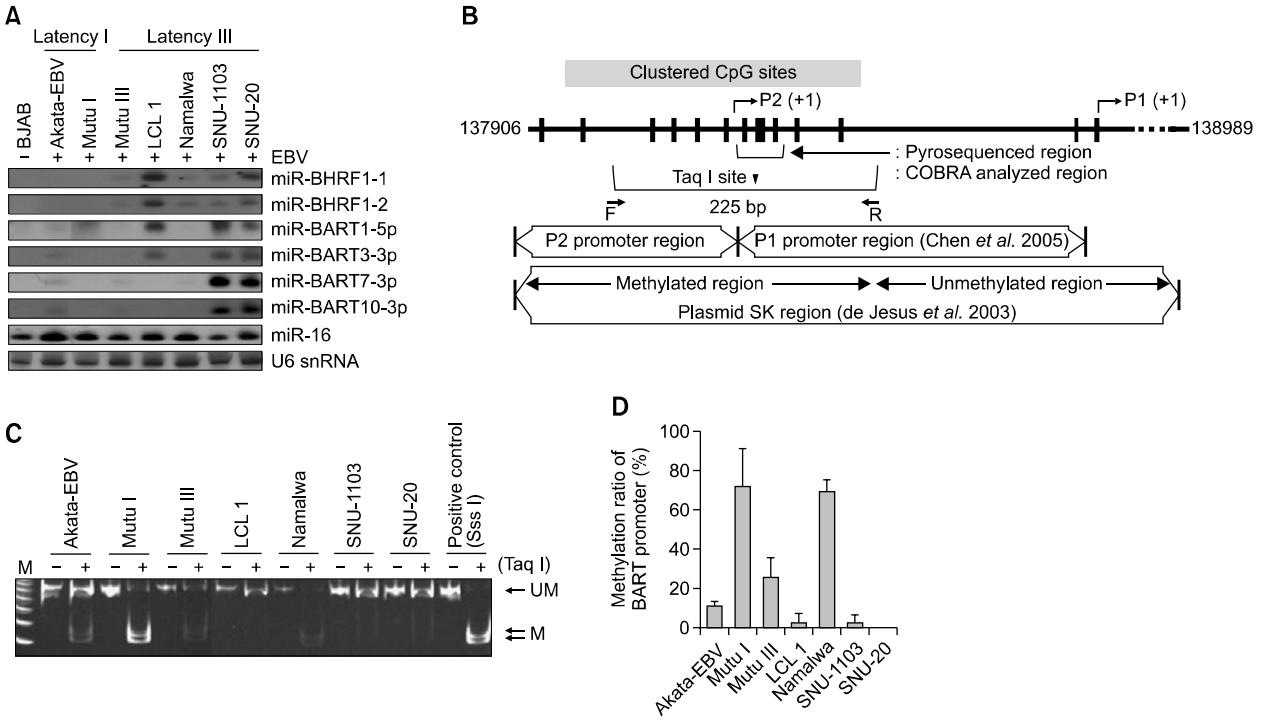

Figure 1. Inverse relationship between the EBV BART miRNAs expression and the promoter methylation. (A) Northern blot for EBV miRNAs was performed using total RNA isolated from the cells. An EBV-negative B cell line, BJAB, was included as a negative control. Loading amount of each RNA sample was monitored by reprobing the blot using a specific probe to U6 snRNA. The expression of human miR-16 was assessed as a reference. (B) Schematic representation of the CpG-rich region of BARTs promoter (GenBank entry AJ507799). CpG sites are indicated with vertical ticks, Taq I restriction site is indicated with the vertical triangle, and PCR primers for COBRA analysis are indicated with arrowheads. BART promoter regions analyzed previously (de Jesus *et al.*, 2003; Chen *et al.*, 2005) are also shown for comparison. (C) The methylation status of the BART promoter analyzed by COBRA. SNU-719 DNA treated with Sss I methyl transferase was used as a positive control for methylated DNA. (D) CpG methylation of BART promoter region in EBV positive cell lines was assessed by pyrosequencing. The mean ± standard deviation values for individual cell lines shown in Supplemental Data Table S1, are plotted in a bar graph for easy comparison.

Results

Inverse correlation between BART miRNAs expression and methylation status of the promoter

The expression of EBV miRNAs in two latency I and five latency III EBV-infected B cell lines were analyzed by Northern blot. As expected, BHRF1 miRNAs were readily detectable in latency III cells but were not detected in any of the latency I cells (Figure 1A). Although BART miRNAs are known to be expressed in all the EBV infected cells, their expression level varied greatly in different cells. In general, lymphoblastoid cell lines (LCL) (LCL1, SNU-1103 and SNU-20) expressed BART miRNAs at markedly higher level than Burkitt's lymphoma cell lines (Akata-EBV, Mutu I, Mutu III and Namalwa). LCL1 did not express miR-BART7-3p and miR-BART10-3p as it is infected with B95-8 EBV strain which has a large deletion in these regions (Wei *et al.*, 1994).

To test if the expression level of BART miRNAs was related with epigenetic status of the BART promoter, methylation level of the BART promoter was analyzed by COBRA using a primer set capable of amplifying the sequence encompassing the clustered CpG sites in this region (Figure 1B). BART promoters of LCL1, SNU-1103, and SNU-20 were unmethylated for the most part, while those of Akata-EBV, Mutu I, Mutu III, and Namalwa were methylated at variable degrees (Figure 1C).

To confirm the results of COBRA, pyrosequencing was carried out for the four consecutive CpG sites (Figure 1B) within the BART promoter of the cells. Methylation ratios of the four CpG sites in the BART promoter region were not appreciably different from each other within each cell line (Supplemental Data Table S1). However, different cells showed differential methylation ratios in their BART promoters (Supplemental Data Table S1; Figure 1D).

LCLs that showed a high level miRNA expression such as LCL1, SNU-1103, and SNU-20 exhibited relatively low BART promoter methylation, while Mutu I and Namalwa which had lowest BART miRNAs expression displayed the highest promoter methylation ratios among the tested cell lines (Figure 1A vs. Figure 1D). Akata-EBV and Mutu III showed intermediate levels of BART promoter methylation and BART miRNAs expression.

Upregulation of BART miRNAs expression following demethylation of the BART promoter using 5-aza-Cd

If hypermethylation of the CpG-rich area was one of the cause of transcriptional repression of the BART miRNAs, demethylation of the CpGs should upregulate their expression. To check whether BART miRNAs expression could be induced by BART promoter demethylation, cells were treated with 5-aza-2'-deoxycytidine (5-aza-Cd) for 72 h. BART promoter methylation decreased significantly following 0.5-5 μM 5-aza-Cd treatment of Mutu I cells compared to the untreated control (Figure 2A, Supplemental Data Table S2). This coincided with a large induction of BART miRNAs expression in this cell line (Figure 2B). BART promoter methylation decreased (Figure 2A) and BART miRNAs expression increased noticeably (Figure 2B) only when these cells were treated with higher concentrations of 5-aza-Cd than 5 μM. In Akata-EBV, which showed relatively low basal BART promoter methylation, 5-aza-Cd treatment reduced methylation ratio of the promoter only slightly and BART miRNAs expression was induced nominally. 5-Aza-Cd treatment of the cells which have hypomethylated promoters such as LCL1, SNU-1103, and SNU-20 failed to alter the levels of BART miRNAs expression as well as the promoter methylation (data not shown).

To investigate whether the expression of EBV miRNAs could also be regulated by histone acetylation, BART and BHRF1 miRNAs expression was

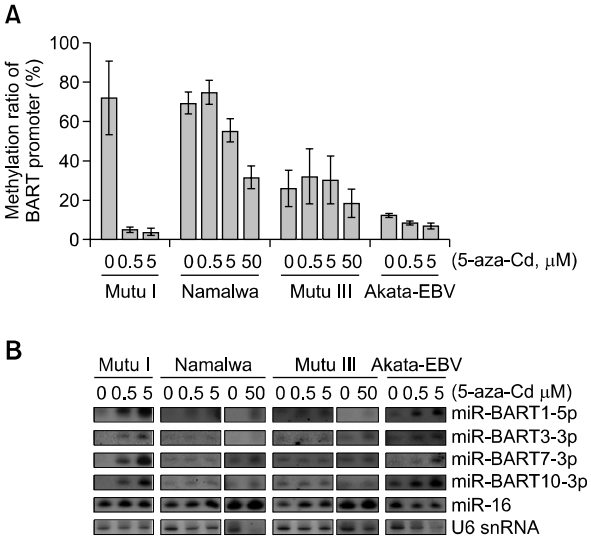

Figure 2. Effect of 5-aza-Cd on the expression of BART miRNAs and BART promoter. (A) CpG methylation status of the BART promoter was analyzed by pyrosequencing in Mutu I, Namalwa, Mutu III, and Akata-EBV cell lines which were treated with 5-aza-Cd for 72 h. (B) Northern blot was performed for EBV miRNAs using total RNA isolated from the cells treated with the indicating concentrations of 5-aza-Cd for 72 h. To facilitate analysis of the changed expression levels of BART miRNAs following 5-aza-Cd treatment, different optimal exposure time was used for each cell line and for each individual BART miRNA. Loading amount of each RNA sample was monitored by reprobing the blot with a specific probe to U6 snRNA. The expression of human miR-16 was assessed as a reference.

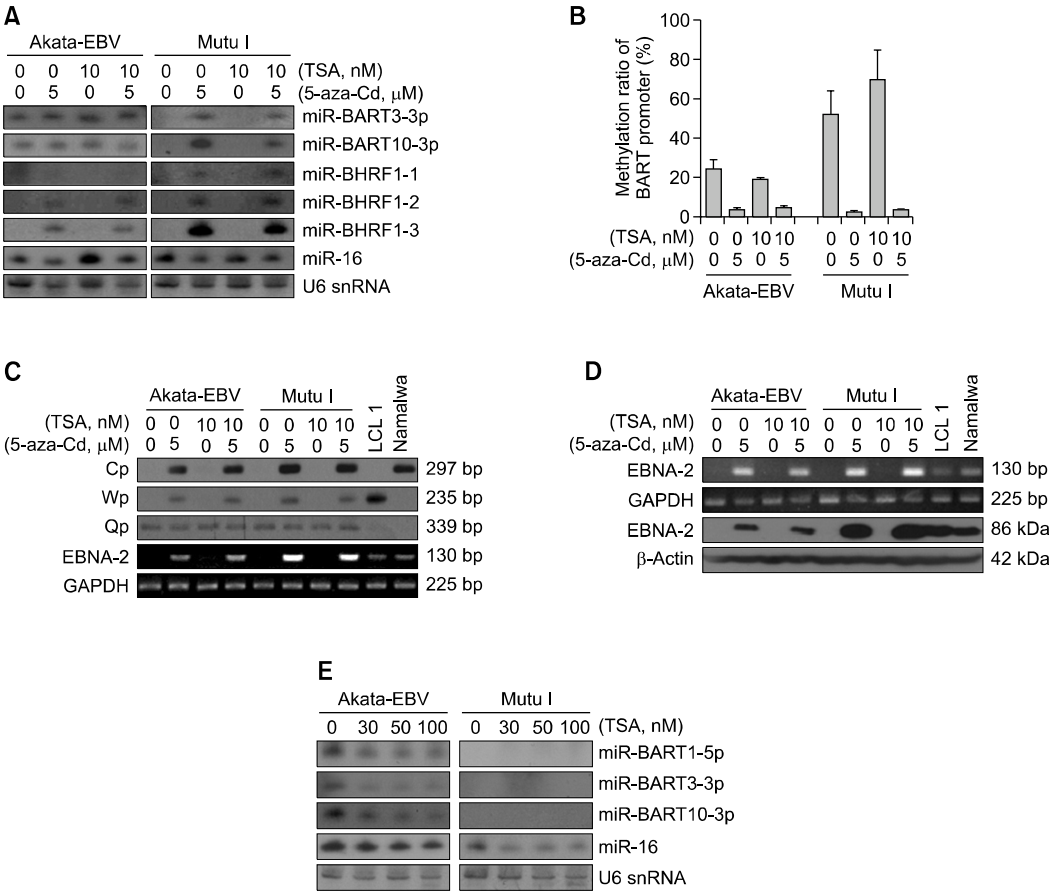

Figure 3. Effect of 5-aza-Cd and/or TSA on the expression of EBV miRNAs in Akata-EBV and Mutu I cell line. (A) Northern blot for EBV miRNAs was performed using total RNA isolated from the cells treated with 5-aza-Cd (5 μM) and/or TSA (10 nM) for 72 h. (B) Pyrosequencing was carried out for the BART promoter of Akata-EBV and Mutu I cell lines treated with 5-aza-Cd and/or TSA. (C) A specific 3′-primer for each transcript initiating at the Cp, Wp, or Qp was used for cDNA synthesis. cDNA was subjected to latent EBV promoter specific PCR/Southern blot. cDNA of EBNA-2 was also amplified by RT-PCR to confirm latency type. GAPDH mRNA was amplified to compare the quantity and quality of the RNA samples. (D) Expression of EBNA-2 protein was detected using PE2 monoclonal antibody. β-Actin was used as a loading control for Western blot. LCL1 and Namalwa were used as positive controls for the transcripts initiating at Wp and Cp, respectively. (E) Northern blot was performed for BART miRNAs using total RNA isolated from Akata-EBV and Mutu I cells which were treated with TSA (0, 30, 50, or 100 nM) for 24 h. Loading amount of each RNA sample was monitored by reprobing the blot with a specific probe to U6 snRNA. The expression of human miR-16 was assessed as a reference.

analyzed for latency I cells treated with trichostatin A (TSA) alone or in combination with 5-aza-Cd. TSA cotreatment with 5-aza-Cd or TSA alone had no effect on the BART and BHRF1 miRNAs expression (Figure 3A) as well as on the promoter methylation (Figure 3B).

Not unexpectedly, the expression of BHRF1 miRNAs was detected in latency I Akata-EBV and Mutu I cells treated with 5-aza-Cd (Figure 3A). As BHRF1 miRNAs are reported to be expressed in only latency III, we checked whether treatment with 5-aza-Cd caused activation of Cp/Wp in these latency I cells. Cp and Wp transcripts were detected in Akata-EBV and Mutu I cells following treatment with 5-aza-Cd but not with TSA (Figure 3C). A transcript initiating at Qp was detected in these cells regardless of the treatment. Expression

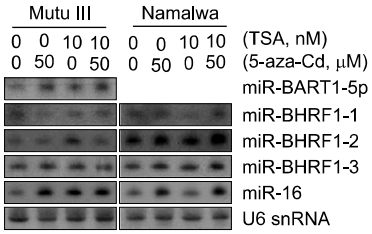

Figure 4. Effect of 5-aza-Cd and/or TSA on the expression of EBV miRNAs in Mutu III and Namalwa cell line. Northern blot was performed for EBV miRNAs using total RNA isolated from Mutu III and Namalwa cells which were treated with 5-aza-Cd (50 μM) and/or TSA (10 nM) for 72 h. To facilitate analysis of the changed expression levels of BART and BHRF1 miRNAs following 5-aza-Cd and/or TSA treatment, different optimal exposure time was used for each cell line and for each individual BART miRNA. Loading amount of each RNA sample was monitored by reprobing the blot with a specific probe to U6 snRNA. The expression of human miR-16 was assessed as a reference.

of EBNA-2 was also observed by RT-PCR and Western blot in these cells after treated with 5-aza-Cd (Figure 3D), confirming the activation of latency III promoters. We did not noticed significant alteration in the size and shape of Mutu I and Akata-EBV cell following 5-aza-Cd treatment using bright-field microscopy.

To make sure TSA was functioning properly, cell cycle was accessed by flow cytometric analysis following TSA-treatment of the Akata-EBV and Mutu I cells. Cell cycle was inhibited when these two cell lines were treated with increasing concentrations of TSA for 24 h (Supplemental Data Figure S1). However, the expression of BART miRNAs was not induced in the same cells following up to 100 nM TSA treatment (Figure 3E). TSA had no effect on the expression of BART and BHRF1 miRNAs in latency III cell lines, either (Figure 4).

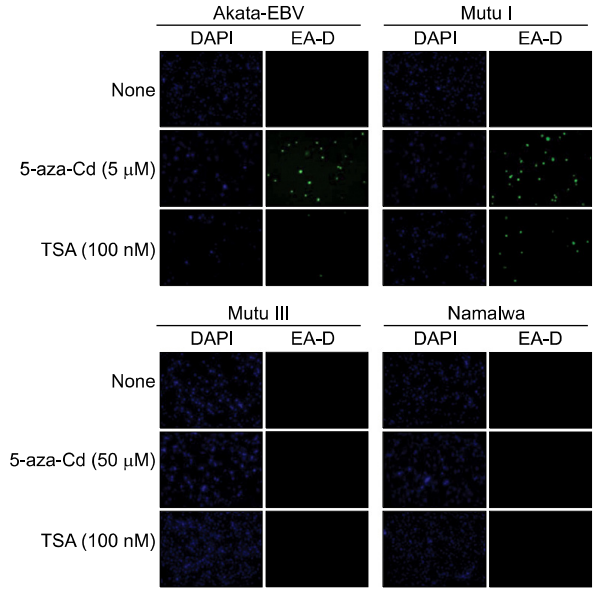

Figure 5. Induction of EBV lytic gene expression by 5-aza-Cd and TSA in latency I and latency III cells. The cells were treated with 5-aza-Cd (5 or 50 μM) for 72 h and TSA (100 nM) for 24 h. For immunofluorescence assay, anti-EA-D Ab (1:500) was used. Nuclei was stained with DAPI.

Induction of EBV lytic cycle by 5-aza-Cd and TSA treatment

The effect of 5-aza-Cd and TSA on the induction of EBV lytic cycle was analyzed by detecting the expression of BMRF1 gene product (early antigen diffuse component; EA-D) (Figure 5). Akata-EBV, Mutu I, Mutu III, and Namalwa cells were treated with 5-aza-Cd (5 or 50 μM) for 72 or TSA (100 nM) for 24 h. 5-Aza-Cd and TSA treatment caused EA-D expression in small portions of Akata-EBV (5-aza-Cd, 27%; TSA, 7%) and Mutu I (5-aza-Cd, 41%; TSA, 12%) cells but not in Mutu III and Namalwa (Figure 5). 5-Aza-Cd induced EA-D expression seemed to coincide with the enhanced BART miRNA expression (Figure 5 vs. Figure 2B). However, there were no parallels between the extent of lytic cycle induction and the extent of upregulated miRNA expression by TSA (Figure 5 vs. Figure 3E).

Repression of BART promoter and Cp activity via *in vitro* methylation

Cells exhibiting hypermethylated promoter region showed enhanced expression of BART miRNAs when they were treated with 5-aza-Cd. To determine whether DNA methylation directly activates the BART promoter, *in vitro* methylation assays were performed. The CpG residues within the BART promoter was methylated *in vitro* using the CpG-

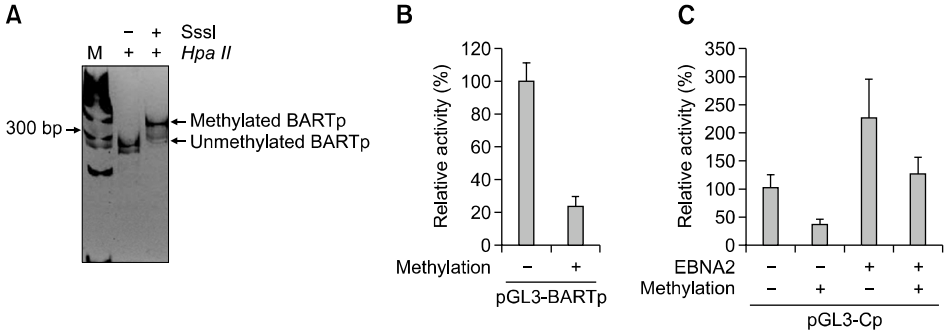

Figure 6. Effects of the CpG methylation on BART promoter or Cp activity. The BART promoter and Cp fragments were methylated *in vitro* using SssI methylase. (A) The methylation efficiency was monitored by *Hpa*II digestion and gel electrophoresis (M, size marker). The unmethylated or methylated BART promoter (BARTp) fragment (B) and Cp fragment (C) was ligated with pGL3-basic vector and transfected into AGS cells. Forty-eight hours after transfection, luciferase activities were measured. To calculate the relative luciferase activity, the unmethylated pGL3-BART promoter or pGL3 Cp-driven luciferase activity was set to 100%.

specific SssI methylase, which was confirmed by digestion with the methylation sensitive restriction endonuclease *Hpa*II (Figure 6A). Each of the unmethylated and methylated BART promoter fragment was ligated with pGL3-basic vector and transfected into AGS cells. Forty-eight hours after transfection, luciferase activities were measured to determine the BART promoter activity. *In vitro* methylation of the CpG residues within the BART promoter repressed the basal BART promoter activity by 77% (Figure 6B). As previously reported (Robertson *et al.*, 1995; Robertson and Ambinder, 1997), Cp methylation also significantly reduced the basal and EBNA2-induced Cp activation (Figure 6C).

Discussion

In our experiments, BL cell lines expressed BART miRNAs at lower levels than LCLs. In general, BL biopsies and BL cell lines show a high level EBV DNA methylation, whereas LCLs show unmethylated or hypomethylated EBV DNA (Minarovits *et al.*, 1991). We found that BART promoters present in BL cell lines were more heavily methylated than those in LCLs. In B cell lines, 5-aza-Cd treatment reduced BART promoter methylation and induced BART miRNAs expression. Furthermore, in vitro methylation of the CpG residues within the BART promoter significantly reduced BART promoter activity, supporting direct link between BART promoter methylation and BART miRNAs expression.

Epigenetic status of the EBV BART promoter region has been analyzed by several investigators previously (Smith *et al.*, 2000; de Jesus *et al.*, 2003; Chen *et al.*, 2005; Al-Mozaini *et al.*, 2009). One group reported that BART promoter activity was found in about 1 kb region encompassing just upstream of the transcription start site to a distance downstream of exon I of the BART gene (de Jesus *et al.*, 2003; Al-Mozaini *et al.*, 2009). The upstream sequences of the BART promoter including the CpG residues (Figure 1B) was shown to be methylated in DNA from C15 NPC tissue and argued that this region was not essential for BART transcripts expression (de Jesus *et al.*, 2003). However, their data showed that deletion of the plasmids containing this upstream sequences, including the CpG residues that we analyzed in this study, resulted in enhanced BART promoter activity (de Jesus *et al.*, 2003). This supports that the upstream sequences including the CpG residues indeed down-regulated the BART promoter, possibly by DNA methylation as we suggested in this paper.

Another group mapped two different TATA-less promoter regions (P1 and P2) for BART transcripts (Chen *et al.*, 2005). Both P1 and P2 are located upstream of exon I. P1 appears to be the dominant start site for BARTs expression in Akata cell line and NPC tumor tissues. The region encompassing both P1 and P2 promoters is hypomethylated in C15 NPC-tumor-associated virus. The BART promoter region analyzed in our experiments expands from 100 bp upstream of the P2 transcription start site to 259 bp upstream of the P1 transcription start site (Figure 1).

In some cells such as Akata-EBV, BART miRNAs expression was not significant even though the BART promoter methylation was relatively low. Thus, the expression of BART miRNAs may also be regulated by other mechanisms (Palii *et al.*, 2008; Dickerson *et al.*, 2009) in addition to the BART promoter methylation in these cells. For example, P1 promoter was downregulated by IRFs, while P2 promoters were positively regulated by C-Myc and C/EBP family members (Chen *et al.*, 2005). Differential expression level of these transcription factors in different cell lines could also affect BART miRNAs expression. Treatment with 5-aza-Cd may have induced those transcription factors (Palii *et al.*, 2008) to affect EBV miRNAs expression in addition to directly affected the methylation status of EBV miRNA promoters. miRNA processing and degradation might also be affected by 5-aza-Cd treatment (Saito *et al.*, 2006; Chuang and Jones, 2007).

We found that Cp/Wp of latency I cells became activated and BHRF1 miRNAs were expressed after 5-aza-Cd treatment. Our results corroborate that BHRF1 miRNAs are primarily processed from the long EBNA transcripts produced from Cp/Wp in latency III (Cai *et al.*, 2006; Xing and Kieff, 2007; Amoroso *et al.*, 2011).

Latency I cells treated with TSA showed a high level of acetylated histone H4 at Cp, but the transcripts initiated at Cp were detected at very low levels (Fejer *et al.*, 2008). This may explain why we could not detect the expression of BHRF1 miRNAs in latency I cells following TSA treatment.

It is controversial whether BART miRNA expression is affected by EBV lytic induction. Pratt *et al.* (Pratt *et al.*, 2009) showed that the level of BART miRNAs were not changed by lytic induction of EBV infected Akata and B95-8 cells using IgG and TPA treatment, respectively. Xing and Kieff (Xing and Kieff, 2007) also reported that miR-BART1 and miR- BART-2 expression was not affected following induction of EBV replication in Akata cells. However Cai *et al.* (Cai *et al.*, 2006) argued that the expression BART miRNAs was increased significantly in Mutu I cells lytically induced with TPA and n-butyrated. In this study, treatment with

5-aza-Cd or TSA caused EBV lytic cycle in latency I cells as we previously reported (Jung *et al.*, 2007; Seo *et al.*, 2008). Even though, the extent of EBV lytic induction by 5-aza-Cd seem to correlate with the increase of miRNA expression, the extent of EBV lytic induction by TSA did not parallel the extent of upregulated miRNA expression.

There are two plausible reasons for these seemingly contradictory results. First, achieving a certain level of EBV lytic induction may be important to upregulate BART miRNAs. Second, not the lytic induction *per se*, but some factor(s) affected by 5-aza-Cd or TSA treatment may regulate BART miRNA transcription, procession, or degradation. Further studies are required to clarify these possibilities.

Taken together, our data suggest that promoter methylation, but not histone acetylation, plays a partial role in the regulation of EBV BART and BHRF1 miRNAs expression in EBV infected B cells.

Methods

Cell lines and culture medium

EBV-positive (Akata-EBV, Mutu I, Mutu III, Namalwa, LCL 1, SNU-1103, and SNU-20) and EBV-negative (BJAB and AGS) cell lines were used. Akata was provided by Dr. K. Takada (Hokkaido University, Japan). Mutu I, Mutu III, Namalwa, LCL1, and BJAB were provided by Dr. R. Longnecker (Northwestern University). AGS, SNU-20, and SNU-1103 were obtained from Korea Cell Line Bank (Seoul, Korea). The cells were maintained at 37°C in RPMI-1640 plus 10% fetal bovine serum (FBS; Gibco, Grand Island, NY), antibiotics (penicillin 100 units/ml and streptomycin 100 μg/ml, Gibco), and Fungizone (100 μg/ml, Gibco). To assess the effect of epigenetic modulation on miRNA expression, cells (5×10^5/ml) were treated with increasing concentrations of 5-aza-2'-deoxycytidine (5-aza-Cd) and/or TSA for 24 or 72 h.

Northern blot of EBV miRNAs

RNA extraction was conducted with RNAzol™ B reagent (Tel-Test, Friendswood, TX) according to the manufacturer's instruction. Equal amounts of total RNA (30 μg) were resolved in a 15% polyacrylamide-8 M urea gel and transferred to a Zeta-Probe Blotting membrane (Bio-Rad Laboratories, Hercules, CA). An oligonucleotide complementary to each mature EBV miRNA (Kim *et al.*, 2007; Jun *et al.*, 2008) was end-labeled with [γ-^{32}P] ATP and T4 kinase. Pre-hybridization and hybridization were carried out using ExpressHyb Hybridization Solution (BD Biosciences Clontech, Palo Alto, CA). Quality and quantity of the loaded RNA was assessed by reprobing the blot for U6 snRNA using a [γ-^{32}P]-labeled oligodeoxynucleotide probe (5'-GCAGGGGCCATGCTAATCTTCTCTGTATCG-3').

Analysis of EBV latent promoter usage

First-strand cDNA synthesis was performed using 1 μg total RNA (Lee *et al.*, 2008) and a 3′-primer specific for each transcript initiating at Cp, Wp, or Qp EBV promoter. The cDNA samples were then subjected to 30 cycles of PCR using appropriate primer pairs for the transcripts initiating at Cp, Wp, or Qp (Sugiura *et al.*, 1996). Each cycle consisted of denaturation for 30 s at 94°C, annealing for 30 s at 59°C, and extension for 30 s at 72°C. The PCR products were then electrophoresed in a 1.5% agarose gel, blotted onto a Hybond N+ nylon membrane (Amersham, Buckinghamshire, UK), and analyzed with an enhanced chemiluminescence 3′-oligolabeling and detection system (Amersham). To detect EBNA2 gene expression, Western blot and RT-PCR were performed as described elsewhere (Oh *et al.*, 2007; Kubota *et al.*, 2008).

DNA extraction and bisulfite treatment

DNA was extracted from the cells by standard phenol-chloroform extraction. Bisulfite treatment was performed for 1 μg genomic DNA using an EZ DNA™ methylation kit (Zymo Research, Orange, CA). Bisulfite-treated DNA was used for Combined Bisulfite Restriction Analysis (COBRA) and pyrosequencing.

Combined bisulfite restriction analysis (COBRA)

PCR primers were designed to amplify a series of CpG dinucleotides within the reported BART promoter region (Smith *et al.*, 1993; de Jesus *et al.*, 2003; Chen *et al.*, 2005): forward primer 5'-AGGGTAAGGTTATAATTGTAATTTT-3' and reverse primer 5'-(Biotin) ATCTATTTATACATTTTTCT CAAATCTAAT-3'. The PCR product (225 bp) was digested with TaqI (New England Biolabs, Ipswich, MA), separated in a 10% acrylamide gel, and visualized by ethidium bromide staining.

Pyrosequencing

A separate PCR was carried out using the same primers and templates as for COBRA and the PCR product was used for pyrosequencing. Pyrosequencing was performed using the PSQ96MA system (Biotage AB, Uppsala, Sweden) and PyroGold reagents (Biotage AB). The primer used for pyrosequencing was 5'-TCCCCGGGAGTGTATC-3'.

Plasmids

pGL3-BART promoter was constructed by PCR amplification of the BART promoter with the following primers: 5'-GGGGTACCGTAGCTACGGCCAAGGGCAG-3' and 5'-CCCAAGCTTGCAGCTTGAAAAATGGCAAC-3' (Robertson and Ambinder, 1997; Chen *et al.*, 2005) The PCR product was digested with *Kpn*I and *Hind*III, and then cloned into the corresponding sites of pGL3-basic vector (Promega, Madison, WI). pSG5-EBNA2 (wild type) was kindly provided by Dr. Elliott Kieff (Harvard Medical School).

Immunofluorescence assay

Mutu I and Mutu III were seeded in a 10-well slide. The cells were fixed in 100% ice-methanol for 5 min at -20°C. For immunofluorescence staining, EA-D (mouse monoclonal antibody (Novocastra, Newcastle upon Tyne, UK, 1:500) was used. Neclei were stained with Prolong Gold Anti-fade Reagent (Molecular probe, Invitrogen, Carlsbad, CA) containing 4', 6-diamino-2-phenylinodole (DAPI). The fluorescence signal was visualized using a Fluorescence Attached Microscope (AX70, TR-62A02, Olympus, Tokyo, JAPAN).

In vitro methylation, transfection, and luciferase assay

The BART promoter fragment was excised from pGL3-BART promoter using *Kpn*I and *Hind*III, and gel purified with the QIAquick gel extraction kit (QIAGEN, Valencia, CA). The BART promoter fragment was then incubated overnight with *Sss*I methylase (New England Biolabs). The methylation efficiency was monitored by *Hpa*II (New England Biolabs) digestion and gel electrophoresis. Each of the unmethylated and methylated BART promoter fragment was ligated into the pGL3-basic vector and transfected along with *Renilla* luciferase reporters (pRL-TK) into AGS cells using Lipofectamine 2000 transfection reagents (Invitrogen, Carlsbad, CA). AGS cells were used for this analysis as they are widely used for EBV research and easily transfected. In addition, AGS cells infected with recombinant EBV were shown to express BART miRNAs at high levels (Kim *et al.*, 2007). Forty-eight hours after transfection, luciferase activities were measured by Dual-Glo Luciferase assay system (Promega). Each transfection was performed in triplicate, and experiments were repeated three times. Luciferase activity was normalized for transfection efficiency by pRL-TK *Renilla* luciferase activity.

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Supplemental data

Supplemental data include a figure and two tables and can be found with this article online at http://e-emm.or.kr/article/article_files/ SP-43-7-04.pdf.

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