MAJOR ARTICLE

Plasma Viral MicroRNA Profiles Reveal Potential Biomarkers for Chronic Active Epstein–Barr Virus Infection

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Background. Chronic active Epstein–Barr virus (CAEBV) infection has high mortality and morbidity, and biomarkers for disease severity and prognosis are required. MicroRNAs (miRNAs) are small noncoding RNAs, and EBV encodes multiple miRNAs. Because plasma contains sufficiently stable miRNAs, circulating EBV–associated miRNA profiles were investigated as novel biomarkers in CAEBV infection.

Methods. Plasma miRNA expression was assessed for 12 miRNAs encoded within 2 EBV open reading frames (BART and BHRF). Expression levels were investigated in 19 patients with CAEBV infection, 14 patients with infectious mononucleosis, and 11 healthy controls. Relative expression levels of plasma miRNAs were determined by TaqMan probe-based quantitative assay.

Results. Plasma miR-BART1-5p, 2-5p, 5, and 22 levels in patients with CAEBV infection were significantly greater than those in patients with infectious mononucleosis and in controls. Plasma miR-BART2-5p, 4, 7, 13, 15, and 22 levels were significantly elevated in patients with CAEBV infection with systemic symptoms, compared with levels in patients with no systemic symptoms. The levels of miR-BART2-5p, 13, and 15 showed clinical cutoff values associated with specific clinical conditions, in contrast to plasma EBV loads.

Conclusions. Levels of specific plasma EBV miRNAs were elevated differentially in patients with CAEBV infection. Several EBV miRNAs, particularly miR-BART2-5p, 13, and 15, are potentially biomarkers of disease severity or prognosis.

Keywords. biomarker; chronic active Epstein–Barr virus infection; Epstein–Barr virus; microRNA.

Epstein–Barr virus (EBV) is the causative agent of infectious mononucleosis (IM) and is associated with several malignancies, including Burkitt lymphoma, Hodgkin disease, nasopharyngeal carcinoma, posttransplantation lymphoproliferative disorders, and CAEBV infection [1, 2]. CAEBV infection is characterized by chronic or recurrent IM-like symptoms, such as fever, swelling of lymph nodes, and hepatomegaly, in apparently immunocompetent individuals [3, 4]. Previous studies have indicated that the clonal expansion of EBV-infected T cells and natural killer (NK) cells plays a central role in the pathogenesis of CAEBV infection [5–8], although CAEBV infection in Western countries may not always be associated with the expansion of EBV-infected T or NK cells [9–11]. CAEBV infection is associated with high mortality and morbidity, with various life-threatening complications [11]. Hematopoietic stem cell transplantation (HSCT) has been used as a curative therapy [12–14]. The EBV DNA loads in blood samples have been occasionally used for diagnosis and the evaluation of response to treatment; other biomarkers for disease severity, progression, and prognosis have not yet been identified.

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MicroRNAs (miRNAs) are a family of small (length, 18–25 nucleotides), noncoding RNAs with complementarity to sequences in messenger RNAs. miRNAs function primarily as negative regulators of gene expression and have been implicated in the regulation of cellular differentiation, proliferation, and apoptosis [15, 16]. EBV encodes multiple miRNAs, with the majority of the miRNAs encoded within 2 primary transcripts [17–21], designated *Bam*HI fragment H rightward open reading frame 1 (BHRF1) and *Bam*HI-A region rightward transcript (BART). EBV-transformed cells express viral miRNAs that target viral and cellular genes [22]. BHRF1-derived miRNAs were reported to be highly expressed in EBV-positive lymphoblastoid cell lines (LCLs), whereas BART miRNAs have been found in all EBV-infected cell lines, such as LCL, Burkitt lymphoma, Hodgkin disease, and nasopharyngeal carcinoma [17, 20, 23]. Because miRNAs possess high stability and are easily quantified, these molecules could possibly serve as biomarkers for EBV-associated diseases [24]. Circulating miRNAs have been identified in the serum and plasma of patients with cancer, and the expression profiles of these circulating miRNAs have immense potential for use as novel minimally invasive biomarkers in diagnosing and monitoring human diseases [25, 26]. Gourzones et al reported that miR-BART7-3p levels in plasma samples from patients with nasopharyngeal carcinoma were higher than those seen in samples from control patients [27]. However, the potential of EBV miRNAs as biomarkers in EBV-associated diseases has not yet been fully explored.

MATERIALS AND METHODS

Patients

Nineteen patients (11 males and 8 females) with CAEBV infection, ranging in age from 1 to 65 years (median age, 14 years), were enrolled in this study. CAEBV infection was defined according to the following previously proposed criteria [9]: EBV-related symptoms for at least 6 months, an increased EBV load in either the affected tissue or peripheral blood, and a lack of evidence of previous immunological abnormalities or other recent infections that could explain the condition. On the basis of the infected cell type, 11 patients were identified as having T-cell–type CAEBV infection, while 8 patients were identified as having NK-cell–type CAEBV infection. Peripheral blood was collected at the time of diagnosis, follow-up, or referral to our hospital. For 7 of the 19 patients, blood samples were collected before, during, and after treatment. For 9 of the patients, samples were available only before treatment or with no treatment. The remaining 3 patients were receiving or had already received therapy (ie, steroid therapy or chemotherapy) at the time of blood sample collection. The patients with samples collected before treatment or without treatment were divided into 2 groups on the basis of clinically active or inactive disease. Clinically active disease was defined as the presence of severe symptoms such as high fever and/or an elevated hepatic transaminase level (9 patients). Inactive disease was defined as the absence of symptoms or the presence of exclusively dermal symptoms, including hydroa vacciniforme or hypersensitivity to mosquito bites (7 patients). On the other hand, the 5 patients who underwent HSCT were also divided into 3 groups, as follows: stable disease (0 patients), partial remission (0 patients), and complete remission (5 patients). Patients with partial remission had no symptoms but had substantial EBV DNA loads (ie, $> 10^{2.5}$ copies/μg of total DNA) in peripheral blood mononuclear cells (PBMCs) [9, 28]. Patients with complete remission had no symptoms and continuously low or no EBV loads in PBMCs (ie, $< 10^{2.5}$ copies/μg of DNA). The results were compared to those for 14 patients with diagnosed IM and for 11 healthy volunteers who were seropositive for EBV. IM was defined as fever, pharyngitis, cervical lymphadenopathy, and >10% atypical lymphocytes among peripheral white blood cells. Primary EBV infection was defined as positivity for immunoglobulin M to the antiviral capsid antigen and the presence of EBV DNA in blood samples at diagnosis.

Informed consent was obtained from all patients or their guardians. Three healthy volunteers who were seronegative for immunoglobulin G to the antiviral capsid antigen and in whom EBV DNA was not detected in blood samples also participated. The characteristics of participants are summarized in Tables 1 and 2. The Institutional Review Board of Nagoya University Hospital approved the use of the specimens that were examined in this study.

Cell Lines

The EBV-positive B-cell lines used in this study were LCL-1 and LCL-2. Both LCLs are derived from cells infected by the B95-8 EBV strain, which harbors a 12-kb deletion of a region that spans several BART cluster-1 miRNAs and all BART cluster-2 miRNAs [29, 30]; therefore, these miRNAs were not measured in the LCLs. The EBV-positive T-cell lines used were SNT-13 and SNT-16 [31]. The EBV-positive NK-cell lines used were SNK-6 [31] and KAI-3 [32]. The T-/NK-cell lines used were derived from patients with CAEBV infection or nasal NK-/T-cell lymphomas. Both LCLs were grown in Roswell Park Memorial Institute 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin, and streptomycin (complete medium). The medium for SNT-13, SNT-16, SNK-6, and KAI-3 was complete medium supplemented with 100 U/mL human interleukin 2 (IL-2). To assess the effect of IL-2 on the expression levels of EBV miRNAs, LCL-1 and LCL-2 were also cultured with the same medium as EBV-positive T-/NK-cell lines. Preliminary work (data not shown) demonstrated that the EBVs in the non-LCLs did not carry deletions of any of the miRNAs being measured in this study.

Table 1. Comparison of Characteristics Among 19 Patients With Chronic Active Epstein–Barr Virus (CAEBV) Infection, 14 With Infectious Mononucleosis (IM), and 11 Healthy EBV-Seropositive Controls

Characteristic	CAEBV Group	IM Group	Control Group	*P*[a] CAEBV vs IM Groups	*P*[a] CAEBV vs Control Groups	*P*[a] IM vs Control Groups
Sex						
Male	11	9	8	.710	.341	.496
Female	8	5	3			
Age, y						
Mean ± SD	17.4 ± 15.3	7.7 ± 6.9	30.9 ± 7.9	.007	.002	<.001
Median	14.0	6.0	32.0			
Plasma EBV load, copies (IU)/mL, mean ± SD	419 ± 27	1332 ± 20	. . .	.229		

[a] By the χ^2 test, Fisher exact test, or Mann–Whitney *U* test with Bonferroni correction.

RNA Extraction and Multiplexed Stem-Loop Reverse Transcription Polymerase Chain Reaction (RT-PCR)

For each sample, total RNA was extracted and then enriched for small RNAs (<200 bp), using a mirVana PARIS Kit (Ambion) according to the manufacturer's protocol. For the patients with CAEBV infection, patients with IM, and healthy donors, the specimen size corresponded to 200 μL of plasma or 1×10^6 cells. Detection of EBV miRNAs was performed using reagents and protocols for the TaqMan MicroRNA Reverse Transcription Kit and the TaqMan MicroRNA Assay (Applied Biosystems) [33]. RT-PCR reactions were conducted with custom stem-loop primers (Applied Biosystems) specific to the corresponding mature sequence obtained from miRBase (available at: http://www.miRBase.org [accessed 3 June 2013]) [34]. Amplification reactions then were performed by using the Mx3000P real-time PCR system (Stratagene). Data from quantitative RT-PCR were analyzed using the comparative threshold cycle (C_t) method, with hsa-miR-16 as the endogenous reference. The amount of miRNA is given by the arithmetic formula $2^{\Delta C_t}$. The C_t is the point at which the fluorescence of the TaqMan assay reaction exceeds the threshold limit. ΔC_t is the difference in the C_t values between the target miRNA and the control hsa-miR-16; ΔC_t is given as the C_t of hsa-miR-16 minus the C_t of each miRNA. Note that relative expression levels (ie, normalized to hsa-miR-16 levels in the same sample) are by definition values without units.

Table 2. Characteristics of Patients With Chronic Active Epstein–Barr Virus (CAEBV) Infection

Characteristic	CAEBV Group
Cell type infected	
NK cells	8/19
T cells	11/19
Disease status before/without treatment	
Active	9/16
Inactive	7/16
Treatment	
HSCT	5/19
Chemotherapy	5/19
No treatment	9/19
Disease status after HSCT	
Stable disease	0/5
Partial remission	0/5
Complete remission	5/5
Outcome	
Alive	15/19
Dead	4/19

Data are no. of patients with the characteristic/total no. evaluated.

Abbreviations: NK, natural killer; HSCT, hematopoietic stem cell transplantation.

Quantification of EBV DNA

Viral DNA was extracted (from either 200 μL of plasma or whole blood or from 1×10^6 PBMCs) using QIAamp DNA blood kits (Qiagen). A real-time quantitative PCR assay was performed as previously described [9, 35]. The lower limit of detection was 1 copy(IU)/reaction, which is equivalent to 10 copies(IU)/μg DNA for PBMCs and 50 copies(IU)/mL for plasma. Our system has been standardized using the first World Health Organization international standard for EBV, which consists of a whole-virus preparation of the EBV B95-8 strain (National Institute for Biological Standards and Controls).

Determination of EBV-Infected Cells

To determine which cells harbored EBV, PBMCs were fractionated into $CD3^+$, $CD19^+$, and $CD56^+$ cells by means of IMag (BD Biosciences). The purity of each PBMC subpopulation in our system is usually >92% by flow cytometry analysis. The fractionated cells were analyzed by real-time quantitative PCR.

EBV-infected cell fractions were defined as those having larger amounts of EBV DNA than the unfractionated PBMCs [9].

Statistical Analysis

Values are presented as means with standard errors. Statistical analysis was performed using SPSS 19.0 software (SPSS). The Mann–Whitney *U* test and Wilcoxon signed rank test were used for comparisons of 2 groups of patients. For comparisons of 3 groups, the Mann–Whitney *U* test with the Bonferroni correction was used. For samples without detectable signal (ie, negative samples), a default value, defined as the lower limit of detection for the miRNA assay (10^{-9}), was used for the statistical analysis. Pearson correlation coefficient analysis was used to assess the relationship between the DNA copy numbers in plasma and whole blood and the expression levels of miRNA. Differences with *P* values of < .05 were deemed to be statistically significant.

RESULTS

Levels of EBV miRNAs in Plasma From Patients With CAEBV Infection, Patients With IM, and Control Patients

Twelve miRNAs (miR-BHRF1-1, miR-BHRF1-2, miR-BHRF1-3, miR-BART1-5p, miR-BART2-5p, miR-BART4, miR-BART5, miR-BART7, miR-BART13, miR-BART15, miR-BART16, and miR-BART22) were quantified by real-time RT-PCR in all study groups (16 specimens from patients with CAEBV infection [9 from those with active disease and 7 from those with inactive disease], 14 from patients with IM, and 11 from healthy seropositive controls; Figure 1). The miR-16 microRNA precursor sequence maps to chromosome 13, and the levels of miRNA were not significantly altered in experimental plasma samples [36]. Preliminary experiments were performed using the plasma samples, and miR-16 was considered to be appropriate as an endogenous control (data not shown). The levels of plasma miR-BART1-5p, miR-BART2-5p, miR-BART5, and miR-BART22 in patients with CAEBV infection were significantly greater than those in patients with IM (*P* = .002, .004, <.001, and .004, respectively) and those in controls (*P*<.001, <.001, <.001, and <.001, respectively; all comparisons involved the Mann–Whitney *U* test with Bonferroni correction). In contrast, the levels of plasma miR-BHRF1-1 and miR-BHRF1-2 in patients with IM were significantly greater than those in patients with CAEBV infection (*P*<.001 and <.001, respectively) and those in health controls (*P*<.001 and <.001, respectively; all comparisons involved the Mann–Whitney *U* test with Bonferroni correction). None of the 12 miRNAs exhibited significant differences in expression levels in comparisons between the T-cell type (n = 9) and NK-cell type (n = 7) within the CAEBV infection group (data not shown). Moreover, the direct associations between the EBV DNA copy number (n = 39) in plasma and the expression level of each miRNA were analyzed. A

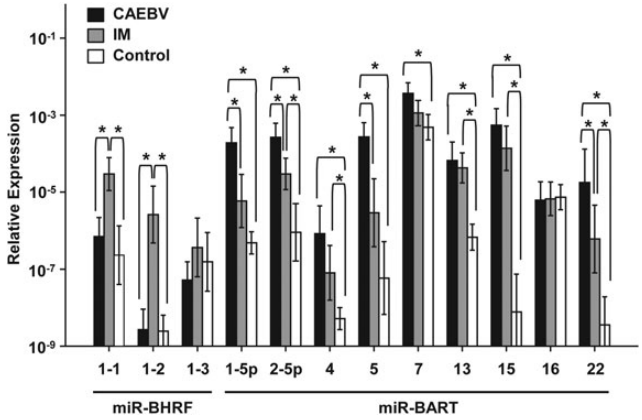

Figure 1. Levels of Epstein–Barr virus (EBV) microRNAs (miRNAs) in patient plasma samples. Levels of miRNAs are presented as the mean and standard error of the values following normalization to the level of hsa-miR-16. Black bars denote data for 16 patients with chronic active EBV (CAEBV) infection (plasma samples were collected before treatment or with no treatment), gray bars denote data for 14 patients with infectious mononucleosis (IM); and white bars denote data for 11 healthy seropositive controls. Statistical significance was determined using the Mann–Whitney *U* test with Bonferroni correction. **P* < .05.

significant correlation was detected between the plasma EBV DNA copy number and the level of each miRNA, except for that of miR-BHRF3 ($0.42 \le r \le 0.74$, $P < .05$). The correlation between the copy number of EBV DNA in whole blood (n = 18), which includes the cell compartment, and the expression level of each miRNA was also analyzed. A significant correlation was detected between EBV DNA copy numbers in whole blood and the levels of 9 of 12 miRNAs (miR-BHRF1-3, miR-BART1-5p, miR-BART2-5p, miR-BART4, miR-BART5, miR-BART7, miR-BART13, miR-BART15, and miR-BART22; $0.51 \le r \le 0.80$, $P < .05$).

Expression Levels of EBV miRNAs in EBV-Positive Cell Lines

The levels of EBV miRNAs in EBV-positive B-cell lines (LCL-1 and LCL-2), T-cell lines (SNT-13 and SNT-16), and NK-cell lines (SNK-6 and KAI-3) were measured. The expression patterns were very similar between T- and NK-cell lines (Figure 2). The levels of expression of miR-BHRF1-1, miR-BHRF1-2, and miR-BHRF1-3 appeared to differ between B-cell lines and T-/NK-cell lines (Figure 2). There were no significant differences in the expression levels of EBV miRNAs in terms of the presence of IL-2 in the culture medium, at least in the case of B-cell lines (Figure 2).

Comparison of the Profile of EBV miRNAs Between Plasma Levels in the Patients and Expression Levels of EBV-Positive Cell Lines

The profiles of EBV miRNAs were investigated to determine whether the patterns of plasma expression of miRNAs in patients with CAEBV infection (with EBV-infected T/NK

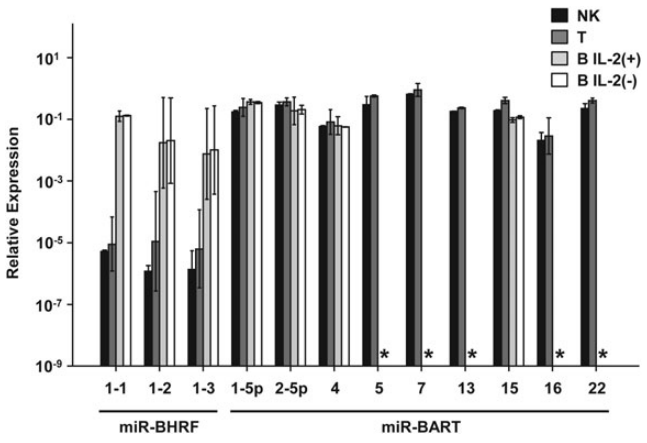

Figure 2. Levels of Epstein–Barr virus (EBV) microRNAs (miRNAs) in EBV-positive cell lines. Levels of miRNAs are presented as the mean and standard error of the values following normalization to the level of hsa-miR-16. Black bars denote data for 2 natural killer (NK)–cell lines (KAI-3 and SNK-6), dark gray bars denote data for 2 T-cell lines (SNT-13 and SNT-16), light gray bars denote data for 2 B-cell lines with interleukin 2 (IL-2; lymphoblastoid cell line 1 [LCL-1] and LCL-2), and white bars denote 2 B-cell lines without IL-2 (LCL-1 and LCL-2). There were no significant differences in the levels of each miRNA among the 4 groups. The asterisk denotes that the miRNA was deleted in the EBV genome of the LCLs.

lymphocytes) matched patterns of expression in EBV-positive T-/NK-cell lines. The patterns of the miRNA profiles appeared to be similar between plasma samples and cell lines (Figure 3*A*). With the lack of the data on 5 EBV miRNAs, the patterns of the miRNA profiles also seemed to be similar between plasma samples in patients with IM and the EBV-positive B-cell lines in terms of miR-BHRF1-1, miR-BHRF1-2, miR-BHRF1-3, miR-BART1-5p, miR-BART2-5p, and miR-BART4 (Figure 3*B*).

Association Between the Profile of EBV miRNAs in Plasma and Clinical Conditions in Patients With CAEBV Infection

Next, the plasma levels of EBV miRNAs were compared among patients with CAEBV infection with different clinical conditions. The levels of miR-BART2-5p, miR-BART4, miR-BART7, miR-BART13, miR-BART15, and miR-BART22 were each significantly higher in the 9 patients with CAEBV infection with active disease than in the 7 patients with inactive disease ($P = .013$, .013, .044, .001, .017, and .030, respectively, by the Mann–Whitney *U* test; Figure 4*A*). The levels of miR-BART1-5p, miR-BART2-5p, miR-BART5, miR-BART7, miR-BART13, miR-BART15, and miR-BART22 were each significantly higher in the 16 patients before treatment or with no treatment than in 11 patients during or after treatment ($P < .001$, .007, <.001, .002, .004, .002, and .002, respectively, by the Mann–Whitney *U* test; Figure 4*B*). In this study, the plasma samples of 5 patients who underwent HSCT and maintained complete remission were collected before and after treatment. Among patients with CAEBV infection with sustained complete remission after HSCT, levels of miR-BHRF1-2, miR-BART1-5p, miR-BART2-5p, miR-BART5, miR-BART7, miR-BART13, miR-BART15, and miR-BART22 after HSCT were significantly lower than levels before HSCT ($P = .043$, .043, .043, .043, .043, .043, .043, and .043, respectively, by the Wilcoxon signed rank test; Figure 4*C*).

Comparison of the Levels of EBV miRNAs to Levels of EBV DNA in Plasma of Patients With CAEBV Infection

The levels of EBV miRNAs and EBV DNA in plasma were compared to determine the usefulness of miRNA levels as biomarkers for disease severity. When patients with CAEBV infection were classified according to the presence of active disease (8 patients; the level of plasma EBV DNA was not measured in one of the patients in this group) or inactive disease (7 patients), plasma EBV DNA levels did not permit a clear discrimination between the 2 groups. In contrast, a clear distinction was observed between patients with active disease and those with inactive disease for plasma levels of miR-BART13, with miR-BART13 levels exceeding $10^{-4.3}$ in the former group (Figure 5*A*). A similar analysis was performed for patients with complete remission, defined as no symptoms and continuously low or no EBV loads in PBMCs after HSCT. As seen in the discussion above (for patients classified by symptomatology), plasma EBV loads did not discriminate among disease statuses. Plasma EBV loads were below the lower limit of detection in 4 samples: 1 sample from a patient with inactive disease before HSCT and 3 samples from patients with inactive disease before or without treatment. In contrast, a clear distinction was observed between patients with complete remission (after HSCT) and others (including before HSCT and before or without treatment) with respect to plasma levels of miR-BART2-5p and miR-BART15, with threshold levels of each of these miRNAs exceeding $10^{-5.0}$ (Figure 5*B* and 5*C*).

DISCUSSION

miRNAs have been found in many body fluids, and the use of such body fluids to assess miRNA levels is expected to be less invasive than typical solid tissue biopsies. Recently, the level of EBV-encoded miR-BART7 in plasma has been suggested to be useful for diagnostic screening [37]. EBV miRNAs are differentially expressed in lymphoid cells and under different virus latency programs. EBV-positive cells exhibit one of 3 latency types, distinguished from each other by the pattern of EBV antigens produced. In latency type I, only EBV-encoded nuclear antigen 1 (EBNA1) is produced, as in Burkitt lymphoma; latent membrane protein 1 (LMP1) and LMP2, as well as EBNA1, are produced in latency type II, as in Hodgkin disease, nasopharyngeal carcinoma, and CAEBV infection. In latency type III,

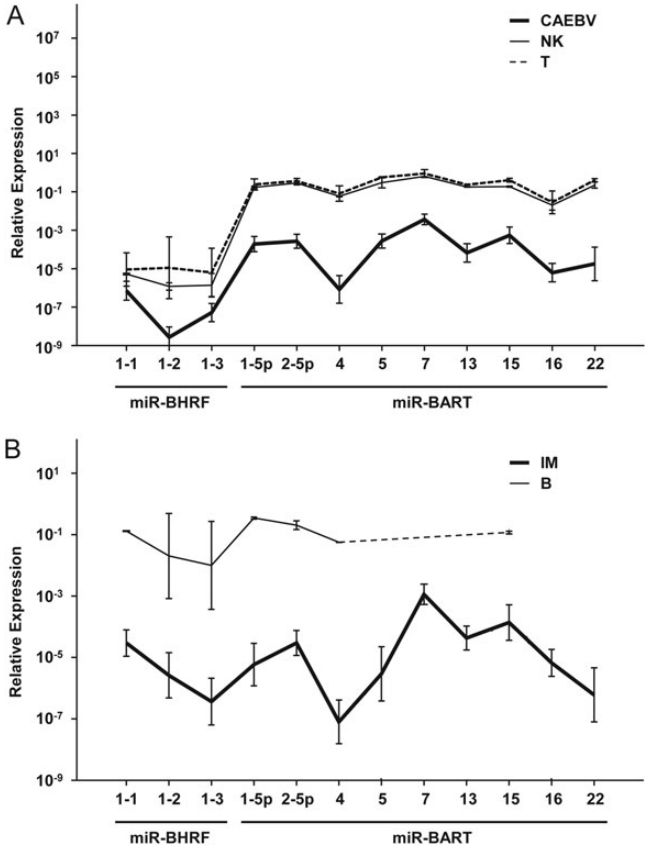

Figure 3. Comparison of the expression profiles of Epstein–Barr virus (EBV) microRNAs (miRNAs) between plasma and cell lines. Concentrations of plasma miRNAs and expression levels of miRNAs in EBV-positive cells are presented as means and standard errors. *A*, Chronic active EBV (CAEBV) infection versus EBV-positive natural killer (NK)–cell lines and T-cell lines. The bold line denotes 16 patients with CAEBV infection (plasma samples were collected before treatment or with no treatment), the thin line denotes 2 NK-cell lines (KAI-3 and SNK-6), and the dotted line denotes 2 T-cell lines (SNT-13 and SNT-16). *B*, Comparison of the EBV miRNA expression profiles in plasma samples from patients with infectious mononucleosis (IM) and B-cell lines (lymphoblastoid cell lines [LCLs]). The bold line denotes data for plasma specimens from 14 patients with IM, and the thin line denotes data for 2 LCLs.

highly immunogenic EBNA3 proteins are produced together with other EBV-latent antigens, as in IM [1]. The BHRF1 miRNAs are exclusively expressed at high levels in cells displaying type III EBV latency [38], and these miRNAs are not detected in infections with latency types I and II [17, 20, 39]. This may explain our results, in which plasma levels of miR-BHRF1-1 and miR-BHRF1-2 were upregulated in patients with IM (latency III), compared with those in patients with CAEBV infection (latency II) [40]. On the other hand, Pratt et al demonstrated that the levels of expression of BART miRNAs were greater in nasopharyngeal carcinoma and gastric carcinoma cell lines (both of which exhibit latency type II), compared with levels in other cell lines with latency I or III [41]. In this study, increases in the plasma levels of BART miRNAs in CAEBV infection with latency II were consistent with the previous experimental results that involved EBV-positive cell lines. The EBV miRNAs appear to come from EBV-infected cells. Our previous report revealed that the plasma viral load was more important to monitor than the PBMC viral load during the follow-up of patients after HSCT [12]. We speculated that the plasma viral load at diagnosis is an indicator of the amount of EBV-infected cells infiltrating organs, such as the liver and spleen. On the basis of this speculation, plasma samples may have an advantage for evaluating disease status in CAEBV infection for endogenous virus–associated miRNAs.

In the present study, the levels of several EBV miRNAs (particularly miR-BART7 and miR-BART16) were unexpectedly elevated in healthy EBV-seropositive controls. In contrast, very low levels of EBV miRNAs were expected in the corresponding plasma samples because the number of EBV-infected B cells was very small. Additionally, in 3 healthy volunteers who were seronegative for EBV and negative for EBV DNA, 6 of 12 miRNAs were detected; these 6 miRNAs included 5 that were detected at levels of $\leq 10^{-7}$ and 1, miR-BART7, that was detected at a level of $10^{-4.5}$ (data not shown). In this context, we note that Chen et al proposed that viral miRNAs act as mimics of or competitors with human cellular miRNAs in EBV-infected cells [42]. This hypothesis was supported by the correlation in expression between several high-abundance EBV BART miRNAs in normal tissue and their cellular seed-sharing orthologues in nasopharyngeal carcinoma tissue, such as miR-BART5-5p versus miR-18, miR-BART1-3p versus miR-29, and miR-BART9-3p versus miR-200 [42]. This redundancy may partly explain the apparent detection of several EBV miRNAs in control patients who were seronegative or seropositive for EBV. They also demonstrated important results in terms of mimicry of miRNAs. They found that EBV miRNAs exist with multiple lengths and nucleotide variants, by using a deep sequencing technique, and decided to use the total reading, including all lengths and nucleotide variants, to represent the abundance of individual EBV miRNAs in nasopharyngeal carcinoma tissue. They also showed that real-time PCR results are highly correlated with the total readings from deep sequencing. Moreover, approximately 0.1% of total miRNAs from non–nasopharyngeal carcinoma biopsy samples were mapped to known EBV miRNAs. They speculated that sequencing data are probably minimal because the sequence similarity among EBV miRNAs is much lower than the sequence similarity observed in human miRNAs from the same miRNA family [42].

miR-BART2-5p downregulates the viral DNA polymerase BALF5, thereby inhibiting the transition from latent to lytic viral replication and thus maintaining EBV latency so as to prevent host immune attack [43]. miR-BART2-5p also protects EBV-infected cells from recognition and killing by NK cells, targeting the major histocompatibility complex class I–related

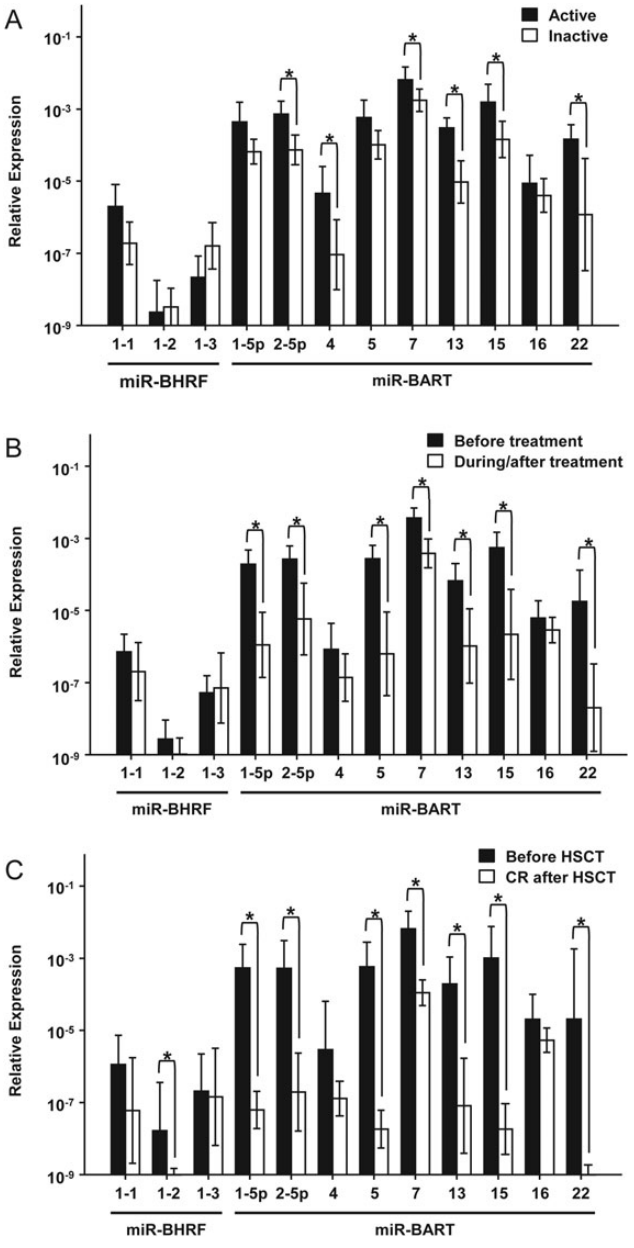

Figure 4. Comparison of expression profiles of Epstein–Barr virus (EBV) microRNAs (miRNAs) from patients with chronic active EBV (CAEBV) infection who had different clinical conditions. *A*, Comparison of 9 patients with active symptoms versus 7 with inactive symptoms before treatment. *B*, Comparison of 16 patient before treatment versus 11 patients during/after treatment. *C*, Patients achieving complete remission (CR). Comparison of 5 patients before treatment and after hematopoietic stem cell transplantation (HSCT), when complete remission was achieved. Statistical significance was determined using the Mann–Whitney *U* test and Wilcoxon signed rank test. **P* < .05.

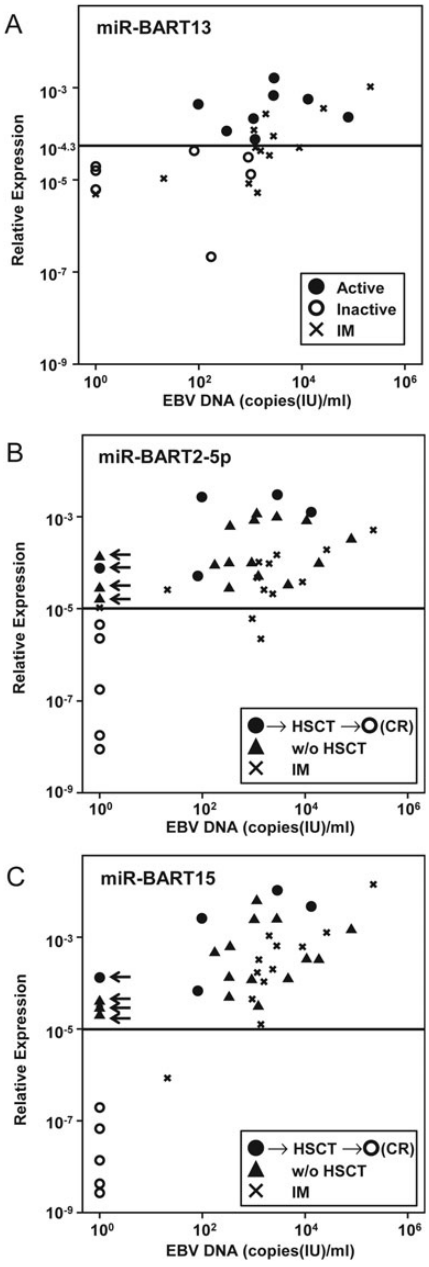

Figure 5. Comparison of the levels of Epstein–Barr virus (EBV) microRNAs (miRNAs) with EBV DNA in plasma of patients with chronic active EBV (CAEBV) infection. *A*, The level of EBV miR-BART13 in comparison with plasma EBV loads in patients with CAEBV infection, classified by the severity of clinical symptoms before treatment. Eight patients had active disease (closed circles), and 7 had inactive disease (open circles). *B* and *C*, The level of EBV miR-BART2–5p (*B*) and miR-BART15 (*C*) in comparison with plasma EBV loads in patients with CAEBV infection, classified by prognosis. For 5 patients, samples were obtained before hematopoietic stem cell transplantation (HSCT; closed circles) and after HSCT (open circles). The remaining 16 patients did not achieve complete remission (CR) without HSCT (triangles). The solid lines indicates the proposed threshold of significantly increased expression for each miRNA, and the arrows indicate the samples that showed more than the threshold level of the miRNA and were negative for EBV DNA.

chain B gene [44]. miR-BART5 has been reported to suppress the production of the p53-upregulated modulator of apoptosis [45]. Inhibition of this protein protects cells from apoptosis,

indicating that this EBV miRNA might be important in promoting tumor cell survival. miR-BART22 causes a reduction of the levels of LMP2, a highly immunogenic protein. Expression of this miRNA protects EBV-infected cells from the host immune response [46]. These miRNAs may contribute to persistent virus infection. In the present study, levels of these miRNAs were significantly higher in patients with CAEBV infection, compared with levels in patients with IM and those in controls. Interestingly, levels of these miRNAs also were significantly higher in patients experiencing the active, progressing state of the disease.

CAEBV infection is a devastating disease, and biomarkers are needed for confirming the diagnosis and evaluating clinical conditions. Viral load in the plasma of patients with CAEBV infection has been reported to increase as the patient's clinical status deteriorates [12, 47, 48]. In the present study, the average plasma levels of EBV DNA differed significantly between patients with CAEBV infection with active or inactive disease (data not shown). However, the data did not yield a threshold level (ie, cutoff) that permitted discrimination between the 2 groups. By contrast, using plasma levels of miR-BART13, patients with active and inactive disease were clearly distinguished. Similarly, plasma miR-BART2-5p and miR-BART15 levels are potentially biomarkers for achieving complete remission of the disease.

Notes

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