Profiling of EBV-Encoded microRNAs in EBV-Associated Hemophagocytic Lymphohistiocytosis

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Epstein-Barr virus-associated hemophagocytic lymphohistiocytosis (EBV-HLH) is a life-threatening complication of EBV infection. MicroRNAs (miRNAs) were small non-coding RNA, and EBV could encode miRNAs that are involved in the progression of infection. However, the profiles of EBV-miRNAs in EBV-HLH were unknown. Here, we aimed to profile the expression of EBV-miRNAs in children with EBV-HLH by analyzing 44 known EBV-miRNAs, encoded within the BamHI fragment H rightward open reading frame 1 (BHRF1) and the BamHI-A region rightward transcript (BART), in plasma and cellular targets by real-time quantitative PCR. The study included 15 children with EBV-HLH, 15 children with infectious mononucleosis (IM), and 15 healthy controls. CD8+ T cells were found to be the cellular target of EBV infection in EBV-HLH, while CD19+ B cells were infected with EBV in IM. We also found the greater levels of several miRNAs encoded by BART in EBV-HLH, compared to those in IM and healthy controls, whereas the levels of BHRF1 miRNAs were lower than those in IM. The profile and pattern of EBV-miRNAs in EBV-HLH indicated that EBV could display type II latency in EBV-HLH. Importantly, the level of plasma miR-BART16-1 continued decreasing during the whole chemotherapy, suggesting that plasma miR-BART16-1 could be a potential biomarker for monitoring EBV-HLH progression. The pathogenesis of EBV-HLH might be attributed to the abundance of EBV-miRNAs in EBV-HLH. These findings help elucidate the roles of EBV miRNAs in EBV-HLH, enabling the understanding of the basis of this disease and providing clues for its treatment.

Keywords: biomarker; children; Epstein-Barr virus; hemophagocytic lymphohistiocytosis; microRNA

Introduction

Epstein-Barr virus (EBV) is a successful gamma herpes virus that infects over 90% of adults and maintains persistent latent infection throughout the lifetime (Cohen 2000; Young and Rickinson 2004). Primary infection with EBV in B cells is usually asymptomatic or causes the self-limited disease infectious mononucleosis (IM) (Cohen 2000). In a limited number of individuals, EBV can target T cells and natural killer (NK) cells, thereby inducing EBV-associated T/NK-cell lymphoproliferative diseases such as chronic active EBV infection, severe mosquito bite allergy, hydroma vacciniforme, and EBV-associated hemophagocytic lymphohistiocytosis (EBV-HLH) (Cohen et al. 2009). EBV-HLH is a life-threatening disorder that progresses rapidly. It is characterized by prolonged fever, hepatosplenomegaly, cytopenias, liver dysfunction, and hyperferritinemia, and it mostly occurs in children (Imashuku 2002; Chandrakasan and Filipovich 2013). The syndrome results from abnormal T-cell activation and inflammatory cytokine production (Jordan et al. 2004). Notably, EBV-HLH has the poorest prognosis of all viruses associated with HLH (Rouphael et al. 2007). The role of EBV infection in the initiation or progression of EBV-HLH remains unclear. In China, most patients receive chemotherapy treatment as per the HLH-2004 protocol regimen once they are diagnosed with EBV-HLH. Not all patients undergoing this therapy show satisfactory outcomes. The most appropriate screening and treatment methods for EBV-HLH have not yet been found.

MicroRNAs (miRNAs) are a subset of 18-25 nucleotides. These non-coding RNAs negatively regulate gene expression by targeting complementary sequences in messenger RNAs (Bartel 2004). They have been demonstrated to play key roles in growth and development, cell cycle regulation, and immunity, and their function have been linked to certain human diseases (Ambros 2004). EBV was the first human virus identified to express miRNAs (Pfeffer et al. 2004). To date, 44 EBV-encoded miRNAs have been
found, among which 4 miRNAs derived from the BamHI fragment H rightward open reading frame 1 (BHRF1), and the remaining 40 are encoded by the BamHI-A region rightward transcript (BART) (Barth et al. 2011). And, EBV displays three latency types (type I, II, and III) in EBV-positive cells undergoing latent infection. Viral genes were differentially expressed in different latency types, such as EBV-encoded nuclear antigen 1 (EBNA1), EBNA2, EBNA3, latent membrane protein 1 (LMP1), LMP2, and some non-coding RNAs, including miRNAs (Cai et al. 2006; Forte and Luftig 2011). MiRNAs encoded by BART are observed in all latent infection types and expressed at high levels in type II EBV latency, while BHRF1 miRNAs are expressed at high levels in cells displaying type III EBV latency (Cai et al. 2006; Pratt et al. 2009).

In EBV-positive Burkitt lymphoma, the level of miR-BART-6-3p was high, and it affected the regulation of cell growth and immune response (Imig et al. 2011). The levels of miR-BART17-5p, -7, -16, -14*, and -22 were high in nasopharyngeal carcinoma and miR-BART7 might be a potential biomarker for monitoring this cancer type (Cosmopoulos et al. 2009; Zhang et al. 2015). However, EBV-miRNAs in EBV-HLH have never been profiled. Such a study is expected to drive the progress of diagnosis and therapy for this disease. The aims of this study were to obtain EBV-miRNA profiles from EBV-HLH patients and compare their profiles with control individuals, to identify potential biomarkers for diagnosis and disease evaluation, and to analyze the potential role of the increased viral miRNAs in EBV-HLH.

Materials and Methods

Patients

Fifteen patients diagnosed with EBV-HLH were enrolled in this study at Beijing Children’s Hospital between September 2012 and September 2014. All patients with EBV-HLH met the HLH-2004 diagnostic criteria (Henter et al. 2007), as well as diagnostic criteria for EBV-HLH (Imashuku 2002). The infection status (primary infection and EBV reactivation) was defined by serological criteria for EBV-HLH (Imashuku 2002). All patients diagnosed with infectious mononucleosis (IM) and 15 healthy children previously infected with EBV were used as control groups (Table 1). All the children diagnosed with IM met criteria for IM (Luzuriaga and Sullivan 2010). The study design was in accordance with the Helsinki Declaration and was approved by the Beijing Children’s Hospital Ethics Committee before the study was initiated. Informed consent was obtained from the parents or legal guardians of the participants.

Cell lines

EBV-positive cell lines with different latency types were used: EBV-positive Burkitt lymphoma cell line Mutu I (latency I), nasopharyngeal carcinoma cell line CNE-2 (latency II), and lymphoblastic cell line LCL (latency III) (Cell bank, Chinese Academy of Sciences, China). EBV-negative Burkitt lymphoma cell line Ramos (ATCC, Manassas, VA, USA) was used as a negative control. Mutu I, LCL, and Ramos were grown in Roswell Park Memorial Institute (RPMI) 1640 medium (HyClone, Logan, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Life Technologies, Grand Island, NY, USA), penicillin, and streptomycin. CNE-2 was cultured in Dulbecco’s Modified Eagle Medium (HyClone) supplemented with 10% heat-inactivated fetal bovine serum (Gibco), penicillin, and streptomycin.

Blood sampling

Peripheral blood samples with EDTA were collected from all 45 subjects. Peripheral blood mononuclear cells were separated from 4 mL fresh whole blood by density gradient centrifugation using the Lymphocyte Separation Medium (Sigma, St. Louis, MO, USA) and then resuspended in RPMI 1640 medium. Cell suspensions were mixed with the respective magnetic bead-conjugated antibodies against CD4*, CD8*, CD19*, and CD56* (BD, Franklin Lakes, NJ, USA) and incubated for 30 min at room temperature. Tagged cells were separated using BD IMagent (BD) according to the manufacturer’s instructions. The quality and amount of cells were evaluated by flow cytometry analysis (BD). The purity of lymphocyte subpopulations was always ≥ 94%, in line with our study requirements.

EBV-DNA quantification

Total DNA was extracted from 200-μL plasma or 1 × 10⁴ cells (as a standard) using either QIAmp DNA Micro Kit (Qiagen, Germany) or QIAamp Viral RNA Mini Kit (Qiagen). The viral load was detected by real-time quantitative PCR (qPCR) using a commercial EBV DNA Quantitative kit (Daan Genes, China). A viral load of ≥ 5 × 10⁶ (10²⁷³⁸) was considered positive. Concentrations of EBV DNA were calculated using standard curves generated with known copy numbers of EBV DNA from cell lines.

Table 1. Characteristics of EBV-HLH patients, IM patients, and healthy controls.

<table>
<thead>
<tr>
<th></th>
<th>EBV-HLH</th>
<th>IM</th>
<th>Healthy Control</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (Male/Female)</td>
<td>7/8</td>
<td>10/5</td>
<td>8/7</td>
<td>0.50a</td>
</tr>
<tr>
<td>Age (Mean ± SD, years)</td>
<td>4.8 ± 3.2</td>
<td>4.9 ± 1.7</td>
<td>5.7 ± 1.9</td>
<td>0.29a</td>
</tr>
<tr>
<td>EBV Cellular Target</td>
<td>CD8⁺</td>
<td>CD19⁺</td>
<td>CD19⁺</td>
<td></td>
</tr>
<tr>
<td>Cellular EBV-DNA copies/µg DNA (Mean, Range)</td>
<td>10⁴⁻⁷¹, 10⁴⁻⁷⁰, 10⁴⁻³⁴</td>
<td>10³⁻⁸⁰, 10²⁻⁷⁸, 10⁵⁻⁴³</td>
<td>&lt; 10⁻⁷⁰</td>
<td></td>
</tr>
<tr>
<td>Plasma EBV DNA copies/ml (Mean, Range)</td>
<td>10⁻⁷⁻⁷³, &lt; 10⁻⁷⁻⁷⁰, 10⁻⁴⁻³⁴</td>
<td>10⁻⁶⁻⁷⁰, &lt; 10⁻⁷⁻⁶⁻⁴⁻¹⁰</td>
<td>&lt; 10⁻⁷⁰</td>
<td></td>
</tr>
</tbody>
</table>

*For healthy controls, CD19⁺ cells were the main EBV-infected cells.
DNA in cell samples were expressed as copies per microgram (μg) of DNA. EBV-infected cells were determined by comparing specific cell-type viral load. The cellular target was the lymphocyte subpopulation that showed the highest viral load.

**RNA extraction, reverse transcription, and relative quantification of viral miRNAs**

For different samples, total RNA was extracted by using either a miRNeasy mini kit (Qiagen) for cell samples or a miRNeasy serum/plasma kit for plasma samples (Qiagen) according to the manufacturer’s protocol. Reverse transcription was performed using a miScript II RT Kit (Qiagen). A miScript SYBR Green PCR kit (Qiagen) was used for the relative quantitation of EBV-miRNA according to the manufacturer’s protocol. qPCR was conducted with miScript primers (Qiagen) (Table 3) specific to the corresponding mature sequence obtained from miRBase (www.mirbase.org), using an Applied Biosystems 7500 real-time PCR system. For cell samples, cell-derived U6 snRNA (Qiagen) was used as an endogenous reference to normalize the relative expression of EBV-miRNAs. In all plasma samples, the same amount of cel-miR-39 (Qiagen) was “spiked-in” at the early stage of RNA extraction as a mimic reference. Data from qPCR were analyzed using the comparative threshold cycle (Ct) method. The method of 2\(^{-ΔCt}\) is used for comparing the relative expression levels of EBV-miRNAs, which is the D-value between the endogenous and the target miRNA.

**Statistical analysis**

SPSS 17.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. According to the relative expression of EBV-miRNAs in the EBV-negative cell line Ramos, \(2^{-12}\) was defined as the cut off value. The Mann-Whitney U-test with Wilcoxon’s signed-rank test was used for comparing EBV-miRNAs between different groups. Pearson correlation coefficient analysis was used to investigate the relationship between plasma viral load and the expression levels of EBV-miRNAs. Differences with \(P\) values of < .01 were considered statistically significant in this study.

**Results**

**Cellular targets of EBV infection**

To identify the cellular targets of EBV in lymphocyte subpopulations, CD4\(^+\) and CD8\(^+\) T cells, CD19\(^+\) B cells, and CD56\(^+\) NK cells from EBV-HLH, IM, and healthy controls were sorted by antibody-coated immunomagnetic beads; EBV load was then detected in each lymphocyte subpopulation. In patients with EBV-HLH, the EBV load was higher in CD8\(^+\) T cells than that in other lymphocyte sub-

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**Table 2. Clinical characteristics of patients with EBV-HLH.**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>EBV-HLH</th>
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<tbody>
<tr>
<td>Infection Status</td>
<td></td>
</tr>
<tr>
<td>Primary Infection</td>
<td>4/15</td>
</tr>
<tr>
<td>Reactivation</td>
<td>11/15</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>3/8</td>
</tr>
<tr>
<td>Outcome (Fatal Case)</td>
<td></td>
</tr>
<tr>
<td>Steroid pulse</td>
<td>3/4</td>
</tr>
<tr>
<td>Plasma exchange</td>
<td>1/1</td>
</tr>
<tr>
<td>Give up treatment</td>
<td>2/2</td>
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<tr>
<td>Overall Case-fatality Rate</td>
<td>9/15 (60%)</td>
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</table>

**Table 3. List of miScript primers (Cat. No.).**

<table>
<thead>
<tr>
<th>EBV-miRNA</th>
<th>Cat. No.</th>
<th>EBV-miRNA</th>
<th>Cat. No.</th>
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<td>EBV-miR-BART22</td>
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populations, indicating that CD8+ T cells were the main cellular target of EBV infection in EBV-HLH (Table 1). In contrast, the EBV viral load was only detectable in CD19+ B cells of patients with IM, suggesting CD19+ B cells were the predominant target of EBV infection in IM (Table 1). The EBV load in plasma of patients with HLH was higher compared to patients with IM (Table 1). The EBV load in lymphocyte subpopulations and the plasma of healthy carriers was below the limit of detection (< 10^2.70 copies/ml) (Table 1).

**EBV-miRNA expression profiling and pattern in CD8+ T cells from EBV-HLH patients**

Forty-four known EBV-encoded miRNAs were observed in CD8+ T cells from EBV-HLH patients (n = 15). As shown in Fig. 1A, 38 EBV-miRNAs from the BART region were expressed. MiR-BART-10*-1 and 20-3p were undetectable. In the BHRF1 cluster, miR-BHRF1-1, 1-2-3p, and 1-3 were observed. But these miRNAs were expressed at low levels. The level of miR-BART13-1 was the highest among all the viral miRNAs measured. The expression patterns of EBV-miRNAs in Mutu I (latency I), CNE-2 (latency II), and LCL (latency III) were also observed (Fig. 1B). In EBV-HLH, BART-miRNAs were expressed at high levels, while BHRF1-miRNAs were expressed at low levels in CD8+ T cells. The pattern of EBV-miRNAs in EBV-HLH was similar to the expression pattern in the CNE-2 cell line (Fig. 1C).

Fig. 1. The expression patterns and profiles of EBV-miRNAs in EBV-HLH and EBV-positive cell lines with different latency types.

The expression levels of EBV-miRNAs in EBV-infected cells of patients with EBV-HLH (Before treatment) are shown as the mean and standard error of values that are normalized to the expression level of U6. The expression levels of EBV-miRNAs in different EBV latency type cell lines are presented as the values that are normalized to the expression level of U6. (A) The expression profile of EBV-miRNAs in CD8+ T cells of 15 patients with EBV-HLH. CD8+ T cells were the EBV-infected cells in EBV-HLH. (B) The expression patterns of EBV-miRNAs differ across different EBV latency type cell lines, including latency I EBV-positive Burkitt lymphoma cell line Mutu I, latency II nasopharyngeal carcinoma cell line CNE-2, and latency III lymphoblastoid cell line LCL. (C) Comparison of the expression patterns of EBV-miRNAs between CD8+ T cells of 15 patients with EBV-HLH and latency II nasopharyngeal carcinoma cell line CNE-2.
Association of EBV-miRNA expression profiles with cellular targets and plasma in patients with EBV-HLH or IM

To explore the association of EBV-miRNA expression profiles between cellular targets and plasma, cellular EBV-miRNA profiles in patients with EBV-HLH or IM were compared with plasma profiles. The EBV-miRNA expression profiles in plasma were similar to those of CD8^+ T cells in EBV-HLH (Fig. 2A). Plasma EBV-miRNA expression profiles resembled those of CD19^+ B cells in IM (Fig. 2B).

Comparison of plasma EBV-miRNA profiling between patients with EBV-HLH or IM and healthy carriers

In this study, the profiles of plasma EBV-miRNA were consistent with those of cellular targets both in EBV-HLH and IM. Comparing the differences of EBV-miRNAs in different cell types is difficult. Therefore, to explore the differences of EBV-miRNAs among EBV-HLH patients, IM patients, and healthy carriers, 44 EBV-miRNAs were quantified in plasma from patients with EBV-HLH (n = 15), patients with IM (n = 15), and healthy carriers (n = 15) by qPCR. The expression levels of EBV-miRNAs from BART Cluster 1 (3-3p, 3-5p, 1-3p, 1-5p, 5-3p, 16-1, 17-3p, 17-5p, 6-3p, and 6-5p) in patients with EBV-HLH were significantly higher than in patients with IM (P < .001, = .004, < .001, < .001, < .001, < .001, < .001, < .001, < .001, < .001, respectively) (Fig. 3A). Among miRNAs derived from BART Cluster 2, miR-BART21-3p, 18-3p, 8-3p, 8-5p, 9-5p, 22, 10-1, 11-3p, 11-5p, 12-1, 19-3p, 19-5p, 20-5p, 13-1, 13*-1, and 14-1 were also expressed at significantly higher levels than those in IM patients (P < .001, = .003, = .004, < .001, = .002, < .001, < .001, < .001, < .001, < .001, < .001, < .001, respectively) (Fig. 3B). The expression levels of miR-BART2-5p were significantly higher in EBV-HLH patients compared to IM patients (P < .001) (Fig. 3A). MiR-BART2-3p was almost undetectable in all three groups. All miRNAs derived from BART in patients with

Fig. 2. Comparison of profiles and patterns of EBV-miRNAs between cellular targets and plasma.

The expression levels of EBV-miRNA in cellular targets are shown as the mean and standard error of values that are normalized to the expression level of U6. The expression levels of plasma EBV-miRNA are presented as the mean and standard error of values that were normalized to the expression level of cel-miR-39. (A) Comparison of EBV-miRNA expression patterns and profiles between CD8^+ T cell and plasma in 15 patients with EBV-HLH. CD8^+ T cells were the cellular target of EBV infection in EBV-HLH. (B) Comparison of EBV-miRNA expression patterns and profiles between CD19^+ B cells and plasma in 15 patients with IM. CD19^+ B cells were infected with EBV in IM.
EBV-HLH were expressed at significantly higher levels compared with healthy carriers \((P < .001)\). In contrast, the expression levels of plasma EBV-miRNAs were significantly higher than those in patients with EBV-HLH \((P = .001, < .001\), respectively\) (Fig. 3A). Plasma miR-BHRF1-1, 1-2-3p, 1-2-5p, and 1-3 in IM were expressed at significantly higher levels than those in healthy controls \((P < .001)\) (Fig. 3A). All comparisons were performed using the Mann-Whitney U-test with the Bonferroni correction.

**Plasma EBV-miRNA expression under different clinical conditions of EBV-HLH**

EBV-HLH can occur in the setting of primary infection or be the result of EBV reactivation. To identify the difference of EBV-miRNA expression in the two infection statuses, EBV-miRNAs were investigated in patients with primary infection \((n = 4)\) and those with EBV reactivation \((n = 11)\). There were no significant differences in the expression levels of each plasma EBV-miRNA between patients with primary infection and EBV reactivation \((P > .05)\) (Fig. 4A). There were no significant differences in plasma viral load between patients with primary infection \((n = 4)\) and those with EBV reactivation (median EBV load in primary infection: \(10^{3.18}\) copies/ml, range \(10^{3.46}-10^{4.79}\) copies/ml; median EBV load in EBV reactivation: \(10^{3.61}\) copies/ml plasma, range \(<10^{2.70}-10^{5.34}\) copies/ml) \((P = .443)\) (Fig. 4).

In this study, eight patients received etoposide-containing chemotherapy. Five patients achieved clinical remissions, the other three got poor outcomes. The expression levels of plasma EBV-miRNAs were observed in patients with EBV-HLH before treatment (Active Stage), during chemotherapy (After 4 weeks Induction Therapy), and after treatment (Clinical Remission) \((n = 5)\). The expression of plasma miR-BART3-3p, 3-5p, 15-1, 5-3p, 5-5p, 16-1, 6-5p, 21-3p, 18-3p, 7-5p, 8-3p, 11-3p, 12-1, 13-1, and 2-5p were significantly lower during the clinical remission status compared with the active stage \((P = .008\) for all) of the five patients (Fig. 4B). The levels of plasma miR-BART16-1 and 12-1 were significantly lower during chemotherapy compared with the clinical remission \((P = .008\) for both) of the five patients (Fig. 4B). However, the expression level of plasma miR-BART12-1 was not significantly lower during chemotherapy compared with the active stage \((P > .05)\).
Notably, the continuing decrease in expression level of plasma miR-BART16-1 was significant during the whole chemotherapy. The viral load of EBV-HLH patients in clinical remission were lower as compared to those at active stage and during chemotherapy (Median EBV load at active stage: $10^{2.67}$ copies/ml, range: $10^{2.70}$-$10^{4.62}$ copies/ml, one patient $<10^{2.70}$ copies/ml; Median EBV load during chemotherapy: $10^{3.2}$ copies/ml, range: $10^{2.70}$-$10^{4.23}$ copies/ml; EBV load in clinical remission: one patient $10^{2.83}$ copies/ml, the other four $<10^{2.70}$ copies/ml). There was no significant correlation detected between plasma viral load and the expression level of plasma miR-BART16-1 ($r = 0.445; P = 0.097$).

**Discussion**

To our knowledge, this is the first study to report the profile and pattern of EBV-encoded miRNAs in children with EBV-HLH. The EBV-miRNA pattern in EBV-HLH was similar to that in the CNE-2 cell line (latency II), indicating that EBV could display type II latency in EBV-HLH. EBV expressed EBNA1, LMP1, and LMP2 in latency II. Ito et al. (2013) have found EBNA1, LMP1, LMP2 and lytic genes BRLF were expressed in CD8$^+$ T cells of one patient with EBV-HLH. The result demonstrated type II latency.
latency in CD8\(^+\) T cells of EBV-HLH. Except for latent infection, lytic infection is another infection type of EBV infection, in which BART miRNAs and BHRF1 miRNAs are both expressed, as well as lytic genes BRLF1 and BZLF1 (Young and Rickinson 2004; Cai et al. 2006). Moreover, BHRF1 miRNAs were also detected in our study. The expression of BHRF1 miRNAs might be associated with EBV lytic replication (Forte and Luftig 2011). EBV lytic genes, such as BZLF1 mRNA, could be tested in further study to explore whether EBV displayed lytic infection in EBV-HLH. It is well known that EBV also displays latency II in EBV-associated T-cell lymphomas (Cohen 2000; Forte and Luftig 2011). EBV-HLH is likely to relapse or progress to T-cell lymphoma over months to years (Su et al. 1993; Yao et al. 1994; Chen et al. 1998). The same latency type may be related to the mechanism of disease progression from EBV-HLH to T-cell lymphoma. This might contribute to the malignant outcome of EBV-HLH. The profiling and pattern of plasma EBV-miRNA resemble those in EBV-infected cells, indicating that circulating viral miRNAs are produced by EBV-infected cells. In patients with nasopharyngeal carcinoma, plasma viral miRNAs have been demonstrated to be secreted from EBV-infected carcinoma cells (Gourz zones et al. 2010). It was presumed that circulating viral miRNAs may serve as signaling molecules between EBV-infected cells and non-EBV-infected cells in EBV-associated diseases, potentially regulating cell function.

In the current study, several plasma miR-BARTs were expressed at higher levels in children with EBV-HLH than those of IM and healthy children. The elevated EBV-miRNAs may be involved in the mechanism of EBV-HLH. Many patients with EBV-HLH manifested with a prolonged atypical IM-like course at the early stage of this disease. However, the prognosis of EBV-HLH was much poorer than that of IM. IM is a self-limiting begin disease. The different outcomes might be related with the different pathogenesis of the two diseases. EBV usually infects B cells, and EBV-specific T cells control the infected B cells in IM patients and healthy carriers (Cohen 2000). For patients with EBV-HLH, EBV mainly infects CD8\(^+\) T cells, leading to a lack of EBV-specific cytotoxic T cells and monoclonal proliferation of EBV-infected CD8\(^+\) T cells (Kasahara et al. 2001). EBV infection of T cells can activate T lymphocytes to secrete cytokines, such as tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) and interferon-\(\gamma\) (IFN-\(\gamma\)) (Lay et al. 1997; Jordan et al. 2004). Apart from TNF-\(\alpha\) and IFN-\(\gamma\), other proinflammatory cytokines, including interleukin (IL)-3, IL-6, and IL-10 have also been proved to be upregulated in HLH patients (Sumegi et al. 2011). The secretion of these cytokines may also be associated with EBV infection of CD8\(^+\) T cells. The level of IL-6 could be upregulated by miR-BART3-3p through targeting importin-7 (IPO7). Knockdown of IPO7 would block the production of IL-6 (Dolken et al. 2010). And, miR-BART3-3p was also expressed greatly in EBV-HLH. Therefore, we speculate that EBV may modulate cytokine storm by generating EBV-miRNAs in EBV-HLH.

Moreover, miR-BART22 was observed to be expressed at higher levels in EBV-HLH than those in IM or healthy children, and the miRNA could reduce LMP2A production (Lung et al. 2009). Because LMP2A is easily recognized by host immune, miR-BART22 might reduce the level of LMP2A to help EBV-infected cells escape host immune response, contributing to persistent EBV infection of CD8\(^+\) T cells (Khanna et al. 1998). MiR-BART16-1 and 17-5p were also found to be overexpressed, thereby reducing LMP1 protein production (Lo et al. 2007). High levels of LMP1 expression will inhibit the proliferation and promote the apoptosis of EBV-infected cells (Liu et al. 2002). Reducing the expression of LMP1 at appropriate levels will promote the proliferation and suppress the apoptosis of EBV-infected cells. EBV-negative T cells lack viral miRNAs to prevent cells apoptosis. Thus, it seems reasonable that EBV-infected T cells have the advantage to survive and proliferate in the cytokine storm of EBV-HLH, unlike non-EBV-infected T cells.

Several EBV BART miRNAs share seed sequences with cellular miRNAs (Chen et al. 2010). Chen et al. (2010) speculated that viral miRNAs act as mimics or competitors of cellular miRNAs in EBV-infected cells to regulate host immunity. MiR-18/miR-BART5-5p and miR-29/miR-BART1-3p were identified to share seed sequences in EBV-infected T cells. miR-BART8-3p was highly expressed in our study, and it has been demonstrated to share seed sequences with miR-513b (Chen et al. 2010). High mobility group-box 3 (HMGB3) is a potential target for miR-513b, which is an oncogene for leukemia and can promote cell proliferation (Chen et al. 2014). It was speculated that miR-BART8-3p may act as a competitor to suppress the expression of miR-513b. When miR-513b is down-regulated, HMGB3 will be overexpressed. The elevated HMGB3 could promote EBV-infected CD8\(^+\) T cell proliferation, contributing to abnormal EBV-infected CD8\(^+\) T cell activation (Chen et al. 2014). We propose that EBV-miRNA might influence immune functions of EBV-infected CD8\(^+\) T cells by sharing seed sequences with cellular miRNAs.

Previous studies have reported that circulating viral miRNAs might be potential markers for early detection, diagnosis, progression, and prognosis of diseases, as well as for response to treatment (Moussay et al. 2011; Fang et al. 2012). Plasma miR-BART2-5p, 13, and 15 were shown to be potential biomarkers of disease severity or prognosis for chronic active EBV infection (CAEBV) (Kawano et al. 2013). Like CAEBV, EBV-HLH is also a serious disease caused by EBV infection. Chemotherapy with etoposide is one of the most effective treatments for EBV-HLH, but it does not always show satisfactory outcomes (Kogawa et al. 2014). It is essential to identify potential biomarkers for screening disease progression during course of chemotherapy in patients with EBV-HLH. EBV load has been used as a marker of disease activity in EBV-HLH, but it is not a
specific marker. In our study, for one EBV-HLH patient at active stage, plasma EBV load was below the limit of detection but with high level of plasma miR-BART16-1. Another patient achieved clinical remission with positive plasma viral load. Notably, his plasma miR-BART16-1 level was observed to continue decreasing during the whole chemotherapy. Therefore, plasma miR-BART16-1 could be a suitable biomarker for monitoring disease progression during chemotherapy.

In conclusion, for the first time, we profiled EBV-encoded miRNAs from EBV-HLH patients. The profiling of EBV miRNA in CD8+ T cells suggests that EBV could display type II latency in EBV-HLH. The elevated EBV-miRNAs in EBV-HLH might help clarify the pathogenesis of EBV-HLH. Biomarkers for etoposide containing chemotherapy and for monitoring disease during remission have not been identified thus far. However, our finding that plasma miR-BART16-1 level was observed to continue decreasing during the whole chemotherapy suggests plasma miR-BART16-1 as a suitable biomarker for monitoring disease progression during chemotherapy.

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Conflict of Interest

The authors declare no conflict of interest.

References


