MAJOR ARTICLE

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

Yoshihiko Kawano,[1] Seiko Iwata,[2] Jun-ichi Kawada,[1] Kensei Gotoh,[3] Michio Suzuki,[1] Yuka Torii,[1] Seiji Kojima,[1] Hiroshi Kimura,[2] and Yoshinori Ito[1]

[1]Department of Pediatrics and; [2]Department of Virology, Nagoya University Graduate School of Medicine, Nagoya; and [3]Department of Pediatrics, Konan Kosei Hospital, Konan, Japan

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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Correspondence: Yoshinori Ito, MD, PhD, Department of Pediatrics, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan (yoshi-i@med.nagoya-u.ac.jp).

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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

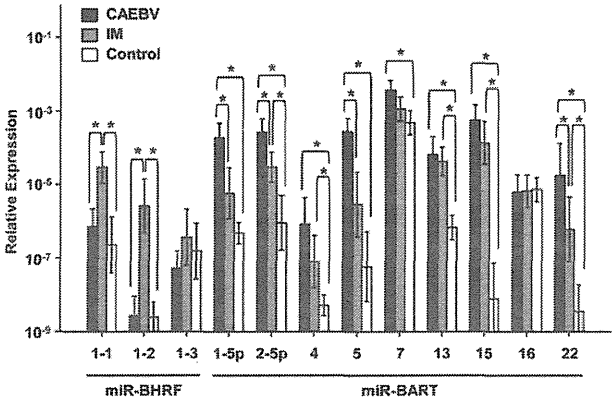

Figure 1. Levels of Epstein–Barr virus (EBV) microRNAs (miRNAs) in patient plasma samples. Levels of miRNAs are presented as the mean and standard error of the values following normalization to the level of hsa-miR-16. Black bars denote data for 16 patients with chronic active EBV (CAEBV) infection (plasma 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 ($0.42 \leq r \leq 0.74$, $P < .05$). 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; $0.51 \leq r \leq 0.80$, $P < .05$).

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

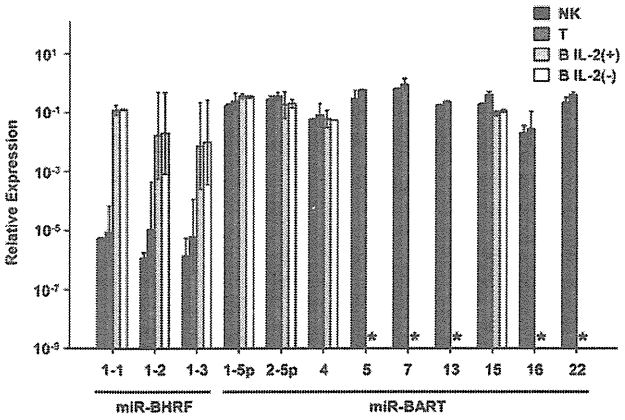

Figure 2. Levels of Epstein–Barr virus (EBV) microRNAs (miRNAs) in EBV-positive cell lines. Levels of miRNAs are presented as the mean and standard error of the values following normalization to the level of hsa-miR-16. Black bars denote data for 2 natural killer (NK)–cell lines (KAI-3 and SNK-6), dark 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,

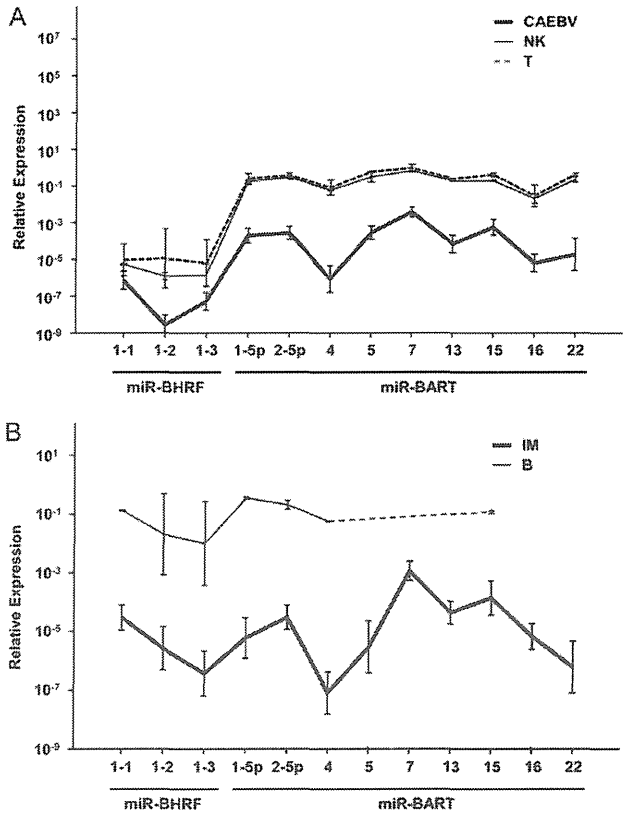

Figure 3. Comparison of the expression profiles of Epstein–Barr virus (EBV) microRNAs (miRNAs) between plasma and cell lines. Concentrations of plasma miRNAs and expression levels of miRNAs in EBV-positive cells are presented as means and standard errors. *A*, Chronic active EBV (CAEBV) infection versus 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

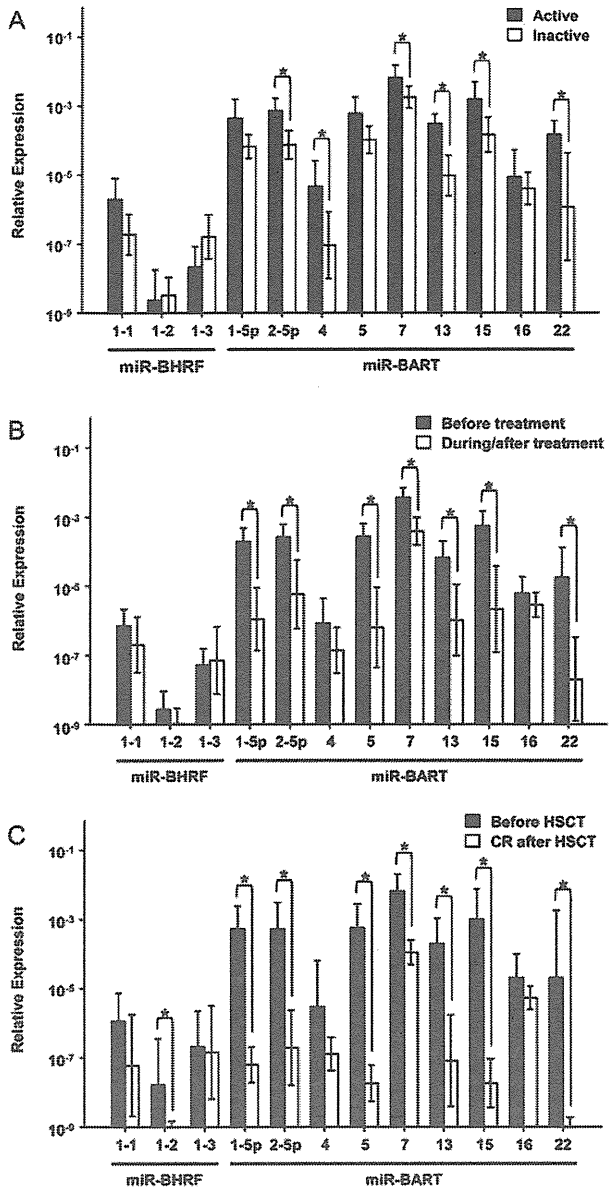

Figure 4. Comparison of expression profiles of Epstein–Barr virus (EBV) microRNAs (miRNAs) from patients with chronic active EBV (CAEBV) infection who had different clinical conditions. *A*, Comparison of 9 patients with active symptoms versus 7 with inactive symptoms before treatment. *B*, Comparison 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. $^{*}P < .05$.

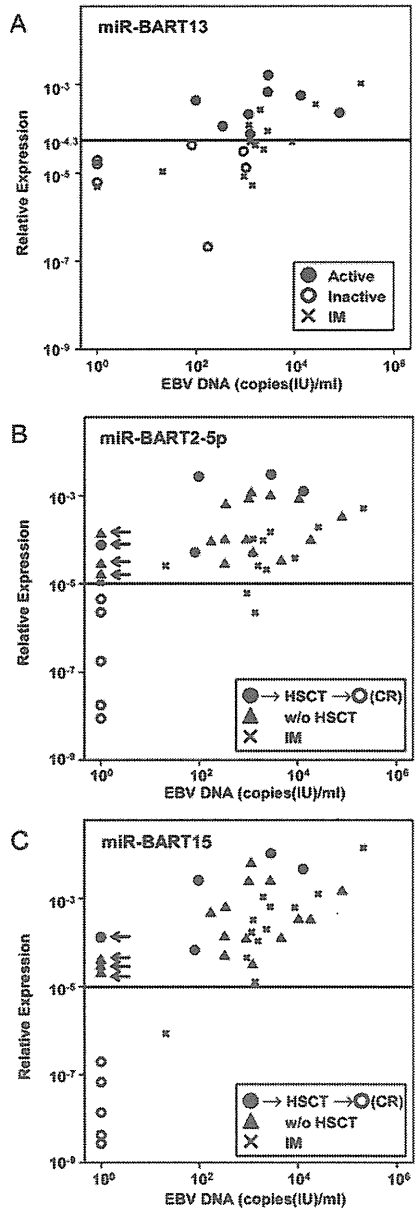

Figure 5. Comparison of the levels of Epstein–Barr virus (EBV) microRNAs (miRNAs) with EBV DNA in plasma of patients with chronic active EBV (CAEBV) infection. *A*, The level of EBV miR-BART13 in comparison with plasma EBV loads in patients with CAEBV infection, classified by the severity of clinical 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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慢性活動性EBV感染症（CAEBV）といわれてきた疾患
―EBV陽性T，NKリンパ増殖症（EBV-T/NK-LPDs）

新井文子*

要旨

・慢性活動性EBV感染症（chronic active Epstein-Barr virus infection：CAEBV）は，当初は抗EBV抗体高値に伴う全身の炎症症状，すなわち発熱，肝機能障害，リンパ節腫脹などが持続する疾患として報告された。
・その中でEBV感染細胞がTもしくはNK細胞で，かつクローナルな増殖をみる例を，皮膚に病変が限局する二つの疾患，種痘様水疱症，蚊刺過敏症，およびEBV陽性血球貪食性リンパ組織球症と合わせて，EBV陽性T，NKリンパ増殖症（EBV-positive T or NK-cell lymphoproliferative diseases；EBV-T/NK-LPDs）とし，リンパ系腫瘍として取り扱う提案がされている。本稿でもそれに基づき記載する。
・EBV-T/NK-LPDsは本邦を中心とする東アジアに報告が集中している。
・ありふれたウイルスがなぜ一部のヒトでT，NK細胞に持続感染し，腫瘍発症に至るのかその仕組みは解明されていない。
・EBV-T/NK-LPDsの診断には，末梢血中のEBV-DNA定量検査とEBV感染細胞の同定検査が必要である。
・EBV-T/NK-LPDsの多くは，臓器障害，より悪性度の高い腫瘍への進行，もしくは血球貪食症候群を発症し，適切な治療がなされないと致死的経過をとる。
・唯一の根治療法は同種造血幹細胞移植である。

疾患概念の変遷

慢性活動性EBV感染症（chronic active Epstein-Barr virus infection：CAEBV）とは，伝染性単核球症（infectious mononucleosis：IM）様の慢性炎症症状が持続する疾患としてVirelizierらにより1978年に最初に報告された[1]。当初は慢性化したIMと考えられていたが，1988年にJonesらによりT細胞にEBVが感染し，かつクローナルに増殖している症例があることが報告された[2]。その後も同様の報告が続き，2008年に改訂された

*東京医科歯科大学大学院医歯学総合研究科血液内科〔〒113-8519 東京都文京区湯島1-5-45〕 *ARAI Ayako*

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WHOのリンパ腫分類ではT細胞性腫瘍の一つとして記載された[3]。

さらに，NK細胞への感染例や，皮膚に特徴的な病変を生じる2疾患，種痘様水疱症(hydroa vacciniforme-like eruption)，蚊刺過敏症(hypersensitivity to mosquito bites)，そしてEBV陽性血球貪食性リンパ組織球症(EBV-positive hemophagocytic lymphohistiocytosis)はEBVがT，NK細胞に感染し，クローナルな増殖を認め，経過中リンパ腫を発症するなどCAEBVと同じような経過をたどることから，これらを一つの疾患単位とし，EBV陽性T，NKリンパ増殖症(EBV-positive T or NK-cell lymphoproliferative diseases；EBV-T/NK-LPDs)として統一する提案がなされている[4]。

CAEBVという診断名について

CAEBVという疾患名はIM様の炎症症状が持続し，抗EBV抗体上昇を伴う疾患を，あくまでEBVの感染症という視点でとらえたものである。しかし，前述のようにCAEBVの中にはEBVのTもしくはNK細胞への感染と，それらのクローナルな増殖を認める例があり，適切な治療を行わないと進行し致死的となることから，EBV-T/NK-LPDsという腫瘍として病名を改めることが提案されている[4]。

一方，IMの中には，症状が数カ月遷延するものがある。さらに最近では，急性期にEBVがT細胞へ感染し，形態的に異型性を伴いリンパ腫との鑑別が困難な例が報告されている[5]。今までCAEBVとして報告された中には，このようなIMの症例が含まれている可能性がある。IMの診断のポイントは，正確な病歴聴取とVCA-IgM抗体検査から初感染の所見を得ることである。IMの多くは自然軽快する。正しい診療のため，これらの鑑別は非常に重要である。

この他に，CAEBVとして報告されたものには，B細胞にEBVの感染を認めるものもある。米国から報告されてきたCAEBVはこのタイプが多いが，低γグロブリン血症などT，NK細胞感染型と異なった所見を示し，T，NK細胞に感染をみるものとは異なった疾患と考えられる[6]。

以上のように，従来CAEBVと診断され，報告されてきた症例には少なくとも以上の三つの病態が含まれている可能性がある。今後はこれらを区別して解析することが，それぞれの病態解明のために必要である。

本稿では，EBV-T/NK-LPDs，すなわちEBVの感染細胞がTもしくはNK細胞と同定され，かつクローナルな増殖を示す疾患について述べる。

疫　学

EBV-T/NK-LPDsの頻度はきわめてまれである。正確な頻度は不明であるが，CAEBVとしての全国調査によると，本邦での2005～2009年の新規患者数は平均して1年に23.8人であった(難治性疾患克服研究事業　H21-難治-一般-094「慢性活動性EBV感染症の実態解明と診断法確立に関する研究」報告書より)。また，近年，成人患者の報告は増えており，特に2009年には50%以上が成人例で，なかには80歳代の例もあった。世界各地から報告例があるが，発症には地域性があり日本など東アジアに報告が集中している。このことから発症には何らかの遺伝的な背景が関与していると考えられている。

発症機序と病態

EBVがB細胞を不死化，腫瘍化させることはよく知られている。しかし，EBVがT細胞の腫瘍化にも関与しているかどうかは証明されていない。EBVは，日本では20歳以上の成人の90%が感染を受けているごくありふれたウイルスである。そもそも，なぜこのようなありふれたウイル

スが一部の人では重篤な疾患の原因になるのだろうか。

EBVの感染標的細胞は通常はB細胞であるが，その感染受容体であるCD21はT細胞，NK細胞には発現していないといわれている。しかし，わずかであるがT細胞にも発現しているという報告[7]や，B細胞との接触による免疫学的シナプスによりCD21がNK細胞へ発現しEBVが感染し得るという報告[8]がなされている。また，IMの急性期にEBVがT細胞やNK細胞に感染し得ることも報告[9]されており，感染自体は起こり得ると考えられる。

また，EBVがT，NK細胞に感染すると，それらはB細胞同様不死化することが最近明らかになってきた。私たちはT細胞株に*in vitro*でEBVを感染させると，抗がん薬etoposideへの感受性が低下することを見いだした(第18回欧州血液学会総会発表)。同じように*in vitro*でEBVをNK細胞株に感染させるとdoxorubicinによるアポトーシスも抑制される[10]。

さらに，EBV-T/NK-LPDs患者の細胞ではactivation-induced cytidine deaminase(AID)といわれる分子の発現が高いことも報告されている[11]。AIDとは，積極的に遺伝子に変異をいれる蛋白質であり，B細胞における免疫グロブリンのクラススイッチや体細胞超変異に関与している。AID発現の亢進は遺伝子に変異が起こりやすくなることを示唆しており，腫瘍の進展に関連している可能性がある。

大島らはEBVが感染したT，NK細胞では遺伝子変異が蓄積し，オリゴクローナルからモノクローナルな増殖をきたした結果，EBV-T/NK-LPDs，リンパ腫発症にいたる発症モデルを提唱している[12]。実際にEBV-T/NK-LPDsから節外性NK/T細胞リンパ腫鼻型やアグレッシブNK細胞リンパ腫へ進展した例の報告は，その説を裏付けている[4]。

以上を総合するとEBVはB細胞腫瘍のみならずT，NK細胞腫瘍の原因にもなり得ると考えられ，このことからも，EBV-T/NK-LPDsという疾患は，感染症，炎症性疾患としてではなく，EBVによるT細胞，NK細胞の腫瘍として扱うことは合理的である。

EBV-T/NK-LPDsの臨床像

発熱を伴う慢性の臓器障害がEBV-T/NK-LPDsの臨床像である。つまり，炎症と腫瘍，二つの所見を示すことが大きな特徴である。

1 炎症症状

もっとも多い症状は発熱である。その他，多発リンパ節腫脹，肝障害などを示すことが多い。血管炎を生じ，それに伴う臓器障害をきたすことや，ぶどう膜炎を合併することもある。よって患者は消化器内科，膠原病内科の門をまず叩くことが多い。

一部の患者では特徴的な皮膚症状を認める。その一つが蚊(ヒトスジシマカ)に刺された後，局所の高度の炎症に加え，高熱をきたす，いわゆる蚊刺過敏症である。刺された場所は潰瘍化し，約1カ月かけて瘢痕を残して治癒する。ヒトスジシマカの唾液成分に対するEBV感染細胞の高度な反応が原因と考えられている。感染細胞の種類と発症は関連はない。また，日光に当たる皮膚に，炎症や水疱を繰り返すことがあり，種痘様水疱症といわれる。これらの皮膚症状は小児例・若年例に多く，思春期に軽快するが感染細胞が除去されることはない。

最近の研究によって，EBV-T/NK-LPDs患者では血中のインターロイキン-6，TNF-α，インターフェロン-γなどの炎症性サイトカイン濃度が上昇していることが明らかとなっている。これらのサイトカインは感染細胞が産生し，炎症の原因となるほか，マクロファージを刺激して，血球貪食症候群をきたすと考えられている[13]。

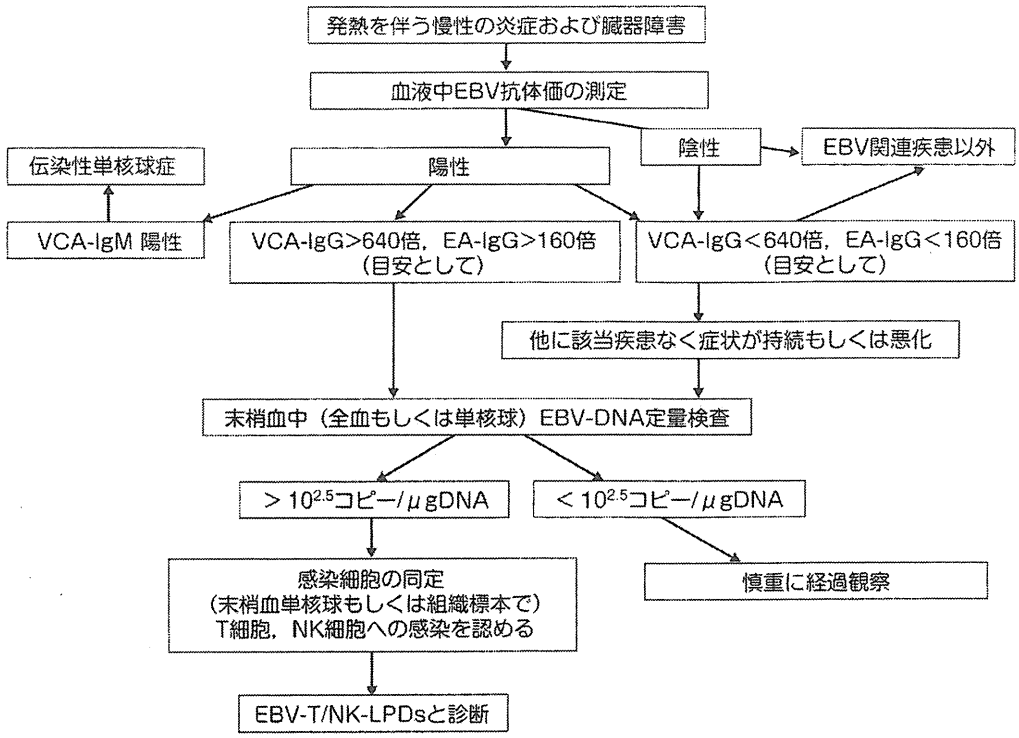

図　EBV陽性T，NKリンパ増殖症診断のフローチャート（EBウイルス感染症研究会診断指針および文献[4]より）

2 腫瘍症状

EBV感染細胞の増殖と浸潤により臓器障害を起こす。リンパ節，肝臓，脾臓などのリンパ系組織のみならず，皮膚，肺，心筋，腸管，中枢および末梢神経など，あらゆる臓器が標的となり得る。EBV-T/NK-LPDsは腫瘤を形成することは多くない。また，浸潤細胞は異型性に乏しい。よって病理組織のみでの診断は困難であることが多い。

予後は非常に悪い。2012年の木村らの108例（1～50歳）の解析では，観察中央値46カ月で44%の症例が重症臓器不全で死亡している[4]。さらに，因子別の予後解析では，発症時年齢が8歳未満の症例の15年生存率は59.7%であったのに対し，8歳以上では27%と有意な差が認められた。筆者らの20歳以上の成人例の解析でも，21例中，移植例2例を含む12例（57%）が死亡しており，特に発症時年齢が50歳以上では75%が発症から平均8カ月で死亡していた[14]。これらの結果から，EBV-T/NK-LPDsは年齢の高い患者ほど予後が悪いと推測される。

診　断

EBV-T/NK-LPDsの診断は容易ではない。筆者らの解析では，成人例では発症から治療開始までの平均期間は20カ月と長かった[14]。その主な原因は，疾患の認知度が低いこと，病理診断が困難であることが考えられる。

本疾患の診断のためのフローチャートを図に示す。以下の順に検査を進めていく。

1 抗体検査

前述の臨床症状から EBV-T/NK-LPDs を疑ったら，まず EBV 抗体検査を行い，既感染であることを確認する。EBV-T/NK-LPDs では，抗 VCA-IgG のみならず抗 EA-IgG 抗体が高値を示すことが多い。一方，抗 EBNA 抗体は陰性もしくは低下とされるが特異的ではない。抗 VCA-IgM 抗体陽性を確認し，IM を除外することは重要である。

2 ウイルス DNA 量測定

抗体が陽性かつ IM が除外されたら，次に末梢血中の EBV の DNA 量を測定する(EBV-DNA 定量検査)。全血もしくは単核球分画での測定を行う。EBV-T/NK-LPDs では EBV 感染細胞が末梢血中に検出されるため，$10^{2.5}$コピー/μgDNA 以上を示す[15]。これは診断に必須の検査であるが，2013 年現在，保険適用外である。多くの検査会社で外注検査として受け付けている。

末梢血中で EBV が増加していて，EBV-T/NK-LPDs の疑いが高くなったら，確定検査には EBV 感染細胞の同定，つまり，どのタイプのリンパ球分画に EBV が感染しているのか(通常通り B 細胞か，それとも T 細胞もしくは NK 細胞か)を調べる。組織標本を用いて *in situ* hybridization 法(*in situ* hybridization of Epstein-Barr virus-encoded mRNA(EBER))と免疫染色法を行い EBV 陽性細胞の表現型を検討するか，末梢血リンパ球を各分画に分け，それぞれの EBV-DNA 量を解析する。後者は研究施設での解析となる。前述のように，EBV-T/NK-LPDs では EBV 感染細胞は T もしくは NK 細胞であり，その確認をもって診断となる。

治　療

EBV-T/NK-LPDs の予後は不良で，慢性に進行し適切に治療されないと致死的経過をとる。本疾患は，炎症と，腫瘍の二つの性質を持つため，両者のコントロールを目的とした治療を行う。炎症に対しては，prednisolone，cyclosporine A，VP-16 の併用療法がもっともよく行われており，症状の制御に有効である[16]。一方で，感染腫瘍細胞を除去し得る有効な化学療法は確立されていない。

しかし，造血幹細胞移植の有効性が指摘されており，木村らの 108 例の解析では，移植施行例の 15 年生存率は 60.6%であったのに対し，未施行例では 25.7%と移植例が有意に生存率が高かった[17]。さらに，2010 年に河らは大阪母子保健総合医療センターの移植例を後方視的に解析し，骨髄非破壊的移植を行った患者では 90%以上の 3 年生存率を得たと報告している[18]。今後は長期予後も含めた多数例での移植成績の解析に加え，至適移植時期の検討，さらには移植が困難な症例に対する治療法の開発が必要である。

おわりに

CAEBV と報告されてきた疾患の中から，EBV が T，NK 細胞に感染し，かつ感染細胞がクローナルに増殖しているものを，同じく EBV 感染 T，NK 細胞のクローナルな増殖をみる蚊刺過敏症，種痘様水疱症と合わせて EBV-T/NK-LPDs とし，一つの疾患として解説した。今後は，これがはたして一つの疾患なのか，感染細胞や発症年齢などで臨床像に差があるのか，さらに詳細な検討が必要である。EBV-T/NK-LPDs の解析は，EBV による T，NK 腫瘍発症機構の解明のみならず EBV 陽性 T，NK 細胞腫瘍というカテゴリーの確立，さらには治療法開発にもつながり得る。本邦を中心とした東アジアの研究者に期待される役割は大きい。

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