Iwata S, Saito T, Ito Y, Kamakura M, Gotoh K, Kawada J, Nishiyama Y, Kimura H.	Antitumor activities of valproic acid on Epstein-Barr virus-associated T and natural killer lymphoma cells.	Cancer Sci	103	375-378	2012
Kimura H, Ito Y, Kawabe S, Gotoh K, Takahashi Y, Kojima S, Naoe T, Esaki S, Kikuta A, Sawada A, Kawa K, Ohshima K, Nakamura S.	Epstein-Barr virus (EBV)-associated T/NK lymphoproliferative diseases in non-immunocompromised hosts: prospective analysis of 108 cases.	Blood	119	673-686	2012
新井文子	Chronic active EBV infection (CAEBV) 正しい診断のために内科医が知っておくべき事	内科	110	263-266	2012

研究成果の刊行に関する一覧表（平成25年度）

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
新井文子	血液疾患	宮坂信之	ポケットサイズのステロイド診療マニュアル	新興医学出版社	東京	2013	p113-121
新井文子	フローサイトメトリー、染色体分析、FISH、遺伝子診断	定平吉都、北川昌伸	腫瘍病理鑑別診断アトラス	文光堂	東京	2013	p23- 33
北條浩彦、清水則夫	基本編－原理と基本知識－ リアルタイムPCRを使った解析の基	北條浩彦	原理からよくわかるリアルタイムPCR完全実験ガイド	株式会社羊土社	東京	2013	72-74
清水則夫、渡邊健、外丸靖浩	実践編－プロトコールを中心に－ Ⅳ章　遺伝子量解析	北條浩彦	原理からよくわかるリアルタイムPCR完全実験ガイド	株式会社羊土社	東京	2013	192-202

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Fujiwara S, Kimura H, Imadome K, Arai A, Kodama, E, Morio T, Shimizu N, and Wakiguchi H.	Current studies on chronic active Epstein-Barr virus infection in Japan.	Pediatrics International,	in press		2014
Fujiwara S.	Reproduction of Epstein-Barr virus infection and pathogenesis in humanized mice	Immune Network	in press		2014
Nakamura H, Liao H, Minami K, Toyoda M, Akutsu H, Miyagawa Y, Okita H, Kiyokawa N, Umezawa A, Imadome K, Inoue N, and Fujiwara S.	Human cytomegalovirus induces apoptosis in neural stem/progenitor cells derived from induced pluripotent stem cells by generating mitochondrial dysfunction and endoplasmic reticulum stress.	Herpesviridae	4	2	2013

Wang L, Sato-Otsubo A, Sugita S, Takase H, Mochizuki M,Usui Y, Goto H, Koyama T , Akiyama H, Miura O, Ogawa S, Arai A.	High-resolution genomic copy number profiling of primary intraocular lymphoma by single nucleotide polymorphism microarrays.	Cancer Science	in press		2014
Arai A, Yamaguchi T, Komatsu H, Imadome K, Kurata M, Nagata K, Miura O.	Infectious mononucleosis accompanied by clonal proliferation of EBV-infected cells and by the infection on CD8-positive cells.	Int J Hematol,	in press		2014
福田祥子、神田紗也香、高瀬博、小林大輔、新井文子、望月學	急速に進行した劇症の原発性眼内リンパ腫の一例	眼科臨床紀要	in press		2014
Yagi K, Yamamoto K, Umeda S, Abe S, Suzuki S, Onishi I, Kirimura S, Fukayama M, Arai A, Kitagawa M, Kurata M.	Expression of multidrug resistance 1 gene in B-cell lymphomas: association with follicular dendritic cells.	Histopathology.	62	414-20	2013
新井文子	PLLの診断と治療	血液内科	66	351-358	2013
新井文子	EBウイルス陽性リンパ腫の発症機構	最新医学	68	48-55	2013
新井文子	慢性活動性EBV感染症 Chronic active EBV infection (CAEBV) と言われてきた疾患 ～EBV陽性T,NKリンパ増殖症 EBV-T/NK-LPDs～	成人病と生活習慣病	43	1073-78	2013
Kazuki Izumi, Kumi Kawaji, Fusasko Miyamoto, Kazuki Shimane, Kazuya Shimura, Yasuko Sakagami, Toshio Hattori, Kentaro Watanabe, Shinya Oishi, Nobutaka Fujii, Masao Matsuoka, Mitsuo Kaku, Stefan G. Sarafianos, and Eiichi N. Kodama	Mechanism of Resistance to S138A Substituted Enfuvirtide and its Application to Peptide Design.	International Journal of Biochemistry and Cell Biology	45	908-915	2013

Kazuki Shimane, KumiKawaji, Fusako Miyamoto, Shinya Oishi, Kentaro Watanabe, YasukoSakagami, Nobutaka Fujii, Kazuya Shimura, Masao Matsuoka, Mitsuo Kaku, Stefan Sarafianos, and Eiichi Kodama	HIV-1 resistance mechanism to an electrostatically constrained peptide fusion inhibitor that is active against T-20-resistant strains.	Antimicrobal Agents and Chemotherapy	57	4035-4038	2013
Eleftherios Michailidis, Emily M Ryan, Atsuko Hachiya, Karen A Kirby, Bruno Marchand, Maxwell D Leslie, Andrew D Huber, Yee T Ong, Jacob C Jackson, Kamalendra Singh, Eiichi N Kodama, Hiroaki Mitsuya, Michael A Parni	Hypersusceptibility Mechanism of Tenofovir-Resistant HIV to EFdA.	Retrovirology	10	65 doi:10.1186/1742-4690-10-65	2013
Atsuko Hachiya, Aaron Reeve, Bruno Marchand, Eleftherios Michailidis, Yee Ong, Karen Kirby, Maxwell Leslie, Shinichi Oka, Eiichi Kodama, Lisa Rohan, Hiroaki Mitsuya, Michael Parniak, and Stefan Sarafianos	Evaluation of combinations of 4'-ethynyl-2-fluoro-2'-deoxyadenosine with clinically used antiretroviral drugs.	Antimicrobal Agents and Chemotherapy	57	4554-4558	2013
Kirby KA, Michailidis E, Fetterly TL, Steinbach MA, Singh K, Marchand B, Leslie MD, Hagedorn AN, Kodama EN, Marquez VE, Hughes SH, Mitsuya H, Parniak MA, Sarafianos SG	Effects of substitutions at the 4' and 2 positions on the bioactivity of 4'-ethynyl-2-fluoro-2'-deoxyadenosine.	Antimicrobal Agents and Chemotherapy	57	6254-64	2013
Kenji Maeda, Darshan V Desai, Manabu Aoki, Hirotomo Nakata, Eiichi N Kodama, Hiroaki Mitsuya.,	Delayed emergence of HIV-1 variants resistant to 4'-ethynyl-2-fluoro-2'-deoxyadenosine: comparative sequential passage study with lamivudine tenofovir, emtricitabine and BMS-986001.	Antiviral Therapy	In press	doi: 10.3851/IMP2697	2013

Fusako Miyamoto and Eiichi N Kodama	Development of small molecule HIV-1 fusion inhibitors: linking Biology to Chemistry.	Current Pharmaceutical Design	19	1827-34	2013
児玉栄一、宮本総子	新しい抗ウイルス剤開発の考え方	臨床と微生物	40	51-55	2013
Ogawa M, Sugita S, Shimizu N, Watanabe K, Nakagawa I, Mochizuki M.	Broad-range real-time PCR assay for detection of bacterial DNA in ocular samples from infectious endophthalmitis.	Japanese Journal of Ophthalmology.	56(6)	529-535	2012
Sugita S, Shimizu N, Watanabe K, Ogawa M, Maruyama K, Usui N, Mochizuki M.	Virological analysis in patients with human herpes virus 6-associated ocular inflammatory disorders.	Investgative Ophthalmology and Visual Science.	53(8)	4692-4698	2012
Ogawa M, Sugita S, Watanabe K, Shimizu N, Mochizuki M.	Novel diagnosis of fungal endophthalmitis by broad-range real-time PCR detection of fungal 28S ribosomal DNA.	Graefes's Archive of Clinical and Experimental Ophthalmol.ogy	250(12)	1877-1883	2012.
Kobayashi Z, Akaza M, Numasawa Y, Ishihara S, Tomimitsu H, Nakamichi K, Saijo M, Morio T, Shimizu N, Sanjo N, Shintani S, Mizusawa H.	Failure of mefloquine therapy in progressive multifocal leukoencephalopathy:Report of two Japanese patients without human immunodeficiency virus infection.	Journal of the Neurological Sciences	324	190–194	2013
Yan J, Ng SB, Tay JL, Lin B, Koh TL, Tan J, Selvarajan V, Liu SC, Bi C, Wang S, Choo SN, Shimizu N, Huang G, Yu Q, Chng WJ.	EZH2 overexpression in natural killer/T-cell lymphoma confers growth advantage independently of histone methyltransferase activity.	blood	121	4512-4520	2013
Tachikawa R, Tomii K, Seo R, Nagata K, Otsuka K, Nakagawa A, Otsuka K, Hashimoto H, Watanabe K, Shimizu N.	Detection of Herpes Viruses by Multiplex and Real-Time PolymeraSEQhain Reaction in Bronchoalveolar Lavage Fluid of Patients with Acute Lung Injury or Acute Respiratory Distress Syndrome.	Raspiration		Epub ahead of print	2013

Ito K, Shimizu N, Watanabe K, Saito T, Yoshioka Y, Sakane E, Tsunemine H, Akasaka H, Kodaka T, Takahashi T.	Analysis of viral infection by multiplex polymera SEQhain reaction assays in patients with liver dysfunction.	Internal Medicine.	52(2)	201-211	2013
Kamae C, Nakagawa N, Sato H, Honma K, Mitsuiki N, Ohara O, Kanegane H, Pasic S, Pan-Hammerstrom Q, van Zelm MC, Morio T, Imai K, Nonoyama S.	Common variable immunodeficiency classification by quantifying T-cell receptor and immunoglobulin κ-deleting recombination excision circles.	J Allerg Clin Immunol	131	1437-40	2013
Machida S, Tomizawa D, Tamaichi H, Okawa T, Endo A, Imai K, Nagasawa M, Morio T, Mizutani S, Takagi M.	Successful Treatment of Diffuse Large B-Cell Lymphoma in a Patient With Ataxia Telangiectasia Using Rituximab.	J Pediatr Hematol Oncol	35	482-5	2013
山田尚、森尾友宏、古川恵一	EBウイルス感染症のピットホール	成人病と生活習慣病	43	1021-32	2013
Kawano Y, Iwata S, Kawada J, Gotoh K, Suzuki M, Torii Y, Kojima S, Kimura H, Ito Y.	Plasma viral MicroRNA profiles reveal potential biomarkers for chronic active Epstein-Barr virus infection.	J Infect Dis	208	771-9	2013
Murata T, Iwata S, Siddiquey NA, Kanazawa T, Goshima F, Kimura H, Tsurumi T.	Heat shock protein 90 inhibitors repress latent membrane protein 1 (LMP1) expression and proliferation of Epstein-Barr virus-positive natural killer cell lymphoma.	PLoS One	8	e63566	2013
Ito Y, Kawamura Y, Iwata S, Kawada J, Yoshikawa T, Kimura H.	Demonstration of type II latency in T lymphocytes of Epstein-Barr Virus-associated hemophagocytic lymphohistiocytosis.	Pediatr Blood Cancer	60	326-328	2013

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

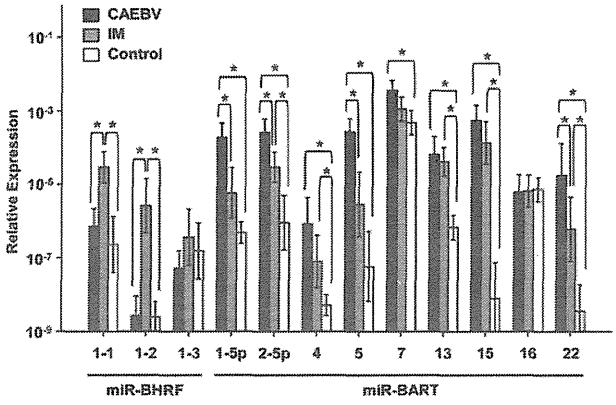

Figure 1. Levels of Epstein–Barr virus (EBV) microRNAs (miRNAs) in patient plasma samples. Levels of miRNAs are presented as the mean and standard error of the values following normalization to the level of hsa-miR-16. Black bars denote data for 16 patients with chronic active EBV (CAEBV) infection (plasma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

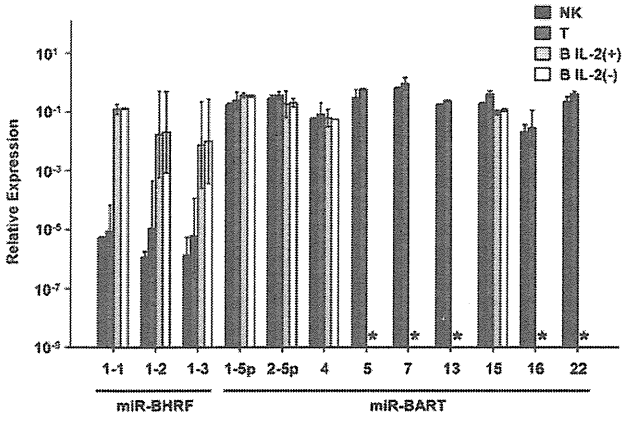

Figure 2. Levels of Epstein–Barr virus (EBV) microRNAs (miRNAs) in EBV-positive cell lines. Levels of miRNAs are presented as the mean and standard error of the values following normalization to the level of hsa-miR-16. Black bars denote data for 2 natural killer (NK)–cell lines (KAI-3 and SNK-6), dark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

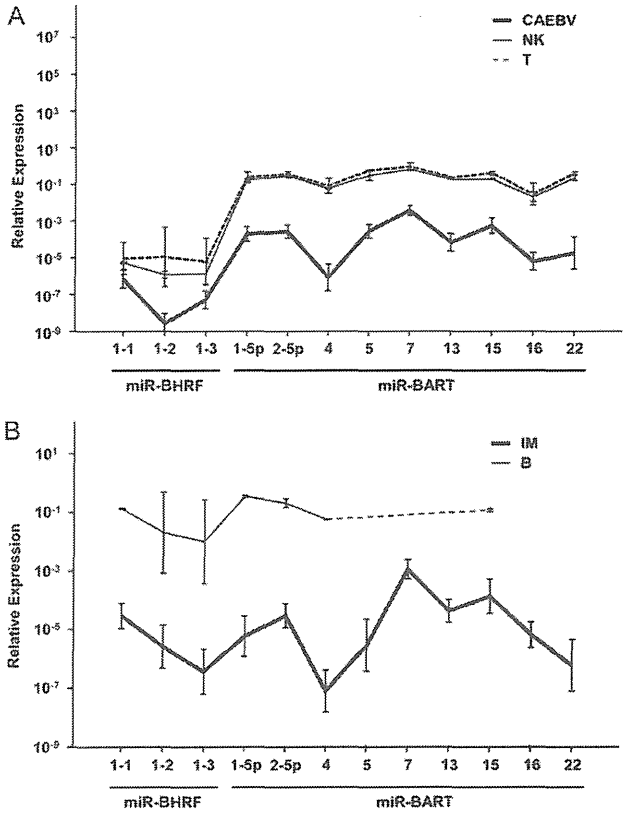

Figure 3. Comparison of the expression profiles of Epstein–Barr virus (EBV) microRNAs (miRNAs) between plasma and cell lines. Concentrations of plasma miRNAs and expression levels of miRNAs in EBV-positive cells are presented as means and standard errors. *A*, Chronic active EBV (CAEBV) infection versus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

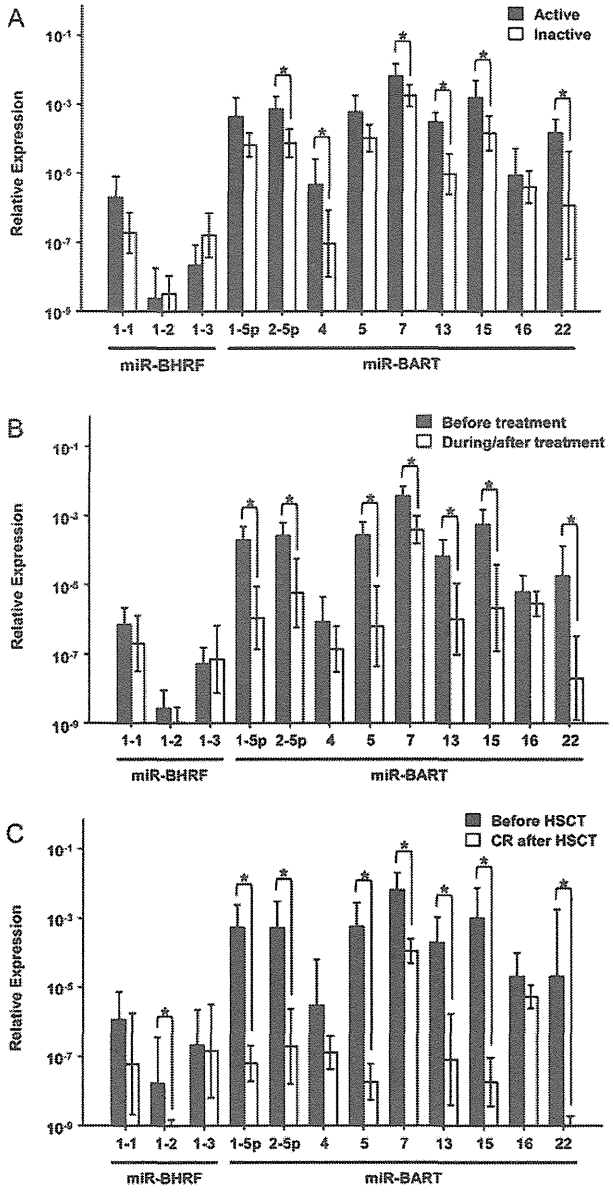

Figure 4. Comparison of expression profiles of Epstein–Barr virus (EBV) microRNAs (miRNAs) from patients with chronic active EBV (CAEBV) infection who had different clinical conditions. *A*, Comparison of 9 patients with active symptoms versus 7 with inactive symptoms before treatment. *B*, Comparison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

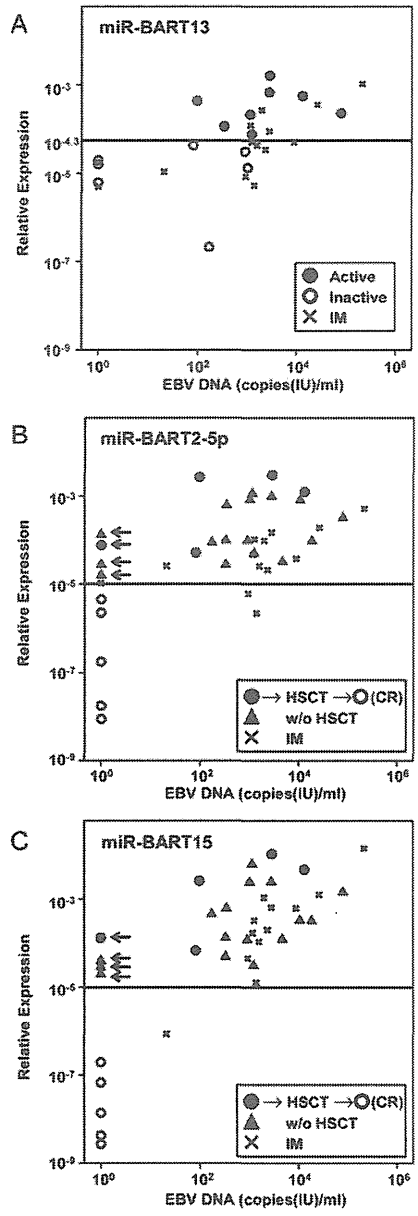

Figure 5. Comparison of the levels of Epstein–Barr virus (EBV) microRNAs (miRNAs) with EBV DNA in plasma of patients with chronic active EBV (CAEBV) infection. *A*, The level of EBV miR-BART13 in comparison with plasma EBV loads in patients with CAEBV infection, classified by the severity of clinical 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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Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. Cohen JI. Epstein-Barr virus infection. N Engl J Med **2000**; 343: 481–92.
2. Williams H, Crawford DH. Epstein-Barr virus: the impact of scientific advances on clinical practice. Blood **2006**; 107:862–9.
3. Tosato G, Straus S, Henle W, Pike SE, Blaese RM. Characteristic T cell dysfunction in patients with chronic active Epstein-Barr virus infection (chronic infectious mononucleosis). J Immunol **1985**; 134:3082–8.
4. Kimura H. Clinical and virologic characteristics of chronic active Epstein-Barr virus infection. Blood **2001**; 98:280–6.
5. Jones JF, Shurin S, Abramowsky C, et al. T-cell lymphomas containing Epstein-Barr viral DNA in patients with chronic Epstein-Barr virus infections. N Engl J Med **1988**; 318:733–41.
6. Kikuta H, Taguchi Y, Tomizawa K, et al. Epstein-Barr virus genome-positive T lymphocytes in a boy with chronic active EBV infection associated with Kawasaki-like disease. Nature **1988**; 333:455–7.
7. Kawa-Ha K, Ishihara S, Ninomiya T, et al. CD3-negative lymphoproliferative disease of granular lymphocytes containing Epstein-Barr viral DNA. J Clin Invest **1989**; 84:51–5.
8. Kimura H, Morishima T, Kanegane H, et al. Prognostic factors for chronic active Epstein-Barr virus infection. J Infect Dis **2003**; 187: 527–33.
9. Kimura H. Pathogenesis of chronic active Epstein-Barr virus infection: is this an infectious disease, lymphoproliferative disorder, or immunodeficiency? Rev Med Virol **2006**; 16:251–61.
10. Cohen JI, Jaffe ES, Dale JK, et al. Characterization and treatment of chronic active Epstein-Barr virus disease: a 28-year experience in the United States. Blood **2011**; 117:5835–49.
11. Kimura H, Ito Y, Kawabe S, et al. EBV-associated T/NK-cell lymphoproliferative diseases in nonimmunocompromised hosts: prospective analysis of 108 cases. Blood **2012**; 119:673–86.
12. Gotoh K, Ito Y, Shibata-Watanabe Y, et al. Clinical and virological characteristics of 15 patients with chronic active Epstein-Barr virus infection treated with hematopoietic stem cell transplantation. Clin Infect Dis **2008**; 46:1525–34.
13. Okamura T, Hatsukawa Y, Arai H, Inoue M, Kawa K. Blood stem-cell transplantation for chronic active Epstein-Barr virus with lymphoproliferation. Lancet **2000**; 356:223–4.
14. Kawa K, Sawada A, Sato M, et al. Excellent outcome of allogeneic hematopoietic SCT with reduced-intensity conditioning for the treatment of chronic active EBV infection. Bone Marrow Transplant **2011**; 46: 77–83.
15. Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell **2009**; 136:215–33.
16. Mattick JS, Makunin IV. Small regulatory RNAs in mammals. Hum Mol Genet **2005**; 14(Spec No 1):R121–32.
17. Cai X, Schafer A, Lu S, et al. Epstein-Barr virus microRNAs are evolutionarily conserved and differentially expressed. PLoS Pathog **2006**; 2: e23.
18. Cosmopoulos K, Pegtel M, Hawkins J, et al. Comprehensive profiling of Epstein-Barr virus microRNAs in nasopharyngeal carcinoma. J Virol **2009**; 83:2357–67.
19. Grundhoff A, Sullivan CS, Ganem D. A combined computational and microarray-based approach identifies novel microRNAs encoded by human gamma-herpesviruses. RNA **2006**; 12:733–50.
20. Pfeffer S, Zavolan M, Grasser FA, et al. Identification of virus-encoded microRNAs. Science **2004**; 304:734–6.
21. Zhu JY, Pfuhl T, Motsch N, et al. Identification of novel Epstein-Barr virus microRNA genes from nasopharyngeal carcinomas. J Virol **2009**; 83:3333–41.
22. Barth S, Meister G, Grasser FA. EBV-encoded miRNAs. Biochim Biophys Acta **2011**; 1809:631–40.
23. Qiu J, Cosmopoulos K, Pegtel M, et al. A novel persistence associated EBV miRNA expression profile is disrupted in neoplasia. PLoS Pathog **2011**; 7:e1002193.
24. Ferracin M, Veronese A, Negrini M. Micromarkers: miRNAs in cancer diagnosis and prognosis. Expert Rev Mol Diagn **2010**; 10:297–308.
25. Brase JC, Wuttig D, Kuner R, Sultmann H. Serum microRNAs as non-invasive biomarkers for cancer. Mol Cancer **2010**; 9:306.
26. Moussay E, Wang K, Cho JH, et al. MicroRNA as biomarkers and regulators in B-cell chronic lymphocytic leukemia. Proc Natl Acad Sci U S A **2011**; 108:6573–8.
27. Gourzones C, Gelin A, Bombik I, et al. Extra-cellular release and blood diffusion of BART viral micro-RNAs produced by EBV-infected nasopharyngeal carcinoma cells. Virol J **2010**; 7:271.
28. Kimura H, Morita M, Yabuta Y, et al. Quantitative analysis of Epstein-Barr virus load by using a real-time PCR assay. J Clin Microbiol **1999**; 37:132–6.
29. Edwards RH, Marquitz AR, Raab-Traub N. Epstein-Barr virus BART microRNAs are produced from a large intron prior to splicing. J Virol **2008**; 82:9094–106.
30. Amoroso R, Fitzsimmons L, Thomas WA, Kelly GL, Rowe M, Bell AI. Quantitative studies of Epstein-Barr virus-encoded microRNAs provide novel insights into their regulation. J Virol **2011**; 85:996–1010.
31. Zhang Y, Nagata H, Ikeuchi T, et al. Common cytological and cytogenetic features of Epstein-Barr virus (EBV)-positive natural killer (NK) cells and cell lines derived from patients with nasal T/NK-cell

lymphomas, chronic active EBV infection and hydroa vacciniforme-like eruptions. Br J Haematol **2003**; 121:805–14.
32. Tsuge I, Morishima T, Morita M, Kimura H, Kuzushima K, Matsuoka H. Characterization of Epstein-Barr virus (EBV)-infected natural killer (NK) cell proliferation in patients with severe mosquito allergy; establishment of an IL-2-dependent NK-like cell line. Clin Exp Immunol **1999**; 115:385–92.
33. Chen C, Ridzon DA, Broomer AJ, et al. Real-time quantification of microRNAs by stem-loop RT-PCR. Nucleic Acids Res **2005**; 33: e179.
34. Griffiths-Jones S, Saini HK, van Dongen S, Enright AJ. miRBase: tools for microRNA genomics. Nucleic Acids Res **2008**; 36:D154–8.
35. Wada K, Kubota N, Ito Y, et al. Simultaneous quantification of Epstein-Barr virus, cytomegalovirus, and human herpesvirus 6 DNA in samples from transplant recipients by multiplex real-time PCR assay. J Clin Microbiol **2007**; 45:1426–32.
36. Mitchell PS, Parkin RK, Kroh EM, et al. Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci U S A **2008**; 105:10513–8.
37. Chan JY, Gao W, Ho WK, Wei WI, Wong TS. Overexpression of Epstein-Barr virus-encoded microRNA-BART7 in undifferentiated nasopharyngeal carcinoma. Anticancer Res **2012**; 32:3201–10.
38. Xia T, O'Hara A, Araujo I, et al. EBV microRNAs in primary lymphomas and targeting of CXCL-11 by ebv-mir-BHRF1-3. Cancer Res **2008**; 68:1436–42.
39. Xing L, Kieff E. Epstein-Barr virus BHRF1 micro- and stable RNAs during latency III and after induction of replication. J Virol **2007**; 81:9967–75.
40. Iwata S, Wada K, Tobita S, et al. Quantitative analysis of Epstein-Barr virus (EBV)-related gene expression in patients with chronic active EBV infection. J Gen Virol **2010**; 91:42–50.
41. Pratt ZL, Kuzembayeva M, Sengupta S, Sugden B. The microRNAs of Epstein-Barr Virus are expressed at dramatically differing levels among cell lines. Virology **2009**; 386:387–97.
42. Chen SJ, Chen GH, Chen YH, et al. Characterization of Epstein-Barr virus miRNAome in nasopharyngeal carcinoma by deep sequencing. PLoS One **2010**; 5:e12745.
43. Barth S, Pfuhl T, Mamiani A, et al. Epstein-Barr virus-encoded microRNA miR-BART2 down-regulates the viral DNA polymerase BALF5. Nucleic Acids Res **2008**; 36:666–75.
44. Nachmani D, Stern-Ginossar N, Sarid R, Mandelboim O. Diverse herpesvirus microRNAs target the stress-induced immune ligand MICB to escape recognition by natural killer cells. Cell Host Microbe **2009**; 5:376–85.
45. Choy EY, Siu KL, Kok KH, et al. An Epstein-Barr virus-encoded microRNA targets PUMA to promote host cell survival. J Exp Med **2008**; 205:2551–60.
46. Lung RW, Tong JH, Sung YM, et al. Modulation of LMP2A expression by a newly identified Epstein-Barr virus-encoded microRNA miR-BART22. Neoplasia **2009**; 11:1174–84.
47. Yamamoto M, Kimura H, Hironaka T, et al. Detection and quantification of virus DNA in plasma of patients with Epstein-Barr virus-associated diseases. J Clin Microbiol **1995**; 33:1765–8.
48. Kanegane H, Wakiguchi H, Kanegane C, Kurashige T, Miyawaki T, Tosato G. Increased cell-free viral DNA in fatal cases of chronic active Epstein-Barr virus infection. Clin Infect Dis **1999**; 28:906–9.

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Heat Shock Protein 90 Inhibitors Repress Latent Membrane Protein 1 (LMP1) Expression and Proliferation of Epstein-Barr Virus-Positive Natural Killer Cell Lymphoma

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Abstract

Epstein-Barr virus (EBV) LMP1 is a major oncoprotein expressed in latent infection. It functions as a TNFR family member and constitutively activates cellular signals, such as NFκB, MAPK, JAK/STAT and AKT. We here screened small molecule inhibitors and isolated HSP90 inhibitors, Radicicol and 17-AAG, as candidates that suppress LMP1 expression and cell proliferation not only in EBV-positive SNK6 Natural Killer (NK) cell lymphoma cells, but also in B and T cells. Tumor formation in immunodefficient NOD/Shi-scid/IL-2Rγ^{null} (NOG) mice was also retarded. These results suggest that HSP90 inhibitors can be alternative treatments for patients with EBV-positive malignancies.

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☯ These authors contributed equally to this work.

Introduction

The Epstein-Barr virus (EBV) is a human gamma-herpesvirus that mainly infects and establishes latent infection in B lymphocytes, but can also infect other types of cells, including NK, T and epithelial cells. EBV infection has been implicated as a causal factor in a variety of malignancies, and the expression pattern of viral latent genes varies depending on the tissue of origin and the state of the tumors. Neoplasms such as Burkitt's lymphomas or gastric carcinomas express only EBER and EBNA1 (type I latency), whereas some Hodgkin lymphomas, nasopharyngeal carcinomas (NPC) and NK/T lymphomas express EBER, EBNA1, LMP1 and LMP2 genes (type II latency). In addition to the type II genes, EBNA2, EBNA3 and EBNA-LP are also expressed in immunosuppression-related lymphomas or lymphoblastoid cell lines (LCLs) (type III latency).

EBV is associated with various types of T or NK cell lymphoproliferative diseases (T/NK LPDs). A severe form of chronic active EBV disease (CAEBV), mainly found in East Asia including Japan, is caused by clonal expansion of EBV-infected T or NK cells [1–3]. Others include extranodal NK/T lymphoma, nasal type (ENKL), and aggressive NK cell leukemia (ANKL). Although such EBV-positive T/NK LPDs are relatively rare, therapeutic treatment for those disorders is challenging, and the prognosis of those patients often can be dismal [4,5]. Therefore, development of effective and specific drugs is an important goal.

The EBV latent infection integral membrane protein 1 (LMP1) is frequently expressed in latent EBV infections, including NK/T lymphomas. Since it functions as a constitutive TNFR family member by aggregation in the plasma membrane, resulting in constitutive activation of cellular signaling through NFκB, MAPK, JAK/STAT and AKT, LMP1 is assumed to be a major oncogene encoded by EBV [6–15].

Heat-shock protein 90 (HSP90) is an ATP-dependent molecular chaperone that is important for stability, quality control, protein interaction and functional maturation of cellular or viral client proteins. Because HSP90 is occasionally overexpressed and present in an activated form in cancer cells, and thereby supports proliferation of activated oncoproteins, including many cancer-associated kinases and transcription factors, it is regarded as an essential factor for oncogenic transformation [16,17]. Radicicol and 17-AAG are HSP90 inhibitors which interact directly within its ATP-binding pocket, preventing ATP binding and interaction with client proteins [18]. These inhibitors might thus have potential as anti-cancer drugs for malignancies that depend on particular driver oncogene products that are sensitive HSP90 clients [16,17,19]. For example, HSP90 inhibitors have shown

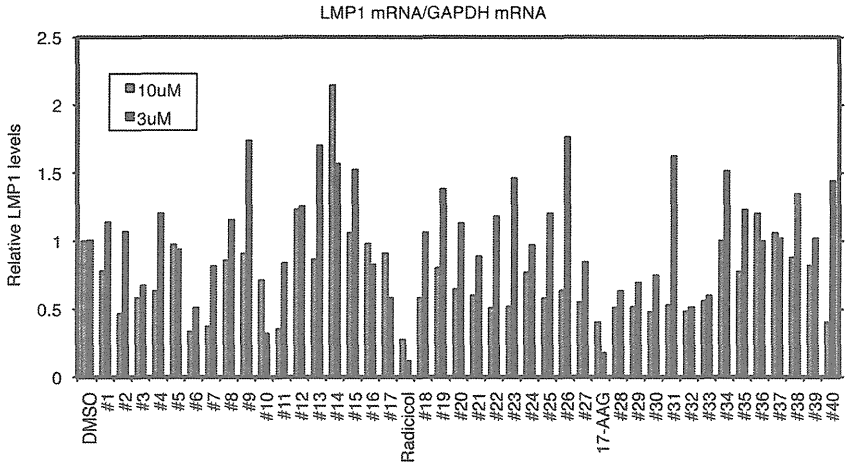

Figure 1. Representative example of screening for small molecule inhibitors that repress LMP1 expression. The EBV-positive NK lymphoma cell line, SNK6, was seeded and small molecule inhibitors were added to the media at concentrations of 10 and 3 μM. After 72 h, cell RNA was collected and subjected to Real-Time RT-PCR using specific primers for LMP1 and GAPDH mRNAs. Relative LMP1 mRNA levels are shown after normalization to GAPDH mRNA levels.
doi:10.1371/journal.pone.0063566.g001

promise as anti-myeloma agents in pre-clinical settings and are currently being evaluated in clinical trials [20].

In the present study, we screened small molecule inhibitors and isolated HSP90 inhibitors as candidates that suppress LMP1 expression and cell proliferation in EBV-positive SNK6 NK cell lymphoma cells. The inhibitors not only retarded tumor proliferation at the culture level but also tumor formation in immunodefficient NOD/Shi-scid/IL-2Rγ^{null} (NOG) mice. HSP90 inhibitors therefore may offer alternative treatments for EBV-positive malignancies.

Materials and Methods

Cell Culture and Reagents

An EBV-positive NK cell lymphoma line, SNK6, and an EBV-positive T cell line, SNT13, were maintained in RPMI1640 medium supplemented with 10% human serum (MP Biomedicals), 2 mM of Glutamax (GIBCO), 0.88 mM Oxalicacetic acid (SIGMA), 1 mM Sodium Pyruvate (GIBCO) and 700 U/ml of IL-2 (Primmune Inc.). SNK6 was originally established from a patient with ENKL and characterized by Nagata and others [21]. SNT13 is a γδ T-cell clone established from a patient with CAEBV [22]. These cell lines of low passage numbers were kindly provided by N. Shimizu in December 2009. B95-8 cells [23] were maintained in RPMI1640 medium supplemented with 10% fetal bovine serum. The Screening Committee of Anticancer Drugs (SCADS), Japan, kindly provided a library of small molecule inhibitors.

Real-time PCR

For real-time RT-PCR, total cell RNA was purified using TriPure Isolation Reagent (Roche) and subjected to Real-Time RTPCR using a One Step SYBR PrimeScript RT-PCR Kit II (TaKaRa) with the Real-Time PCR System 7300 according to the manufacturer's instructions. PCR was performed as described earlier [24]. Primers used for the RT-PCR were as follows [25]: for GAPDH mRNA, 5′-TGCACCACCAACTGCTAGC-3′ and 5′-GGCATGGACTGTGGTCATGAG-3′; for LMP1 mRNA, 5′-CTATTCCTTTGCTCTCATGC-3′ and 5′-TGAGCAGGAGGGTGATCATC-3′; and for EBNA1 mRNA, 5′-AGGTACAGGACCTGGAAATG-3′ and 5′-CCTCGTCCATGGTTATCACC-3′. Real-Time PCR with GAPDH primers was also performed to serve as an internal control for input RNA.

Antibodies and Immunoblotting

Anti-tubulin antibody was from Cell Signaling, and anti-LMP1 monoclonal antibody was reported previously [25]. For immunoblotting, cell proteins lysed in sample buffer were subjected to SDS-PAGE, followed by immunoblotting with the indicated antibodies as described previously [25].

Transplantation of SNK6 into NOD/Shi-scid/IL-2Rγ^{null} (NOG) mice

Female NOG mice were purchased from the Central Institute for Experimental Animals, Japan. Twenty four NOG mice at the age of 10 weeks were subcutaneously implanted with 5×10^6 SNK6 cells per mouse on day 0. From day 14, DMSO (vehicle) was injected into 12 mice, and the rest were treated with 17-AAG (6 times in two weeks, 50 mg/kg in total) intra-peritoneally. Peripheral blood was collected from the tail veins once in two weeks, and levels of EBV genomic DNA in whole blood were measured by Real-Time PCR as described previously [26]. Subcutaneous tumor masses were also measured with an external caliper and tumor volume was calculated using the formula: π × short axis × long axis × height/6. Animal experiments were approved by the University Committee in accordance with the Guidelines for Animal Experimentation at Nagoya University.

Statistical analysis

Data shown are means ± standard errors and were analyzed using SPSS for Windows version 18.0 (IBM Corporation, Chicago, IL, USA). The therapeutic results were analyzed using the Mann-Whitney *U* test between groups.

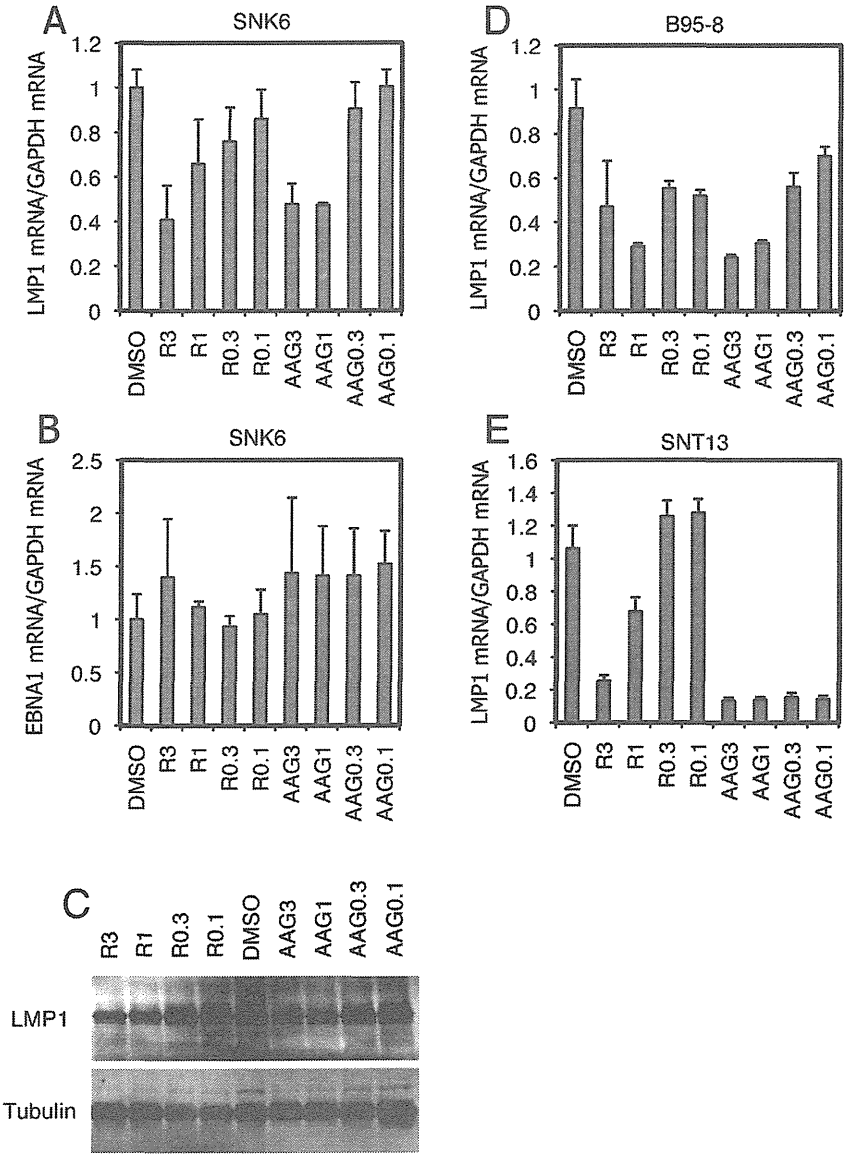

Figure 2. HSP90 inhibitors decrease LMP1 expression. (A, B) SNK6 cells were treated with Radicicol (R) or 17-AAG (AAG) at the concentrations of 3, 1, 0.3 or 0.1 μM. After 72 h, cell RNA was collected and subjected to Real-Time RT-PCR. Relative LMP1 levels (A) and EBNA1 levels (B) are shown after normalization to GAPDH mRNA levels. (C) SNK6 cells were treated with Radicicol (R) or 17-AAG (AAG) at the concentrations of 3, 1, 0.3 or 0.1 μM. After 72 h, cell proteins were collected and subjected to immunoblotting using anti-LMP1 and -Tubulin antibodies. (D, E) As in Fig. 2A, B95-8 (D) or SNT13 (E) cells were treated and subjected to Real-Time RT-PCR. Relative levels of LMP1 mRNA are shown after normalization to GAPDH mRNA levels. Each bar represents the mean and SD of three independent transfections.
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Results

Identification of HSP90 inhibitors on screening for small molecule inhibitors that repress LMP1 transcription

CAEBV and ENKL committed to type II latency express the major EBV oncogene, LMP1. We therefore conducted a search for small molecular inhibitors that repress expression of LMP1. As an initial screen, the EBV-positive NK cell lymphoma, SNK6, was treated with chemicals or the vehicle DMSO at concentrations of 3 or 10 μM for 3 days. Cellular RNAs were harvested and subjected to Real-Time RT-PCR. Among 300 small molecule substances with identified targets, we found Radicicol and 17-AAG, inhibitors of HSP90, to decrease LMP1 transcripts (Fig. 1). In order to further evaluate the effect, SNK6 cells were administered 3, 1, 0.3, 0.1 μM of Radicicol or 17-AAG (Fig. 2A–C). LMP1 levels decreased to 41% and 48% of control (DMSO) with 3 μM of Radicicol and 17-AAG, respectively (Fig. 2A), with EBNA1 levels appearing relatively unaffected (Fig. 2B). We then examined protein levels of LMP1 in Fig. 2C. After 72 h with 3 or 1 μM of Radicicol or 17-AAG, LMP1 protein levels were decreased in SNK6 cells (Fig. 2C), in a similar fashion with the transcript levels. LMP1 expression in B95-8 or EBV-positive T cell

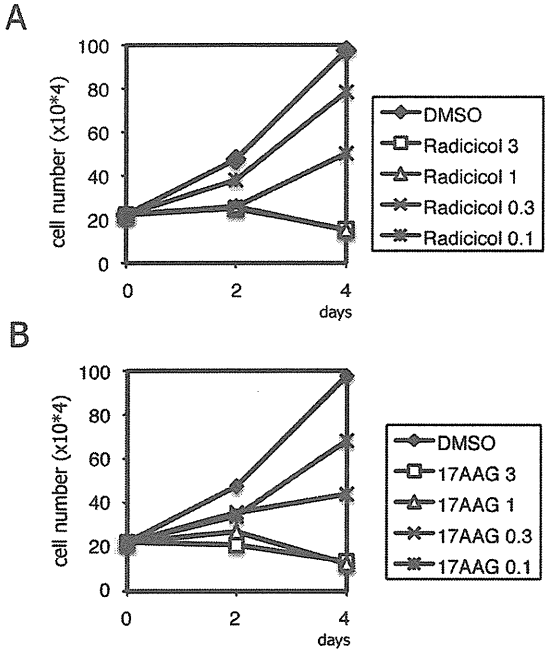

Figure 3. HSP90 inhibitors suppress cell proliferation of the EBV-positive SNK6 NK lymphoma line. SNK6 cells were cultured with Radicicol (A) or 17-AAG (B) at concentrations of 3, 1, 0.3 or 0.1 μM and cell numbers were counted on days 0, 2 and 4. Data are shown as the means of three independent replicates.
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line (SNT13) was also reduced by these HSP90 inhibitors (Fig. 2D, E). For unknown reasons, 17-AAG markedly suppressed LMP1 expression from SNT13 cells even at lower concentrations (Fig. 2E).

Suppression of cell proliferation *in vitro* and *in vivo*

After confirming that HSP90 inhibitors repress LMP1 expression in EBV-positive lymphoma cells, we then examined whether the inhibitors might actually suppress cancerous growth. We first tested at the cell culture level (Fig. 3). Three or 1 μM of Radicicol (Fig. 3A) or 17-AAG (Fig. 3B) could fully block proliferation of SNK6 cells depending on the concentration. Partial but appreciable inhibition was also exhibited at lower concentrations.

It has been already reported that HSP90 inhibitors block proliferation of EBV-positive malignancies, including NK/T lymphomas [27,28], although the earlier studies did not test LMP1 levels. For examination of effects *in vivo*, we here adopted a novel mouse xenograft model using severely immuno-deficient mice of the NOG strain [29], allowing assessment of whether the HSP90 inhibitors might repress EBV-positive malignant cells without seriously affecting other normal part of tissues and organs. The NOG mice were injected subcutaneously with 5×10^6 of SNK6 cells at day 0, and low doses of 17-AAG (50 mg/kg in total) or DMSO were administered into the abdominal cavity for 6 times between days 14 and 25. EBV genomic DNA titers in whole blood (Fig. 4A) and tumor sizes (Fig. 4B) were measured. As shown in Fig. 4, 17-AAG markedly reduced the viral titer in blood and growth of the NK lymphoma cells in NOG mice. Because engraftment of NK lymphomas in mice is not very efficient [29], only 50% of the mice developed subcutaneous tumor masses and others failed to nurture the lymphoma cells even in the DMSO

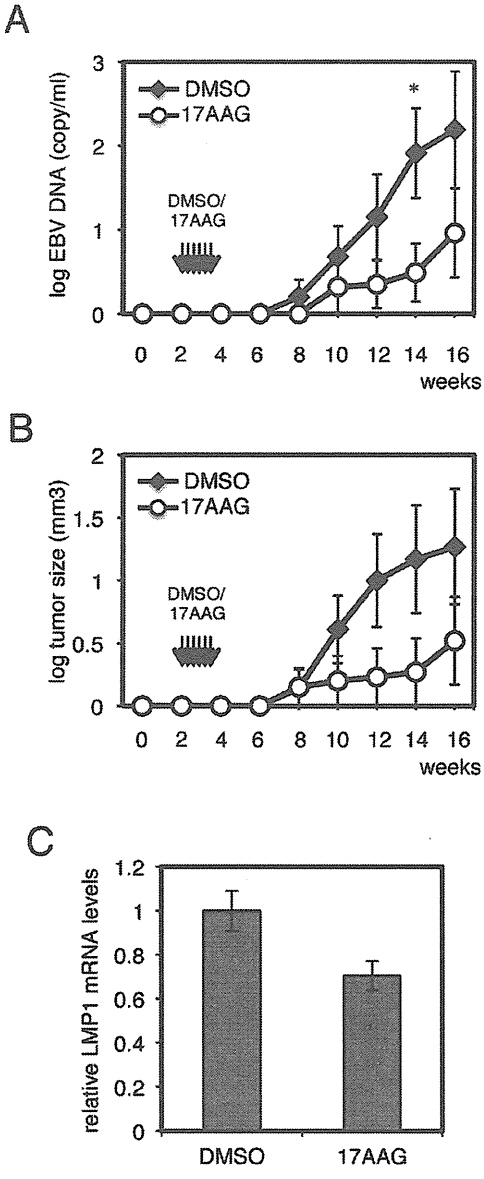

Figure 4. Effects of HSP90 inhibitors *in vivo*. HSP90 inhibitors repress EBV copy numbers in the whole blood (A) and tumor size (B) in mice implanted with EBV-positive NK cell lymphoma. Twenty-four NOG mice were subcutaneously implanted with 5×10^6 of SNK6 cells per mouse on day 0. From day 14, DMSO (vehicle) or 17-AAG (6 times in two weeks, 50 mg/kg in total) was injected to twelve mice each, intraperitoneally. Peripheral blood was collected from the tail veins once in two weeks, and levels of EBV genomic DNA in whole blood were measured by Real-Time PCR (A). Subcutaneous tumor masses (B) and LMP1 mRNA levels in the tumors (C) were also measured. *$p<0.05$ (Mann-Whitney *U* test).
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treatment group. Due to this low efficiency of transplantation, statistical significance could be exhibited only once for EBV DNA load (Fig. 4A, 14 weeks), but we gained the clear impression that 17-AAG worked more efficiently. This is an important difference when compared to LCLs, with which 100% of injected mice develop tumors, and thereby the efficacy of 17-AAG was exhibited more significantly [28]. We then compared the LMP1 expression

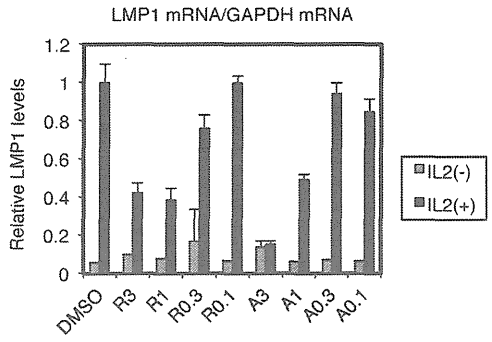

Figure 5. Suppression of LMP1 expression in EBV-positive NK cells by HSP90 inhibitors is likely dependent on IL-2. SNK6 cells, routinely cultured with IL-2, were washed extensively with PBS, and then cultured in the presence (black bars) or absence (gray bars) of IL-2 with Radicicol (R) or 17-AAG (A) at concentrations of 3, 1, 0.3 or 0.1 μM. After 72 h, cell RNAs were collected and subjected to Real-Time RT-PCR. Relative levels of LMP1 mRNA are shown after normalization to GAPDH mRNA levels. Each bar represents the mean and SD of three independent transfections.
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levels in the tumor developed in the absence or presence of 17-AAG (Fig. 4C). LMP1 transcript was lower in the tumor treated with 17-AAG, indicating that the inhibitor reduced LMP1 transctiption *in vivo*, too. To summarize, our results imply high potential of HSP90 inhibitors for treating EBV-positive cancers, including EBV-positive NK lymphomas.

Discussion

ENKL is an aggressive type of cancer often associated with resistance to chemotherapy, and accordingly a poor prognosis. Therefore, treatments are needed that specifically target its molecular determinants. We here focused on expression of the major oncogene of EBV, LMP1, and found two HSP90 inhibitors, Radicicol and 17-AAG, to both decrease LMP1 expression and cancerous growth of the EBV-positive SNK6 NK cell lymphoma line, *in vitro* and *in vivo*. Although the reduction in LMP1 levels by the inhibitors correlated with cell growth inhibition, we still cannot tell if low levels of LMP1 is responsible for the growth inhibition.

Jeon et al. previously reported that HSP90 inhibitors induce apoptotic cell death in EBV-positive NK/T lymphoma cells in vitro [27], but did not examine effects *in vivo*. Elsewhere, Sun et al. reported that HSP90 inhibitors block proliferation *in vitro* and *in vivo* mostly using EBV-positive LCLs [28], but NK/T lymphomas were not tested. Importantly, unlike ours, both of the previous papers did not include data on LMP1 levels.

LMP1 gene transcription differs between type II and type III latency infection. In the latter, LMP1 transcription is turned on by EBNA2 [30–32], whereas LMP1 expression is independent of EBNA2 in type II. In the previous work of Jeon and others induction of apoptosis by HSP90 inhibitors was considered to be through AKT signaling inhibition [27]. The decreased expression of LMP1 gene we observed here (Fig. 1, 2) might have been brought about through suppression of the AKT pathway. In latency II, including the ENKL case, it has been frequently reported that cytokines, such as IL-4, IL-6, IL-10, IL-13 and IL-21, activate the JAK/STAT pathway, thereby inducing LMP1 gene expression through STAT [33–38]. Because JAK/STAT signaling can also be blocked by HSP90 inhibitors [39], it may also be involved. Actually, since our EBV-positive NK/T cells are cultured routinely with IL-2 and it is involved in LMP1 expression [40], the cytokine and its downstream signaling may also be implicated in the reduction by HSP90 inhibitors. To examine this, we cultured SNK6 cells with or without IL-2 and HSP90 inhibitors, and measured the levels of LMP1 mRNA (Fig. 5). As expected from the previous report [40], depletion of IL-2 down-regulated LMP1 levels to 5.5% of control, in DMSO-treated cells. Treatment with Radicicol (R) or 17-AAG (A) at 1 μM or higher caused reduction of LMP1 levels in the presence of IL-2 (black bars), but it did not significantly decrease the levels without IL-2 (gray bars). This result suggests that HSP90 inhibitors suppress LMP1 expression, which is activated by IL-2, and that the cell signalings elicited by IL-2, such as JAK/STAT, are likely be responsible for the LMP1 reduction by HSP90 inhibitors. Besides JAK/STAT pathways, NFκB signaling must also be notified, because it is known that IL-2 elicits NFκB signaling [41], and HSP90 inhibitors can repress NFκB [42,43]. In addition, C/EBP contributes to LMP1 expression in type II [25], and may also be regulated by HSP90 inhibitors.

In type III latency, although EBNA2 does not feature DNA binding activity, it enhances LMP1 promoter activity by acting as a cofactor. It associates with cellular transcriptional factors, including the Recombination signal Binding Protein Jκ (RBP-Jκ [32,44] and PU-box 1 (PU.1) (also known as Spleen Forming Virus (SFV) Proviral Integration 1 (SPI1)) [30,31,45,46], which are then recruited onto the LMP1 promoter for transactivation. Because we found LMP1 expression was decreased by HSP90 inhibitors even in type III B95-8 cells (Fig. 2B), RBP-Jκ or PU.1 may also be under the control of HSP90. In fact, PU.1 is known to be inhibited by HSP90 inhibitors [47,48].

In summary, we here observed reduced expression of LMP1 and simultaneous growth suppressive effects of Radicicol and 17-AAG in EBV-positive lymphomas, especially NK lymphoma. Although the molecular mechanisms of how the HSP90 inhibitors block cell proliferation are elusive because HSP90 has a number of client proteins, our observation suggests that they could be potent therapeutic drugs for EBV-positive lymphomas. Rapid progress in the field of Hsp90 biology has brought about development of more potent and less toxic inhibitors. It is to be hoped that these may become useful as antiviral drugs against EBV-associated disorders.

Acknowledgments

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Author Contributions

Conceived and designed the experiments: TM SI HK TT. Performed the experiments: TM SI MNAS TK FG DK. Analyzed the data: TM SI HK TT. Contributed reagents/materials/analysis tools: TM SI DK HK TT. Wrote the paper: TM HK TT.