the subcutaneous tissue.[10] Recently, we reported three cases of HV-like lymphoma with EBV-infected γδ T cells using the FISH assay.[18] In five of seven patients in the present study (the three cases in the previous report were included), the EBER-positive cells were $CD3^+$ $CD4^-$ $CD8^-$ $TCR\gamma\delta^+$ T cells. The other two cases were of NK and possible NK T cell infection, respectively. These results indicate that γδ T cells play a central role in the formation of HV-like eruptions, although other types of cells can also be involved. This observation accords with other recent reports.[43,44] The γδ T cells are the major T cell population in the skin and mucosal epithelium. The γδ T cells secrete various cytokines and have cytolytic properties.[45] In the present study, EBER-positive γδ T cells were positive for Vδ2, suggesting that they were Vγ9Vδ2 T cells. The Vγ9Vδ2 T cells are the predominant γδ T cell subtype in human peripheral blood.[46] The γδ T cells sense not only infection, but also cellular stress. In patients with HV-like lymphoma, circulating EBV-positive Vγ9Vδ2 T cells may sense and react to cells damaged by ultraviolet radiation. Furthermore, EBER-positive γδ T cells were negative for CD122. A recent study showed that $CD122^-$ $\gamma\delta^+$ T cells produce interleukin (IL)-17.[47] Thus, EBER-positive γδ T cells may produce IL-17 and then induce and activate neutrophils and the epithelium, resulting in the formation of papulovesicular eruptions.

In conclusion, we applied the FISH assay to peripheral blood from 26 patients with EBV-associated T/NK LPD and confirmed that this assay was useful for the diagnosis of this condition. Furthermore, we found that two lymphocyte lineages were present in some patients with EBV-associated T/NK LPD. We showed that γδ T cells were present in peripheral blood from most cases of HV-like lymphoma. Thus, this assay is a direct and reliable method for quantifying and characterizing EBV-infected lymphocytes and can be used not only to complement pathological diagnosis, but also to clarify the pathogenesis of EBV-associated diseases and expand the spectrum of conditions known to be associated with this virus.

Acknowledgments

This study was supported, in part, by the Health and Labour Science Research Grant on Intractable Diseases (H22-Nanchi-080). The authors thank Syuko Kumagai and Fumiyo Ando for excellent technical support. They also thank Dr Atsushi Ogawa (Niigata Cancer Center Hospital), Dr Tsuyoshi Ito (Toyohashi Municipal Hospital), Dr Yoshinobu Beppu (Ooita Prefectural Mie Hospital), Dr Erina Sakamoto (Osaka City General Hospital), Dr Ayako Takusagawa (Ibaraki Children's Hospital), Dr Hirosada Miyake (Kumamoto University), Drs Sae Nishisho and Yasuo Horikoshi (Shizuoka Children's Hospital), Dr Yoji Sasahara (Tohoku University), Dr Mana Nishikawa (Sinnittestu Hirohata Hospital), Dr Rie Kanai (Shimane University), Dr Yumi Tomura (Yamaguchi University), Dr Utako Kaneko (Yokohama City University), Dr Yuka Okura (Hokkaido University), Dr Yasushi Isobe (Jyuntendo University), Dr Fumihiro Ishida (Shinshyu University), Dr Hajime Sakai (Teine Keijinkai Hospital), Drs Sumitaka Dono and Akihiko Maeda (Kochi University), Dr Koji Kato (Nagoya First Red Cross Hospital), and Dr Keiji Iwatsuki (Okayama University) for providing clinical specimens.

Disclosure Statement

The authors have no conflict of interest to declare.

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 Rickinson AB, Kieff E. Epstein–Barr virus. In: Knipe DM, Howly PM, eds. *Virology*, 5th edn. Philadelphia: Wolters Kluwer/Lippincott Williams & Wilkins, 2006; 2655–700.
4 Oshimi K. Progress in understanding and managing natural killer-cell malignancies. *Br J Haematol* 2007; **139**: 532–44.
5 Cohen JI, Kimura H, Nakamura S, Ko YH, Jaffe ES. Epstein–Barr virus-associated lymphoproliferative disease in non-immunocompromised hosts: a status report and summary of an international meeting, 8–9 September 2008. *Ann Oncol* 2009; **20**: 1472–82.
6 Kawa K, Okamura T, Yagi K, Takeuchi M, Nakayama M, Inoue M. Mosquito allergy and Epstein–Barr virus-associated T/natural killer-cell lymphoproliferative disease. *Blood* 2001; **98**: 3173–4.
7 Kimura H, Hoshino Y, Kanegane H *et al.* Clinical and virologic characteristics of chronic active Epstein–Barr virus infection. *Blood* 2001; **98**: 280–6.
8 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.
9 Ohshima K, Kimura H, Yoshino T *et al.* Proposed categorization of pathological states of EBV-associated T/natural killer-cell lymphoproliferative disorder (LPD) in children and young adults: overlap with chronic active EBV infection and infantile fulminant EBV T-LPD. *Pathol Int* 2008; **58**: 209–17.
10 Quintanilla-Martinez L, Kimura H, Jaffe ES. EBV+ T-cell lymphoma of childhood. In: Swerdlow SH, Campo E, Harris NL *et al.*, eds. *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*, 4th edn. Lyon: WHO Press, 2008; 278–80.
11 Park S, Lee DY, Kim WS, Ko YH. Primary cutaneous Epstein–Barr virus-associated T-cell lymphoproliferative disorder: 2 cases with unusual, prolonged clinical course. *Am J Dermatopathol* 2010; **32**: 832–6.
12 Takahashi E, Ohshima K, Kimura H *et al.* Clinicopathological analysis of the age-related differences in patients with Epstein–Barr virus (EBV)-associated extranasal natural killer (NK)/T-cell lymphoma with reference to the relationship with aggressive NK cell leukaemia and chronic active EBV infection-associated lymphoproliferative disorders. *Histopathology* 2011; **59**: 660–71.
13 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.
14 Kimura H, Ito Y, Suzuki R, Nishiyama Y. Measuring Epstein–Barr virus (EBV) load: the significance and application for each EBV-associated disease. *Rev Med Virol* 2008; **18**: 305–19.
15 Randhawa PS, Jaffe R, Demetris AJ *et al.* Expression of Epstein–Barr virus-encoded small RNA (by the EBER-1 gene) in liver specimens from transplant recipients with post-transplantation lymphoproliferative disease. *N Engl J Med* 1992; **327**: 1710–4.
16 Chuang SS, Lin CN, Li CY. Malignant lymphoma in southern Taiwan according to the revised European–American classification of lymphoid neoplasms. *Cancer* 2000; **89**: 1586–92.
17 Middeldorp JM, Brink AA, van den Brule AJ, Meijer CJ. Pathogenic roles for Epstein–Barr virus (EBV) gene products in EBV-associated proliferative disorders. *Crit Rev Oncol Hematol* 2003; **45**: 1–36.
18 Kimura H, Miyake K, Yamauchi Y *et al.* Identification of Epstein–Barr virus (EBV)-infected lymphocyte subtypes by flow cytometric *in situ* hybridization in EBV-associated lymphoproliferative diseases. *J Infect Dis* 2009; **200**: 1078–87.
19 Ito Y, Kawabe S, Kojima S *et al.* Identification of Epstein–Barr virus-infected CD27+ memory B-cells in liver or stem cell transplant patients. *J Gen Virol* 2011; **92**: 2590–5.
20 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.
21 Hoshino Y, Kimura H, Tanaka N *et al.* Prospective monitoring of the Epstein–Barr virus DNA by a real-time quantitative polymerase chain reaction after allogenic stem cell transplantation. *Br J Haematol* 2001; **115**: 105–11.
22 Chan JKC, Jaffe ES, Ralfkiaer E, Ko YH. Aggressive NK-cell leukeamia. In: Swerdlow SH, Campo E, Harris NL *et al.*, eds. *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*, 4th edn. Lyon: WHO Press, 2008; 276–7.
23 Chan JKC, Quintanilla-Martinez L, Ferry JA, Peh S-C. Extranodal NK/T-cell lymphoma, nasal type. In: Swerdlow SH, Campo E, Harris NL *et al.*, eds. *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*, 4th edn. Lyon: WHO Press, 2008; 285–8.
24 Pileri SA, Weisenburger DD, Sng I, Jaffe ES. Peripheral T-cell lymphoma, not otherwise specified. In: Swerdlow SH, Campo E, Harris NL *et al.*, eds.

WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues, 4th edn. Lyon: WHO Press, 2008; 306–8.
25 Henter JI, Horne A, Arico M *et al.* HLH-2004: diagnostic and therapeutic guidelines for hemophagocytic lymphohistiocytosis. *Pediatr Blood Cancer* 2007; **48**: 124–31.
26 Okano M, Kawa K, Kimura H *et al.* Proposed guidelines for diagnosing chronic active Epstein–Barr virus infection. *Am J Hematol* 2005; **80**: 64–9.
27 Kimura H, Hoshino Y, Hara S *et al.* Differences between T cell-type and natural killer cell-type chronic active Epstein–Barr virus infection. *J Infect Dis* 2005; **191**: 531–9.
28 Gotoh K, Ito Y, Ohta R *et al.* Immunologic and virologic analyses in pediatric liver transplant recipients with chronic high Epstein–Barr virus loads. *J Infect Dis* 2010; **202**: 461–9.
29 Shibata Y, Hoshino Y, Hara S *et al.* Clonality analysis by sequence variation of the latent membrane protein 1 gene in patients with chronic active Epstein–Barr virus infection. *J Med Virol* 2006; **78**: 770–9.
30 van Dongen JJ, Langerak AW, Bruggemann M *et al.* Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98–3936. *Leukemia* 2003; **17**: 2257–317.
31 Sandberg Y, van Gastel-Mol EJ, Verhaaf B, Lam KH, van Dongen JJ, Langerak AW. BIOMED-2 multiplex immunoglobulin/T-cell receptor polymerase chain reaction protocols can reliably replace Southern blot analysis in routine clonality diagnostics. *J Mol Diagn* 2005; **7**: 495–503.
32 Kuzushima K, Hoshino Y, Fujii K *et al.* Rapid determination of Epstein–Barr virus-specific CD8(+) T-cell frequencies by flow cytometry. *Blood* 1999; **94**: 3094–100.
33 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.
34 Kanegane H, Wado T, Nunogami K, Seki H, Taniguchi N, Tosato G. Chronic persistent Epstein–Barr virus infection of natural killer cells and B cells associated with granular lymphocytes expansion. *Br J Haematol* 1996; **95**: 116–22.
35 Kasahara Y, Yachie A. Cell type specific infection of Epstein–Barr virus (EBV) in EBV-associated hemophagocytic lymphohistiocytosis and chronic active EBV infection. *Crit Rev Oncol Hematol* 2002; **44**: 283–94.
36 Endo R, Yoshioka M, Ebihara T, Ishiguro N, Kikuta H, Kobayashi K. Clonal expansion of multiphenotypic Epstein–Barr virus-infected lymphocytes in chronic active Epstein–Barr virus infection. *Med Hypotheses* 2004; **63**: 582–7.
37 Ohga S, Ishimura M, Yoshimoto G *et al.* Clonal origin of Epstein–Barr virus (EBV)-infected T/NK-cell subpopulations in EBV-positive T/NK-cell lymphoproliferative disorders of childhood. *J Clin Virol* 2011; **51**: 31–7.
38 Calattini S, Sereti I, Scheinberg P, Kimura H, Childs RW, Cohen JI. Detection of EBV genomes in plasmablasts/plasma cells and non-B cells in the blood of most patients with EBV lymphoproliferative disorders using Immuno-FISH. *Blood* 2010; **116**: 4546–9.
39 Barrionuevo C, Anderson VM, Zevallos-Giampietri E *et al.* Hydroa-like cutaneous T-cell lymphoma: a clinicopathologic and molecular genetic study of 16 pediatric cases from Peru. *Appl Immunohistochem Mol Morphol* 2002; **10**: 7–14.
40 Chen HH, Hsiao CH, Chiu HC. Hydroa vacciniforme-like primary cutaneous CD8-positive T-cell lymphoma. *Br J Dermatol* 2002; **147**: 587–91.
41 Cho KH, Lee SH, Kim CW *et al.* Epstein–Barr virus-associated lymphoproliferative lesions presenting as a hydroa vacciniforme-like eruption: an analysis of six cases. *Br J Dermatol* 2004; **151**: 372–80.
42 Iwatsuki K, Satoh M, Yamamoto T *et al.* Pathogenic link between hydroa vacciniforme and Epstein–Barr virus-associated hematologic disorders. *Arch Dermatol* 2006; **142**: 587–95.
43 Tanaka C, Hasegawa M, Fujimoto M *et al.* Phenotypic analysis in a case of hydroa vacciniforme-like eruptions associated with chronic active Epstein–Barr virus disease of gammadelta T cells. *Br J Dermatol* 2012; **166**: 216–8.
44 Hirai Y, Yamamoto T, Kimura H *et al.* Hydroa vacciniforme is associated with increased numbers of Epstein–Barr virus-infected γδT cells. *J Invest Dermatol* 2012; **132**: 1401–8.
45 Kaufmann SH. Gamma/delta and other unconventional T lymphocytes: what do they see and what do they do? *Proc Natl Acad Sci USA* 1996; **93**: 2272–9.
46 Bonneville M, O'Brien RL, Born WK. Gammadelta T cell effector functions: a blend of innate programming and acquired plasticity. *Nat Rev Immunol* 2010; **10**: 467–78.
47 Shibata K, Yamada H, Nakamura R, Sun X, Itsumi M, Yoshikai Y. Identification of CD25+ gamma delta T cells as fetal thymus-derived naturally occurring IL-17 producers. *J Immunol* 2008; **181**: 5940–7.

doi: 10.1111/j.1349-7006.2012.02305.x

Published OnlineFirst June 6, 2012; DOI:10.1158/1078-0432.CCR-12-1064

Predictive Biomarkers and Personalized Medicine

Clinical Cancer Research

Pretreatment EBV-DNA Copy Number Is Predictive of Response and Toxicities to SMILE Chemotherapy for Extranodal NK/T-cell Lymphoma, Nasal Type

Yoshinori Ito[1], Hiroshi Kimura[2], Yoshinobu Maeda[5], Chizuko Hashimoto[6], Fumihiro Ishida[7], Koji Izutsu[8], Noriyasu Fukushima[10], Yasushi Isobe[9], Jun Takizawa[11], Yuichi Hasegawa[12], Hajime Kobayashi[13], Seiichi Okamura[14], Hikaru Kobayashi[16], Motoko Yamaguchi[17], Junji Suzumiya[15], Rie Hyo[3], Shigeo Nakamura[4], Keisei Kawa[18], Kazuo Oshimi[9], and Ritsuro Suzuki[3]

Abstract

Purpose: Extranodal NK/T-cell lymphoma, nasal type (ENKL) is an Epstein–Barr virus (EBV)–associated lymphoma for which a new chemotherapeutic regimen called SMILE (steroid, methotrexate, ifosfamide, L-asparaginase, and etoposide) recently showed promising results.

Experimental Design: The amount of EBV-DNA was prospectively measured in whole-blood and plasma samples by real-time quantitative PCR from 26 patients registered in the SMILE phase II study.

Results: Before treatment, the EBV-DNA was detected in 22 samples of whole blood with a median number of 3,691 copies/mL (range: 0–1.14×10^7), but 15 samples of plasma with a median of 867 copies/mL (range: 0–1.27×10^7). Results of these 2 measurements of EBV-DNA well correlated ($R^2 = 0.994$, $P < 0.001$). The overall response rate to SMILE was significantly higher in patients with less than 10^5 copies/mL of EBV-DNA in whole blood at enrollment (90% vs. 20%, $P = 0.007$) and in patients with less than 10^4 copies/mL of EBV-DNA in plasma (95% vs. 29%, $P = 0.002$). The incidence of grade 4 toxicity of SMILE other than leukopenia/neutropenia was significantly higher in patients with 10^5 copies/mL of EBV-DNA or more in whole blood (100% vs. 29%, $P = 0.007$) than that of others and in patients with 10^4 copies/mL or more in plasma (86% vs. 26%, $P = 0.002$).

Conclusions: These findings suggest that whole blood is more sensitive for clinical use than plasma. The EBV-DNA amount in whole blood was useful for predicting tumor response, toxicity, and prognosis after SMILE chemotherapy for ENKL. *Clin Cancer Res; 18(15); 4183–90. ©2012 AACR.*

Introduction

Epstein–Barr virus (EBV) causes a variety of benign and neoplastic diseases, including infectious mononucleosis, posttransplantation lymphoproliferative disorder (PTLD) and EBV-associated malignancies such as lymphomas, including extranodal NK/T-cell lymphoma, nasal type (ENKL), Hodgkin lymphoma, Burkitt lymphoma, age-associated large B-cell lymphoma, and several other T-cell lymphomas (1–3). ENKL is a rare subtype of non-Hodgkin lymphoma, mainly occurs in the nasal/paranasal area, skin, or gastrointestinal tract and is much more common in Asia and Latin America than in Western countries (4–6). The prognosis of ENKL was poor under conventional radiotherapy and/or chemotherapy (4, 7) but has recently improved by concurrent chemoradiotherapy (8, 9) or newly developed SMILE chemotherapy, comprising the steroid dexamethasone, methotrexate, ifosfamide, L-asparaginase, and etoposide (10, 11).

This type of lymphoma is invariably associated with episomal infection of EBV in the tumor cells, which implies its tumorigenic role. The presence of EBV-DNA in peripheral blood has been used as a surrogate marker for estimating tumor amount in several EBV-associated malignancies

Authors' Affiliations: Departments of [1]Pediatrics, [2]Virology, [3]Data Management and Biostatistics, and [4]Pathology, Nagoya University Graduate School of Medicine, Nagoya; [5]Department of Hematology and Oncology, Okayama University Graduate School of Medicine, Okayama; [6]Department of Medical Oncology, Kanagawa Cancer Center, Yokohama; [7]Department of Hematology and Oncology, Shinshu University School of Medicine, Matsumoto; [8]Division of Hematology, NTT Medical Center; [9]Department of Hematology, Juntendo University School of Medicine, Tokyo; [10]Department of Hematology and Oncology, Faculty of Medicine, Saga University, Saga; [11]Division of Hematology, Niigata University Graduate School of Medical and Dental Science, Niigata; [12]Department of Hematology, Tsukuba University School of Medicine, Tsukuba; [13]Department of Hematology, Obihiro Kosei Hospital, Obihiro; [14]Department of Hematology, National Hospital Organization Kyushu Medical Center; [15]Department of Hematology, Fukuoka University Chikushi Hospital, Fukuoka; [16]Department of Hematology, Japanese Red Cross Nagano Hospital, Nagano; [17]Department of Hematology and Oncology, Mie University Graduate School of Medicine, Tsu; and [18]Department of Pediatrics, Osaka Medical Center and Research Institute for Maternal and Child Health, Osaka, Japan

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Corresponding Author: Ritsuro Suzuki, Department of HSCT Data Management & Biostatistics, Nagoya University Graduate School of Medicine, 1-1-20 Daiko-Minami, Higashi-ku, Nagoya 461-0047, Japan. Phone: 81-52-719-1974; Fax: 81-52-719-1973; E-mail: r-suzuki@med.nagoya-u.ac.jp

doi: 10.1158/1078-0432.CCR-12-1064

Published OnlineFirst June 6, 2012; DOI:10.1158/1078-0432.CCR-12-1064

Translational Relevance

Peripheral blood of patients with extranodal NK-cell lymphoma, nasal type (ENKL) contains fragmented Epstein–Barr virus (EBV)-DNA. The amount of EBV-DNA can be a good marker for estimating the tumor burden and prognosis of ENKL patients. We recently developed a novel chemotherapeutic regimen, SMILE, comprising steroid, methotrexate, ifosfamide, L-asparaginase, and etoposide. The tumor response rate and survival rate was dramatically improved. However, it is known that the prognostic significance of certain factors may vary when the treatment modality changes. Therefore, the significance of EBV-DNA was analyzed in this study. Consequently, pretreatment whole blood and plasma EBV-DNA were predictive of response and prognosis. Multivariate analysis showed that plasma EBV-DNA was a significant prognostic factor. Furthermore, the EBV-DNA load was also predictive of adverse events by chemotherapy. Prediction of toxicity is particularly important for the SMILE regimen because it is excessively toxic for some patients.

(12–14). In particular, after organ transplantation, increasing loads of EBV in whole blood, lymphocytes, and plasma are associated with corresponding increases in the risk of PTLD (14). For nasopharyngeal carcinoma, plasma EBV-DNA load is known to be useful for monitoring disease activity and predicting the outcome of treatment (15, 16). The disease activity and prognosis of ENKL can also be monitored by measuring circulating EBV-DNA in plasma (17). For patients registered to the SMILE phase II study, we simultaneously conducted a prospective observational study, SMILE-EBV study, in which the amounts of EBV-DNA in whole blood and plasma were evaluated for ENKL.

Materials and Methods

Study design

The aim of this study is to evaluate the copy numbers of EBV-DNA from whole blood and plasma in patients with ENKL who received SMILE chemotherapy. The predictive value of EBV-DNA for tumor response, toxicity, and prognosis was analyzed, as well as the preference of samples from whole blood or plasma. The eligibility criteria, treatment, and response were described in the report of the phase II study (11). EBV-encoded small RNA (EBER) *in situ* hybridization positivity was counted in accordance with our previous study (18). A total of 38 patients were enrolled: 26 from Japan, 6 from Hong Kong, and 6 from South Korea. The amounts of EBV-DNA were measured in whole-blood and plasma samples from patients participating in this phase II study at 3 time points: before the treatment, after 2 courses of SMILE and after a series of treatments. Because of the lack of an international standardized method for quantification of EBV-DNA, the 26 patients from Japan were the subjects for this study. All samples were measured in a central laboratory. Registration onto the study was conducted by facsimile from the participating institutes to the C-SHOT Data Center (Nagoya, Japan), simultaneously with the entry into the SMILE study. The study was approved by both the Protocol Review Committee and the Institutional Review Board of each institution in Japan. Written informed consent was obtained from all of the patients. The study was registered with the University Hospital Medical Information Network Clinical Trials Registry (UMIN-CTR number, UMIN000000741), as an associated but separate study of SMILE phase II (UMIN-CTR number, UMIN000000712).

Response and toxicity criteria

Complete response was defined as the complete disappearance of all objective signs of disease, including enlarged lymph nodes or hepatomegaly and splenomegaly at restaging. Partial response was defined as at least a 50% reduction of tumor volume without the occurrence of new lesions at restaging. Progressive disease was defined as a greater than 25% increase in the sum of tumor lesions or the emergence of one or more new lesions or clinical symptoms that indicate disease progression. No response was defined as any response that did not fall into the categories defined above. If a patient died of any cause before day 42 of the second course of SMILE and could not undergo the defined restaging procedure, the patient's response was recorded as early death. The overall response rate (ORR) was defined as the proportion of all patients who could be evaluated for response who experienced complete or partial response. Toxicity was graded according to the Common Terminology Criteria for Adverse Events v3.0.

Quantification of EBV-DNA

A 5-mL patient peripheral blood was obtained, sent to the central laboratory (Nagoya University Graduate School of Medicine), and divided into whole-blood and plasma samples. DNA was extracted from 200 μL of either whole blood or plasma, using QIAamp DNA blood kits (Qiagen K.K.). A real-time quantitative PCR assay was carried out and the result was expressed as copies per 1 mL of sample, as previously described (19, 20). The minimum detection level was 2 copies per reaction that was equivalent to 100 copies/mL for whole blood or plasma.

Statistical analysis

Regression analysis compared the copy numbers in whole blood and plasma. Fisher exact test was used to compare the responses or toxicities to the SMILE chemotherapy. Mann–Whitney *U* test and Kruskal–Wallis test were used to compare the levels of EBV-DNA between patient groups. Cut-off value of the categorization by EBV-DNA levels were determined by the receiver operating characteristic analysis. Patient survival data were analyzed by the method of Kaplan and Meier and were compared by log-rank test. Univariate and multivariate analyses were carried out using Cox proportional hazard model. Data were analyzed with STATA version 11 and SPSS (SPSS) software.

Published OnlineFirst June 6, 2012; DOI:10.1158/1078-0432.CCR-12-1064

Table 1. Baseline patient characteristics (N = 26)

Characteristic	No. of patients (%)
Age, y	
Median	46.5
Range	17–67
Sex	
Male	14 (54)
Female	12 (46)
Disease state	
Newly diagnosed stage IV	12 (46)
First relapse	11 (42)
Refractory to the first-line treatment	3 (12)
"B" symptoms present	11 (42)
Elevated serum LDH	7 (27)
Performance status	
0	16 (61)
1	5 (19)
2	5 (19)
Detection of EBV-DNA in blood samples before treatment	
Whole blood +, Plasma +	14 (54)
Whole blood +, Plasma −	8 (31)
Whole blood −, Plasma +	1 (4)
Whole blood −, Plasma −	3 (12)

Results

Patient characteristics

The baseline characteristics of 26 eligible patients are listed in Table 1. The median age was 46.5 (range: 17–67) years, and the male:female ratio was 14:12. Twelve patients (46%) had newly diagnosed stage IV disease, 11 were in first relapse, and 3 were in the primary refractory status. EBER *in situ* hybridization was positive in all specimens, with a median positivity of 68% (range: 12%–96%) of lymphoma cells.

Amount of EBV-DNA and correlation between whole blood and plasma

EBV-DNA was detected in 22 samples of whole blood (median: 3.7×10^3, range: 0–1.1×10^7 copies/mL) and 15 samples of plasma (median: 8.7×10^2, range: 0–1.3×10^7 copies/mL). The level of EBV-DNA was not different among the 3 disease state (newly diagnosed, relapsed or refractory) groups at enrollment both in whole blood ($P = 0.19$ by Kruskal–Wallis test) and in whole blood ($P = 0.22$). An inconsistent result was seen in 9 patients. EBV-DNA was positive in whole blood but was negative in plasma in 8 patients. Conversely, in another patient, the EBV-DNA was only detected in plasma. EBV-DNA was not detected in either whole blood or plasma in 3 patients (nos. 9, 23, and 25). The concordance rate between whole blood and plasma was 65% (17 of 26). The viral DNA copy numbers were compared between whole blood and plasma before SMILE chemotherapy. A strong correlation was found between the amounts in whole blood and those in plasma ($r = 0.997$, $P < 0.001$, Fig. 1). No differences were found for the EBV-DNA level among patients with newly diagnosed stage IV, relapsed and refractory status ($P = 0.19$ for whole blood and $P = 0.24$ for plasma). No significant correlation was found between EBER positivity and plasma or whole blood EBV-DNA level (Supplementary Fig. S1).

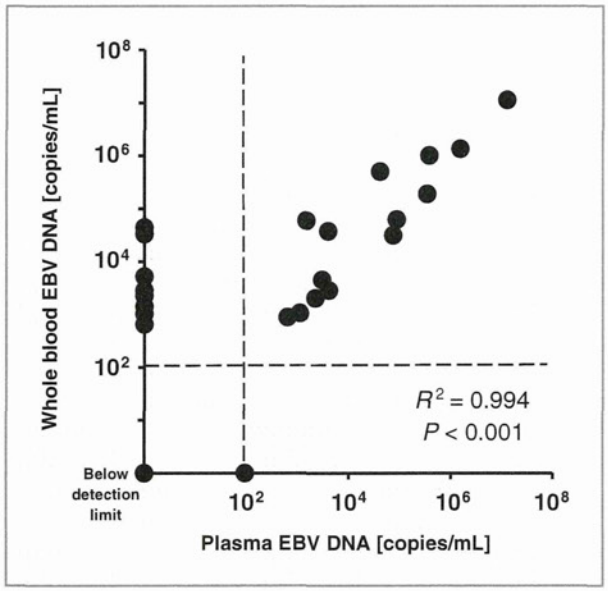

Figure 1. Comparison of EBV-DNA copies between whole blood and plasma in patients with ENKL who received SMILE chemotherapy. The EBV-DNA concentrations in whole blood or in plasma from the patients were measured using real-time PCR assay before SMILE chemotherapy. Dotted lines show the detection limits indicating 100 copies/mL of plasma or whole blood.

Dynamic changes of EBV loads in whole blood and plasma before and after treatment

EBV loads in whole blood or plasma from the 16 patients (8 with complete response, 7 with partial response, and 1 with progressive disease) were measured before the treatment, after 2 courses of SMILE chemotherapy, and after a series of treatments. Viral load declined in most patients with complete response or partial response after 2 courses of SMILE chemotherapy and/or after a series of treatments (Fig. 2). However, 5 patients with complete or partial response did not show the decrease of viral load. Of these, 2 patients experienced disease recurrence, and another patient died of transplant-related mortality in complete response. Other 2 patients maintained response at the time of last follow-up.

Correlation of the amount of EBV-DNA in blood samples and response or toxicities to the therapy

Among the 26 patients, there were 12 patients with complete response, 8 with partial response, 1 with no response, 3 with progressive disease, and 2 with early death (Table 2), and the ORR was 77%. For patients with less than

Published OnlineFirst June 6, 2012; DOI:10.1158/1078-0432.CCR-12-1064

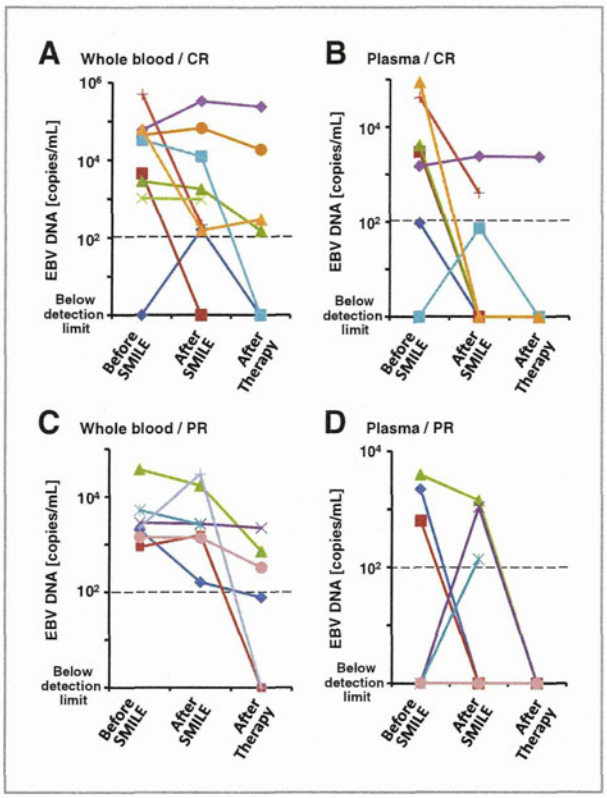

Figure 2. Serial analysis of EBV loads in blood samples from the patients with ENKL. The EBV-DNA concentrations in whole blood or in plasma from the patients were measured using real-time PCR assay before SMILE chemotherapy, after 2 courses of SMILE chemotherapy and after a series of treatments. A, viral loads in whole blood in patients with complete response. B, viral loads in plasma in patients with complete response. C, viral loads in whole blood in patients with partial response. D, viral loads in plasma in patients with partial response. Dotted lines show the detection limits indicating 100 copies/mL of plasma or whole blood. CR, complete response; PR, partial response.

10^5 copies/mL of EBV-DNA in whole blood, the ORR was 90% (19 of 21), but was 20% (1 of 5) in patients with 10^5 copies/mL or more ($P = 0.005$). In addition, the ORR was 95% (18 of 19) in patients with less than 10^4 copies/mL of EBV-DNA in plasma, but was 29% (2 of 7) in patients with 10^5 copies/mL or more ($P = 0.002$). All 3 patients without detectable EBV-DNA in either whole blood or plasma attained complete response. The amounts of EBV-DNA before treatment were not significantly different between patients with complete response and those with partial response (whole blood, $P = 0.82$; plasma, $P = 0.68$).

Grade 4 leukopenia (77%) and neutropenia (88%) were commonly observed. Grade 4 anemia was encountered in one patient and thrombocytopenia was seen in 9 patients. The nonhematologic grade 4 toxicities included infection ($n = 2$), alanine aminotransferase elevation ($n = 1$), and encephalopathy ($n = 1$); 3 patients experienced grade 4 somnolence, which was complicated by a grade 3 infection in one patient and by grade 4 encephalopathy in another patient. One patient experienced grade 2 pancreatitis and had complications from grade 4 hyponatremia, hyperamylasemia, and appetite loss. Grade 4 toxicity other than leukopenia/neutropenia was significantly higher in patients with 10^5 copies/mL of EBV-DNA or more in whole blood (100% vs. 29%, $P = 0.007$). Grade 4 toxicity other than leukopenia/neutropenia was also significantly higher in patients with 10^4 copies/mL of EBV-DNA or more in plasma (86% vs. 26%, $P = 0.002$; Table 2).

Prognostic significance of EBV-DNA

Patients with 10^5 copies/mL of EBV-DNA or more in whole blood showed significantly lower survival than those with less than 10^5 copies/mL (Fig. 3A, $P < 0.0001$). Similarly, the prognosis of patients with 10^4 copies/mL of EBV-DNA or more in plasma was significantly worse than that in those with less than 10^4 copies/mL (Fig. 3B, $P < 0.0001$). EBER positivity of more than 75% was also a factor

Table 2. Correlation of the levels of EBV-DNA and response/adverse events to SMILE chemotherapy for newly diagnosed stage IV, relapsed or refractory ENKL

	Whole blood EBV-DNA			Plasma EBV-DNA (copies/mL)		
	≥10^5 copies/mL	<10^5 copies/mL	*P*	≥10^4 copies/mL	<10^4 copies/mL	*P*
Response						
CR	1	11	0.005	2	10	0.002
PR	0	8		0	8	
NR	0	1		1	0	
PD	2	1		2	1	
ED	2	0		2	0	
Adverse event						
Any grade 4[a]	5	6	0.007	6	5	0.002
No grade 4	0	15		1	14	

Abbreviations: CR, complete response; ED, early death; PD, progressive disease; PR, partial response; NR, No response.
[a]Grade 4 adverse events other than leukopenia and neutropenia.

Published OnlineFirst June 6, 2012; DOI:10.1158/1078-0432.CCR-12-1064

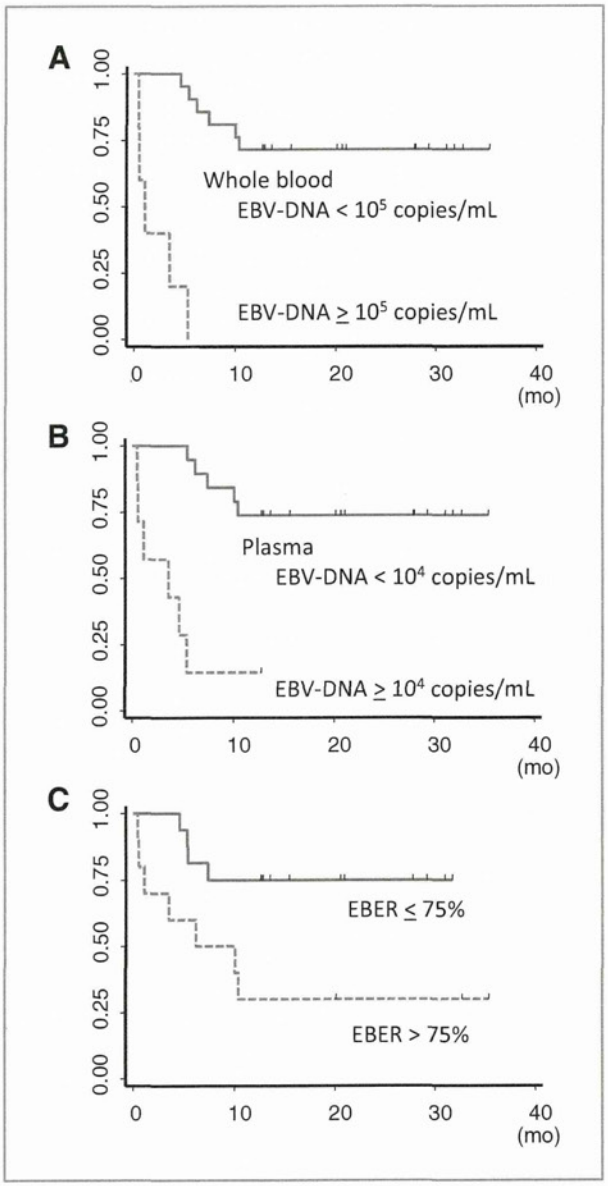

Figure 3. Survival of patients with ENKL who received SMILE chemotherapy by EBV parameters. A, overall survival was significantly lower for patients with a whole blood EBV-DNA level of 10^5 copies/mL or more ($P < 0.0001$). B, overall survival was significantly lower for patients with a plasma EBV-DNA level of 10^4 copies/mL or more ($P < 0.0001$). C, overall survival was significantly lower for patients with EBER positivity of more than 75% ($P = 0.02$).

associated with poor prognosis (Fig. 3C). Plasma and whole blood EBV-DNA before SMILE chemotherapy were significant prognostic factors for overall survival by univariate analysis, as well as serum lactate dehydrogenase (LDH) elevation, B symptom, and EBER positivity (Table 3). Multivariate analysis showed that LDH elevation [HR, 8.5; 95% confidence interval (CI), 1.9–38.0] and pretreatment whole blood EBV-DNA (HR, 65.5; 95% CI, 5.3–813.7) were significant prognostic factors. Plasma EBV-DNA was not prognostic (HR, 3.90; 95% CI, 0.70–21.8) if adjusted by LDH elevation using multivariate analysis. EBER positivity showed marginal significance (HR, 3.3; 95% CI, 0.95–11.8) if included in the model with LDH elevation.

Discussion

For EBV-associated malignancies, the significance placed on EBV-DNA in peripheral blood as a biomarker has increased in recent decades. Previous studies have reported that the level of EBV-DNA in a peripheral blood compartment is a useful biomarker in EBV-associated malignancies (14, 21). Lei and colleagues found a significant reduction of plasma EBV-DNA in patients with EBV-associated lymphoid malignancies (Hodgkin lymphoma, nasal NK/T cell lymphoma, PTLD, and Burkitt lymphoma) during the course of effective therapy (21). In addition, disease progression was associated with a rapid increase in plasma EBV-DNA levels in patients with ineffective therapy. Gandhi and colleagues showed that EBV-DNA is specifically detected in plasma of EBV-positive Hodgkin lymphoma patients before treatment (22). Viral DNA was undetectable following therapy in responsive patients and patients with long-term remission. Patients who experienced relapse had a significantly higher plasma EBV-DNA concentration before treatment. The plasma DNA concentration was persistently low or undetectable in patients with complete clinical remission. Overall survival and relapse-free survival were significantly higher for patients with a pretreatment plasma EBV-DNA level of less than 1,500 copies/mL. Au and colleagues reported that plasma EBV-DNA level is valuable as a tumor biomarker and for prognostication in EBV-positive lymphoma (17). EBV-DNA in plasma became undetectable for patients in remission but was elevated for those with refractory disease. A high level of EBV-DNA was significantly associated with inferior overall survival by multivariate analysis. Subgroup analysis of NK cell lymphoma showed that the level of EBV-DNA was also correlated with disease stage. Presentation of a high level of EBV-DNA was also significantly associated with inferior overall survival by multivariate analysis in their cohort. Prognostic factors of lymphoma may change when the treatment modality changes (23). In this study, however, EBV-DNA copy number in plasma or whole blood was also predictive of response and survival of ENKL patients who received SMILE chemotherapy, in agreement with other observations in the literature.

Another novel finding is that severe adverse events of the chemotherapy were also predictable using the EBV-DNA amount, which has not been identified by other studies in the literature. This analysis is only possible by examining patients who receive exactly the same treatment, ideally subjects of prospective studies. Because the level of EBV-DNA was not different by the 3 disease status groups (newly diagnosed, relapsed, or refractory), we examined the patients together in this study. As an interpretation of this finding, patients with higher tumor burden may experience more severe toxicity because of poor general condition or tissue damage by the tumor. Another hypothesis is that the

Published OnlineFirst June 6, 2012; DOI:10.1158/1078-0432.CCR-12-1064

Table 3. Prognostic factors affecting overall survival

		Univariate		Multivariate[a]	
Variables	**Unfavorable factors**	**Hazard ratio (CI)**	***P***	**Hazard ratio (CI)**	***P***
Age	>50 years	0.5 (0.2–1.9)	0.33	—	
LDH level	Elevated	8.6 (2.4–30.4)	0.001	8.5 (1.9–38.1)	0.005
B symptom	Present	5.0 (1.3–19.0)	0.02	—	
WB EBV-DNA	$\geq 10^5$ copies/mL	53.2 (5.9–482.0)	<0.001	65.5 (5.3–813.7)	0.001
Plasma EBV-DNA	$\geq 10^4$ copies/mL	10.3 (2.9–36.3)	<0.001	—	
EBER	>75%	4.0 (1.2–13.7)	0.03	—	

[a]Final model.

toxicity by chemotherapy is mediated by certain toxic substances in tumor cells. Because NK cells possess cytotoxic activities, almost all ENKL have cytotoxic molecules such as perforin or granzymes. In several EBV-associated malignancies, the high viral load may be explained by the tumor releasing viral components (24, 25). Toxic substances that are released from tumor cells degraded by chemotherapy such as SMILE, although they may not be cytotoxic molecules, may contribute to the high rate of adverse reactions after chemotherapy. Whatever the reason, measurement of EBV-DNA may be helpful for patient stratification to avoid excessive toxicity because the myelosuppressive adverse reaction of SMILE is rather profound for a part of patients.

Plasma is used as samples in most studies for evaluating EBV-DNA as a biomarker in EBV-associated disease (13–17, 22). However, controversies exist as to which blood compartment should be used for measuring EBV because several compartments of blood, whole blood, peripheral blood mononuclear cells, plasma, and serum can be used in the studies. Our previous study compared the usefulness of plasma and mononuclear cells for detecting EBV-DNA in ENKL patients, although the treatment was not unified (16). For the diagnosis of EBV-associated PTLD, earlier studies used peripheral blood mononuclear cells because EBV infection occurs in this cell compartment (26, 27). Plasma or serum samples are readily obtained and widely used for diagnosing EBV-associated PTLD; however, the sensitivity seemed to be low (28, 29). Several reports have revealed that whole blood, containing both cellular and humoral compartments, is better than plasma/serum when testing patients with PTLD (20, 30, 31). Recently, Spacek and colleagues reported that plasma is better than whole blood for the monitoring and estimation of prognosis for Hodgkin lymphoma (32). Plasma samples may be recommended as a biomarker of disease activity rather than peripheral blood mononuclear cells in patients with Hodgkin lymphoma, as shown in another study (20). However, comparison among each blood compartment has not been well investigated. Useful compartments may differ among diseases (14). In this study, the levels of EBV-DNA in plasma were compared with those in whole blood. Although strong correlation was detected between the viral copy numbers in whole blood and those in plasma, EBV-DNA was more frequently detected in whole-blood samples before treatment. Notably, EBV-DNA was only detected in whole blood in 8 patients, whereas it was only positive in plasma in one patient. This suggests that whole blood is more suitable than plasma to examine the EBV-DNA for ENKL. The reason for the phenomenon that EBV-DNA was only detected in whole blood remains undetermined. Among such 8 patients in this study, only 4 patients showed bone marrow involvement, and none accompanied leukemic presentation. The only possible explanation is that EBV-DNA might be lost or degraded in the fractionation procedure. Another point of interest is that EBV-DNA was not detected in either whole blood or plasma in 3 patients, although EBER was positive in tissue samples. Therefore, EBV-DNA detection in peripheral blood cannot be used as an alternative to the histologic detection of EBV or the diagnosis of ENKL. Moreover, the levels of 10^5 copies/mL of EBV-DNA in whole blood and 10^4 copies/mL of EBV-DNA in plasma seem to be cut-off values: the patients with copy numbers lower than these showed significantly better outcome. These 2 copy numbers also showed clinical value to predict severe adverse events.

In conclusion, our study indicates that the level of EBV-DNA in plasma or whole blood can predict response and adverse events of SMILE chemotherapy for newly diagnosed stage IV, relapsed, or refractory ENKL. Whole-blood samples were more suitable for this purpose, although plasma was preferable for other purposes such as diagnosis of EBV infection.

Disclosure of Potential Conflicts of Interest

R. Suzuki received honoraria from Kyowa-Hakko Kirin Company. K. Oshimi is currently an employee of Eisai Pharmaceutical Co., Ltd. (Tokyo, Japan). No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: M. Yamaguchi, J. Suzumiya, K. Kawa, K. Oshimi, R. Suzuki

Development of methodology: H. Kimura, K. Oshimi

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Ito, Y. Maeda, C. Hashimoto, F. Ishida, K. Izutsu, N. Fukushima, Y. Isobe, Y. Hasegawa, S. Okamura, H. Kobayashi, R. Hyo

Published OnlineFirst June 6, 2012; DOI:10.1158/1078-0432.CCR-12-1064

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Ito, F. Ishida, M. Yamaguchi, K. Oshimi, R. Suzuki
Writing, review, and/or revision of the manuscript: Y. Ito, H. Kimura, Y. Maeda, F. Ishida, K. Izutsu, Y. Isobe, M. Yamaguchi, K. Oshimi, R. Suzuki
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Ito, H. Kimura, J. Takizawa, S. Nakamura, K. Oshimi, R. Suzuki
Study supervision: K. Kawa, K. Oshimi, R. Suzuki

Acknowledgments

The authors thank staff at all participating institutions in this study: Tokyo Medical and Dental University, Kanagawa Cancer Center, Yokohama City University, Okayama University, Obihiro Kosei Hospital, Saga University, Yamanashi University, Shinshu University, NTT Medical Center Tokyo, Fukushima Prefectural Medical College, Kurashiki Central Hospital, Niigata University, Kyushu Medical Center, Nagano Red Cross Hospital, Tsukuba University, and Juntendo University.

The authors also thank the members of Central Pathology Review Board (Drs. Koichi Ohshima at Kurume University and Kengo Takeuchi at Cancer Institute), Central Imaging Review Board (Drs. Takao Kodama and Takanori Yano at Miyazaki University, and Yosuke Kakitsubata at Miyazaki Konan Hospital), and Data and Safety Monitoring Committee (Drs. Jin Takeuchi at Nihon University, Keizo Horibe at Nagoya Medical Center, and Keitaro Matsuo at Aichi Cancer Center) and Ms. Fumiyo Ando for excellent technical support for real-time quantitative PCR assay.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received March 30, 2012; revised May 16, 2012; accepted May 17, 2012; published OnlineFirst June 6, 2012.

References

1. Rickinson AB, Kieff E. Epstein-Barr Virus. In:Knipe DM, Howley PM, eds. Fields Virology. Philadelphia: Lippincott Williams & Wilkins; 2007:2655–700.
2. Cohen JI. Epstein-Barr virus infection. New Engl J Med 2000;343: 481–92.
3. Williams H, Crawford DH. Epstein-Barr virus: the impact of scientific advances on clinical practice. Blood 2006;107:862–9.
4. Chan JKC, Quintanilla-Martinez L, Ferry JA, Peh S-C. Extranodal NK/T-cell lymphoma, nasal type. In:Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, et al., eds. WHO classification of tumors of haematopoietic and lymphoid tissues. Lyon, France: IARC; 2008:285–8.
5. Au WY, Weisenburger DD, Intragumtornchai T, Nakamura S, Kim WS, Sng I, et al. Clinical differences between nasal and extranasal natural killer/T-cell lymphoma: a study of 136 cases from the International Peripheral T-Cell Lymphoma Project. Blood 2009;113:3931–7.
6. Suzuki R, Suzumiya J, Yamaguchi M, Nakamura S, Kameoka J, Kojima H, et al. Prognostic factors for mature natural killer (NK) cell neoplasms: aggressive NK cell leukemia and extranodal NK cell lymphoma, nasal type. Ann Oncol 2010;21:1032–40.
7. Oshimi K. Progress in understanding and managing natural killer-cell malignancies. Br J Haematol 2007;139:532–44.
8. Yamaguchi M, Tobinai K, Oguchi M, Ishizuka N, Kobayashi Y, Isobe Y, et al. Phase I/II study of concurrent chemoradiotherapy for localized nasal natural killer/T-cell lymphoma: Japan Clinical Oncology Group Study JCOG0211. J Clin Oncol 2009;27:5594–600.
9. Kim SJ, Kim K, Kim BS, Kim CY, Suh C, Huh J, et al. Phase II trial of concurrent radiation and weekly cisplatin followed by VIPD chemotherapy in newly diagnosed, stage IE to IIE, nasal, extranodal NK/T-Cell Lymphoma: Consortium for Improving Survival of Lymphoma study. J Clin Oncol 2009;27:6027–32.
10. Yamaguchi M, Suzuki R, Kwong YL, Kim WS, Hasegawa Y, Izutsu K, et al. Phase I study of dexamethasone, methotrexate, ifosfamide, L-asparaginase, and etoposide (SMILE) chemotherapy for advanced-stage, relapsed or refractory extranodal natural killer (NK)/T-cell lymphoma and leukemia. Cancer Sci 2008;99:1016–20.
11. Yamaguchi M, Kwong YL, Kim WS, Maeda Y, Hashimoto C, Suh C, et al. Phase II study of SMILE chemotherapy for newly diagnosed stage IV, relapsed, or refractory extranodal natural killer (NK)/T-cell lymphoma, nasal type: the NK-cell tumor study group study. J Clin Oncol 2011;29:4410–6.
12. Lei KI, Chan LY, Chan WY, Johnson PJ, Lo YM. Quantitative analysis of circulating cell-free Epstein-Barr virus (EBV) DNA levels in patients with EBV-associated lymphoid malignancies. Br J Haematol 2000;111: 239–46.
13. Chan KCA, Zhang J, Chan ATC, Lei KIK, Leung S-F, Chan LYS, et al. Molecular characterization of circulating EBV DNA in the plasma of nasopharyngeal carcinoma and lymphoma patients. Cancer Res 2003; 63:2028–32.
14. Kimura H, Ito Y, Suzuki R, Nishiyama Y. Measuring Epstein-Barr virus (EBV) load: the significance and application for each EBV-associated disease. Rev Med Virol 2008;18:305–19.
15. Lin JC, Wang WY, Chen KY, Wei YH, Liang WM, Jan JS, et al. Quantification of plasma Epstein-Barr virus DNA in patients with advanced nasopharyngeal carcinoma. N Engl J Med 2004;350: 2461–70.
16. Chan KC, Leung SF, Yeung SW, Chan AT, Lo YM. Quantitative analysis of the transrenal excretion of circulating EBV DNA in nasopharyngeal carcinoma patients. Clin Cancer Res 2008;14:4809–13.
17. Au WY, Pang A, Choy C, Chim CS, Kwong YL. Quantification of circulating Epstein-Barr virus (EBV) DNA in the diagnosis and monitoring of natural killer cell and EBV-positive lymphomas in immunocompetent patients. Blood 2004;104:243–9.
18. Suzuki R, Yamaguchi M, Izutsu K, Yamamoto G, Takada K, Harabuchi Y, et al. Prospective measurement of Epstein-Barr virus-DNA in plasma and peripheral blood mononuclear cells of extranodal NK/T-cell lymphoma, nasal type. Blood 2011;118:6018–22.
19. Kimura H, Morita M, Yabuta Y, Kuzushima K, Kato K, Kojima S, et al. Quantitative analysis of Epstein-Barr virus load by using a real-time PCR assay. J Clin Microbiol 1999;37:132–6.
20. Wada K, Kubota N, Ito Y, Yagasaki H, Kato K, Yoshikawa T, 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.
21. Lei KI, Chan LY, Chan WY, Johnson PJ, Lo YM. Diagnostic and prognostic implications of circulating cell-free Epstein-Barr virus DNA in natural killer/T-cell lymphoma. Clin Cancer Res 2002;8:29–34.
22. Gandhi MK, Lambley E, Burrows J, Dua U, Elliott S, Shaw PJ, et al. Plasma Epstein-Barr virus (EBV) DNA is a biomarker for EBV-positive Hodgkin's lymphoma. Clin Cancer Res 2006;12:460–4.
23. Sehn LH, Berry B, Chhanabhai M, Fitzgerald C, Gill K, Hoskins P, et al. The revised International Prognostic Index (R-IPI) is a better predictor of outcome than the standard IPI for patients with diffuse large B-cell lymphoma treated with R-CHOP. Blood 2007;109:1857–61.
24. Drouet E, Brousset P, Fares F, Icart J, Verniol C, Meggetto F, et al. High Epstein-Barr virus serum load and elevated titers of anti-ZEBRA antibodies in patients with EBV-harboring tumor cells of Hodgkin's disease. J Med Virol 1999;57:383–9.
25. Gallagher A, Armstrong AA, MacKenzie J, Shield L, Khan G, Lake A, et al. Detection of Epstein-Barr virus (EBV) genomes in the serum of patients with EBV-associated Hodgkin's disease. Int J Cancer 1999;84:442–8.
26. Savoie A, Perpete C, Carpentier L, Joncas J, Alfieri C. Direct correlation between the load of Epstein-Barr virus-infected lymphocytes in the peripheral blood of pediatric transplant patients and risk of lymphoproliferative disease. Blood 1994;83:2715–22.
27. Riddler SA, Breinig MC, McKnight JL. Increased levels of circulating Epstein-Barr virus (EBV)-infected lymphocytes and decreased EBV nuclear antigen antibody responses are associated with the development of posttransplant lymphoproliferative disease in solid-organ transplant recipients. Blood 1994;84:972–84.
28. Wadowsky RM, Laus S, Green M, Webber SA, Rowe D. Measurement of Epstein-Barr virus DNA loads in whole blood and plasma by TaqMan

Published OnlineFirst June 6, 2012; DOI:10.1158/1078-0432.CCR-12-1064

PCR and in peripheral blood lymphocytes by competitive PCR. J Clin Microbiol 2003;41:5245–9.

29. Hakim H, Gibson C, Pan J, Srivastava K, Gu Z, Bankowski MJ, et al. Comparison of various blood compartments and reporting units for the detection and quantification of Epstein-Barr virus in peripheral blood. J Clin Microbiol 2007;45:2151–5.
30. Stevens SJ, Pronk I, Middeldorp JM. Toward standardization of Epstein-Barr virus DNA load monitoring: unfractionated whole blood as preferred clinical specimen. J Clin Microbiol 2001;39: 1211–6.
31. Aalto SM, Juvonen E, Tarkkanen J, Volin L, Haario H, Ruutu T, et al. Epstein-Barr viral load and disease prediction in a large cohort of allogeneic stem cell transplant recipients. Clin Infect Dis 2007;45:1305–9.
32. Spacek M, Hubacek P, Markova J, Zajac M, Vernerova Z, Kamaradova K, et al. Plasma EBV-DNA monitoring in Epstein-Barr virus-positive Hodgkin lymphoma patients. APMIS 2010;119:10–6.

JVI
Journals.ASM.org

Pin1 Interacts with the Epstein-Barr Virus DNA Polymerase Catalytic Subunit and Regulates Viral DNA Replication

Yohei Narita,[a,c] Takayuki Murata,[a] Akihide Ryo,[b] Daisuke Kawashima,[a] Atsuko Sugimoto,[a,c] Teru Kanda,[a] Hiroshi Kimura,[c] Tatsuya Tsurumi[a]

Division of Virology, Aichi Cancer Center Research Institute, Chikusa-ku, Nagoya,[a] Department of Microbiology, Yokohama City University School of Medicine, Kanazawa-ku, Yokohama,[b] Department of Virology, Nagoya University Graduate School of Medicine, Showa-ku, Nagoya,[c] Japan

Peptidyl-prolyl *cis-trans* isomerase NIMA-interacting 1 (Pin1) protein is known as a regulator which recognizes phosphorylated Ser/Thr-Pro motifs and increases the rate of *cis* and *trans* amide isomer interconversion, thereby altering the conformation of its substrates. We found that Pin1 knockdown using short hairpin RNA (shRNA) technology resulted in strong suppression of productive Epstein-Barr virus (EBV) DNA replication. We further identified the EBV DNA polymerase catalytic subunit, BALF5, as a Pin1 substrate in glutathione *S*-transferase (GST) pulldown and immunoprecipitation assays. Lambda protein phosphatase treatment abolished the binding of BALF5 to Pin1, and mutation analysis of BALF5 revealed that replacement of the Thr178 residue by Ala (BALF5 T178A) disrupted the interaction with Pin1. To further test the effects of Pin1 in the context of virus infection, we constructed a BALF5-deficient recombinant virus. Exogenous supply of wild-type BALF5 in HEK293 cells with knockout recombinant EBV allowed efficient synthesis of viral genome DNA, but BALF5 T178A could not provide support as efficiently as wild-type BALF5. In conclusion, we found that EBV DNA polymerase BALF5 subunit interacts with Pin1 through BALF5 Thr178 in a phosphorylation-dependent manner. Pin1 might modulate EBV DNA polymerase conformation for efficient, productive viral DNA replication.

The Epstein-Barr virus (EBV) is a human gammaherpesvirus that mainly infects and establishes latent infection in B lymphocytes, but it also can infect other types of cells, such as NK, T, and epithelial cells.

EBV has both a latent state and a lytic replicative cycle in the nuclei of EBV-infected cells (1). During the latent phase of the EBV life cycle, the EBV genome is maintained as a circular plasmid molecule, which is amplified once in S phase by cellular DNA replication machinery. However, a small percentage of infected cells switch from the latent stage into the lytic cycle, which is triggered by the expression of an immediate-early protein, BZLF1, to produce progeny viruses. This type of activation contributes to the development and maintenance of human cancers (2, 3), suggesting that the EBV switching mechanism is also a key determinant of EBV pathogenesis. After induction of productive viral replication, the EBV genome is amplified 100- to 1,000-fold by viral replication machinery composed of BALF5 DNA polymerase (Pol), BMRF1 polymerase processivity factor, BALF2 single-stranded DNA-binding protein, and BBLF4-BSLF1-BBLF2/BBLF3 (BBLF2/3) helicase-primase complex via a rolling-circle mechanism in discrete sites in nuclei, called replication compartments (4, 5). BALF5 possesses intrinsic DNA polymerase and 3′-to-5′ exonuclease activities (6) and forms a complex with the BMRF1 polymerase accessory protein to exhibit high polymerase processivity (7). The DNA polymerase and exonuclease domains are highly conserved among a variety of DNA polymerases (8, 9). Unlike the case of the eukaryotic chromosomal replication apparatus, the EBV DNA Pol holoenzyme is used in the synthesis of both leading and lagging strands at the replication fork (6).

The peptidyl-prolyl bond has a low rate of spontaneous *cis-trans* isomerization. This is frequently a limiting step for protein folding and usually requires an isomerase to catalyze the process. Phosphorylation on a serine or threonine residue preceding proline (pSer/Thr-Pro) is a key regulatory mechanism, and the conformation of certain phosphorylated Ser/Thr-Pro bonds is regulated specifically by the prolyl isomerase Pin1 (10). The WW domain of Pin1 binds only to specific pSer/Thr-Pro motifs, which are isomerized by the peptidyl-prolyl isomerase (PPIase) domain to induce conformational changes in proteins (11). In this way Pin1 regulates various protein functions, including protein stability, catalytic activity, phosphorylation status, protein-protein interactions, and/or subcellular localization (11–14). Pin1 works in concert with protein kinases that phosphorylate Ser/Thr-Pro motifs, and protein phosphatases, in turn, can also be regulators of the process (15). Pin1 has a pivotal role in a variety of biological processes such as cell cycle control (16), and its deregulation contributes to various pathological conditions, most notably cancer (11, 12, 17, 18). Pin1 is overexpressed in various human cancers, contributing to centrosome amplification, chromosome instability, and tumor development *in vitro* and *in vivo* and correlating with poor clinical outcomes (10, 19–22). In contrast, inhibition of Pin1 suppresses tumorigenesis *in vitro* (23) and prevents cancer development induced by overexpression of oncogenes such as Neu or Ras (24) or by knockout of tumor suppressors such as p53 (25) in mice.

Thus, Pin1 has key roles in control of cellular functions. However, its significance for EBV replication has yet to be clarified in detail. In this study, we show that Pin1 interacts with EBV DNA polymerase BALF5 and modulates productive viral DNA replica-

Received 25 September 2012 **Accepted** 27 November 2012
Published ahead of print 5 December 2012
Address correspondence to Tatsuya Tsurumi, ttsurumi@aichi-cc.jp, or Takayuki Murata, tmurata@aichi-cc.jp.

doi:10.1128/JVI.02634-12

tion. Because there is a very limited number of anti-EBV drugs developed or being developed to date, including acyclic nucleoside analogs, such as acyclovir, and kinase inhibitors, such as maribavir (26, 27), a search for an effective molecular target has been needed. Pin1 may be a potential target for development of novel antiviral drugs.

MATERIALS AND METHODS

Cell culture and reagents. HEK293T and HEK293 EBV-bacterial artificial chromosome (BAC) cells were maintained in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum. B95-8 cells and an EBV-negative cell clone derived from Akata cells [Akata(−)] were cultured in RPMI medium supplemented with 10% fetal bovine serum. To induce lytic EBV replication, tetradecanoyl phorbol acetate (TPA), A23187, and sodium butyrate were added to the culture medium at final concentrations of 20 ng/ml, 1 μM, and 5 mM, respectively.

Antibodies. Rabbit anti-BZLF1, -BMRF1, -BALF2, and -BALF5 antibodies were as reported previously (28). An anti-EBV EA-D-p52/50 (BMRF1 gene product) protein-specific mouse monoclonal antibody, clone R3, was purchased from Chemicon Inc. Anti-Pin1 (H-123 and G-8) and anti-α/β-tubulin (2148) antibodies were purchased from Santa Cruz Biotechnology, Inc., and Cell Signaling, respectively. Horseradish peroxidase (HRP)-linked goat antibodies to rabbit IgG were from Amersham Biosciences.

shRNA and siRNA. Knockdown of Pin1 with short hairpin (shRNA) was carried out as described previously (29). As a control, we targeted the luciferase gene (designated shluc). Oligonucleotide sequences for the Pin1 shRNA (shPin1) were 5′-GATCCGCCGAGTGTACTACTTCAATTCAA GAGATTGAAGTAGTACACTCGGCTTTTTTAT-3′ (shPin1 for) and 5′-CGATAAAAAAGCCGAGTGTACTACTTCAATCTCTTGAATTGA AGTAGTACACTCGGCG-3′ (shPin1 rev), and the sequence for shluc was as noted previously (29). Duplexes of 21-nucleotide small interfering RNAs (siRNAs) were synthesized and annealed (Gene Design, Inc.). The sense and antisense sequences of the duplex were 5′-GCCAUUUGAAGA CGCCUCGdTdT-3′ and 5′-CGAGGCGUCUUCAAAUGGCdTdT-3′ for Pin1 and 5′-GCAGAGCUGGUUUAGUGAAdTdT-3′ and 5′-UUCA CUAAACCAGCUCUGCdTdT-3′ for the control siRNA.

Measurement of the viral genome by qRT-PCR. Cells were harvested at the time indicated in the figure legends and lysed with 200 μl of PCR lysis buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 0.001% Triton X-100, and 0.001% SDS). After treatment with 25 μg of proteinase K at 50°C for 2 h, samples were boiled at 95°C for 10 min. Quantitative real-time PCR (qRT-PCR) was performed in 10 μl of solution containing 1 μM each forward and reverse primer, 5 μl of FastStart Universal Probe Master (Rox) (containing 6-carboxy-X-rhodamine [ROX] dye; Roche Applied Science), 0.5 μl of eukaryotic 18S rRNA endogenous control (Applied Biosystems), and 1 μl of prepared sample DNA in PCR lysis buffer. The intensity of ROX dye was used to compensate for volume fluctuations among the tubes. PCR included 2 min at 50°C and 10 min at 95°C and then 40 cycles at 95°C for 15 s, followed by 1 min at 60°C. Immediately after the PCR, we carried out dissociation curve analysis and confirmed the specificity of each PCR product. A standard curve was constructed using serial dilutions of DNA and was used to quantify the amount of DNA. Primers and a probe for detection of the viral genome were designed using Primer Express (Applied Biosystems) within the BALF2-coding region. The sequences were as follows: 5′-GCCCGTCCG GTTGTCA-3′ (forward primer), 5′-AATATCTGGTTGTTGCCGTTG A-3′ (reverse primer), and 5′-FAM-CTGCCAGTGACCATCAACAAGT ACACGG-TAMRA-3′ (probe; where FAM is 6-carboxyfluorescein and TAMRA is tetramethyl rhodamine).

GST pulldown assays. For bacterial expression of glutathione *S*-transferase (GST)-tagged Pin1 (wild type [WT] or the W34A mutant), *Escherichia coli* strain DH5α was transformed with the pGEX expression vector for each protein (10). Expression of GST fusion proteins was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG; 0.5 mM), followed by incubation at 25°C for 4 h. GST pulldown assays were conducted as described previously (10). In brief, B95-8 or HEK293 cell proteins were lysed in GST lysis buffer at 4°C. After sonication and centrifugation (at 20,000 × *g* for 10 min at 4°C), the supernatant was preincubated with glutathione-Sepharose beads (GE Healthcare) for 30 min at 4°C. Afterwards, the supernatant was mixed with 50 μg of GST fusion protein and glutathione beads for 1 h at 4°C with rotation. The beads were then washed with GST lysis buffer five times and subjected to immunoblotting.

Transfection and IP. Cells were transfected with appropriate plasmids using Lipofectamine 2000 reagent (Invitrogen) or by electroporation using a Microporator (Disital Bio). The total amounts of plasmid DNAs were standardized by addition of an empty vector. For immunoprecipitation (IP), cells were solubilized in 200 μl of 0.5% Nonidet P-40 buffer (10 mM Tris-HCl [pH 7.5], 100 mM NaCl, 0.5% Nonidet P-40, and protease and phosphatase inhibitor mixture). Cell extracts were then diluted with 800 μl of lysis buffer and precleared with protein G-Sepharose (GE Healthcare). Supernatants were then mixed with protein G-Sepharose and antibody and then incubated at 4°C for 4 h with rotation. Immunocomplexes were washed five times with the same buffer. Samples were subjected to SDS-PAGE, followed by immunoblotting with the antibodies indicated in the figures and figure legends. We used TrueBlot anti-rabbit IgG HRP-conjugated antibodies (eBioscience) as the secondary antibody to eliminate the immunoglobulin heavy chain/light chain-specific band.

BALF5 expression plasmid and mutagenesis. The expression vector for BALF5 was made by inserting the BALF5 open reading frame into the EcoRI/HindIII site of pcDNA3.1 (Invitrogen). Mutant vectors were generated by a PCR-based method using the following primers: TTCCATGT CTACGACATACTC (BALF5Δ1 For), ACACTTGGGAATGAGACGC (BALF5Δ1 Rev), AAGGTCACGCGCCGTTCCATT (BALF5Δ2 For), GA GGACTGCAAACTCCACGTC (BALF5Δ2 Rev), AGAAGAGCACAGGC TAGCC (BALF5Δ3 For), TTGTAGAATCCGGACAGGGG (BALF5Δ3 Rev), CTTCGAGTCATCTACGGGGAC (BALF5Δ4 For), GATGGAGAG GCAGGGAAAG (BALF5Δ4 Rev), GCCCCCTGCCGGGTCTCGG (BALF5-T178A For), and CCTGCGGTCGAAGGTGCTGG (BALF5-T178A Rev). The DNA sequence of each vector was confirmed by DNA sequencing using the following primers: GCATCGTCATCAAGCTACTG (BALF5-1), AGCTCGAGTACGACTGTGAG (BALF5-2), CACATCTAC AGCATCAACCC (BALF5-3), GATCCGCGTGTTCTCCTGC (BALF5-4), CCTTCTTGGCTAGTCTGTTG (BALF5-5), and TCCTGCCTGATG CTGATTAC (BALF5-6).

Genetic manipulation of EBV-BAC DNA. EBV-BAC DNA was provided by W. Hammerschmidt (30). Homologous recombination was undertaken in *E. coli* as described previously (28, 29, 31) with the following oligonucleotide primers: 5′-TGTGTGAACGTGTTTGGGCAGCAGGCC TACTTCTACGCCAGCGCGCCTCAGGGTCTGGACGGCCTGGTG ATGATGGCGGGATC-3′ (Neo/stFor), 5′-CGCGTGGCATCCACGTTG GCCTCAAAGATCCGACACCCGTGCTTGTCTTGCCACGTGTCAGA AGAACTCGTCAAGAAGG-3′ (Neo/stRev), 5′-AAGCCCTCTGGACTT CCATG-3′ (Transfer vector For), and 5′-CATTGTCCAGGACAAAGCG G-3′ (Transfer vector Rev). Electroporation was performed using a Gene Pulser III (Bio-Rad), and purification of EBV-BAC DNA was achieved with NucleoBond Bac100 (Macherey-Nagel, Germany).

RESULTS

Productive EBV DNA replication is strongly suppressed by knockdown of Pin1. To determine whether Pin1 might influence productive EBV replication, we first carried out knockdown experiments. In HEK293 EBV-BAC cells featuring EBV latent infection, Pin1 expression was suppressed by shRNA (shPin1) transduction (Fig. 1A). An shRNA against the luciferase gene served as the control. Cells were transfected with empty vector pcDNA3 or pBZLF1, an expression vector for BZLF1, the molecular switch from latent to lytic infection of EBV. After 24 h, levels of viral and cellular proteins were examined by immunoblotting (Fig. 1A). In the control HEK293 EBV-BAC shluc cells, BZLF1 transfection

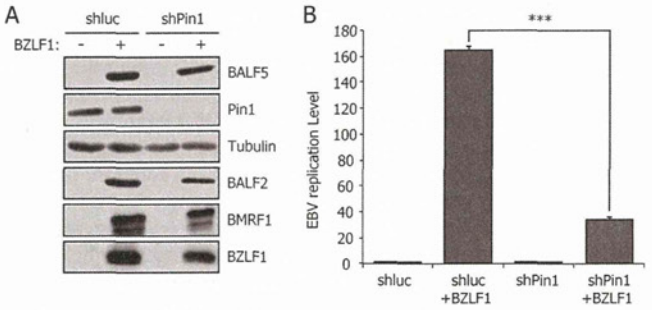

FIG 1 Knockdown of Pin1 decreases the level of EBV viral replication. (A) HEK293 EBV-BAC cells, transduced with control shRNA (shluc) or shRNA for Pin1 (shPin1), were transfected with 50 ng of BZLF1 expression vector or empty vector (pcDNA3). After 24 h, aliquots of cells were harvested and subjected to immunoblotting with the indicated antibodies. (B) Remaining cells transfected in panel A were subjected to qRT-PCR assays 60 h after transfection. The amount of EBV viral DNA was quantified and standardized with an 18S ribosome probe. Each bar represents the mean and standard deviation of three independent transfections and quantifications. ***, $P < 0.002$.

induced expression of viral genes such as BALF5, BALF2, and BMRF1, as expected, and knockdown of Pin1 (shPin1) had little effect on the expression levels.

We next checked the levels of viral DNA by qRT-PCR (Fig. 1B). The amount of synthesized EBV DNA was drastically and significantly reduced in Pin1-depleted cells upon induction compared to that in control cells, while levels of intrinsic, latent EBV genome copy numbers were comparable (Fig. 1B). Similar results were also obtained in lymphocytes (data not shown).

Pin1 interacts with EBV DNA polymerase BALF5. Since Pin1 was shown to contribute to EBV lytic replication (Fig. 1B), we next examined if certain EBV proteins could interact with Pin1 by GST pulldown assay. Whole-cell lysate from B95-8 cells treated with TPA-A23187-butyrate was incubated with purified GST-Pin1, GST-Pin1 W34A or GST alone expressed in bacteria. The W34A mutant of Pin1 served as a negative control because it cannot bind to the Ser/Thr-Pro motif of the substrates due to the mutation in its WW domain. We found that the viral DNA polymerase catalytic subunit, BALF5, was specifically and repeatedly coprecipitated with GST-Pin1 but not with GST alone or GST-Pin1 W34A

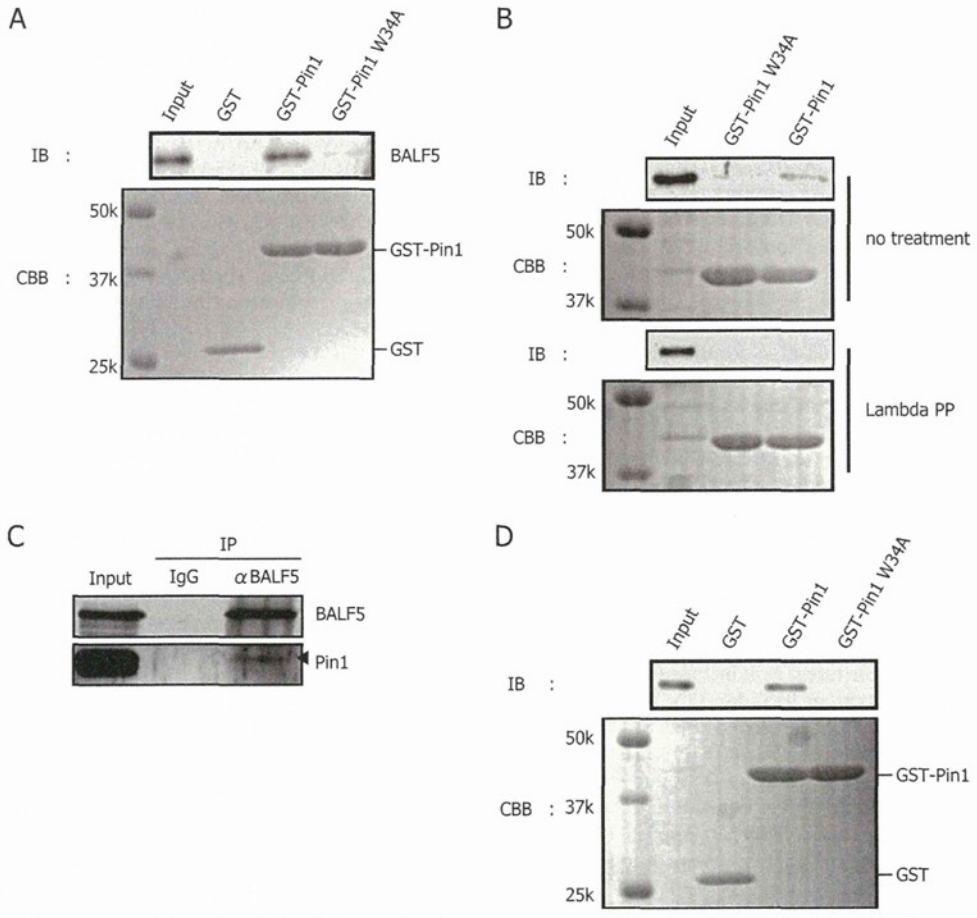

FIG 2 Pin1 interacts with EBV DNA polymerase BALF5. (A) GST-Pin1 binds to BALF5. Proteins from B95-8 cells, induced with TPA, A23187, and sodium butyrate for 24 h, were harvested and lysed in GST lysis buffer. GST pulldown assays were carried out using GST, GST-Pin1, or GST-Pin1 W34A. Pin1 W34A cannot bind with target proteins because of the mutation in its WW domain. (B) Phosphorylation-dependent association of Pin1. A GST pulldown assay was carried out as described for panel A except that the B95-8 cell lysate was incubated with lambda protein phosphatase (PP) (New England BioLabs) for 30 min at 30°C as indicated on the figure. (C) Immunoprecipitation assays confirmed the interaction. Cell proteins from lytic B95-8 lysate were subjected to immunoprecipitation using anti-BALF5 antibodies or normal IgG. The precipitates were then immunoblotted using anti-BALF5 or -Pin1 antibodies. α, anti. Arrowhead indicates size of Pin1. (D) Exogenously overexpressed BALF5 can bind to Pin1. Cell proteins from HEK293T cells transfected with BALF5 expression vector were subjected to GST pulldown assay. IB, immunoblotting; CBB, Coomassie brilliant blue. k, kilodaltons.

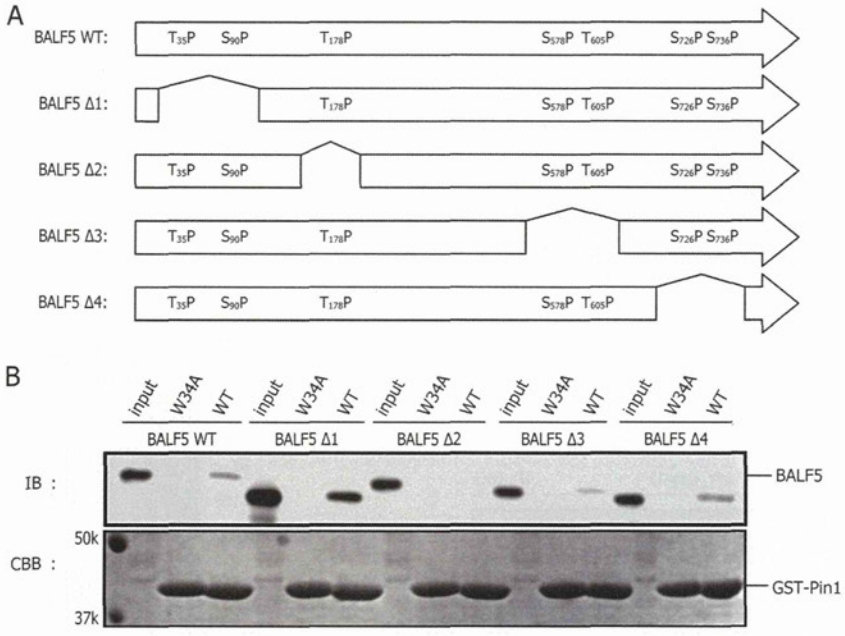

FIG 3 Pin1 interacts with BALF5 Thr178. (A) Scheme of BALF5 truncated mutants, featuring deletion of amino acids 34 to 93 (BALF5Δ1), 167 to 185 (BALF5Δ2), 578 to 607 (BALF5Δ3), and 704 to 748 (BALF5Δ4). Primers used for constructing these mutants are listed in Materials and Methods. (B) Cell proteins lysed from HEK293T cells, transfected with the WT BALF5 expression vector or its derivatives shown in panel A, were subjected to GST pulldown assay.

(Fig. 2A). Other than BALF5, viral factors, such as BZLF1, BMRF1, BBLF4, BBLF2/3, and BSLF1, were not copurified with Pin1 in our GST pulldown assays (data not shown).

To test if the interaction was phosphorylation dependent, an aliquot of the lysate was treated with lambda phosphatase while the rest was left untreated (Fig. 2B). Dephosphorylation by the phosphatase diminished the interaction (Fig. 2B), suggesting that the two proteins associate in a phosphorylation-dependent manner (Fig. 2B).

Immunoprecipitation assays were next conducted in order to confirm the association between endogenous Pin1 and BALF5 in B95-8 cells. Pin1 was coimmunoprecipitated with BALF5, as expected (Fig. 2C).

For the experiments shown in Fig. 2A to C, we collected viral proteins from EBV-positive B95-8 cells after induction of lytic replication and demonstrated that Pin1 interacts with BALF5 in a phosphorylation-dependent manner. Since EBV encodes a protein kinase, BGLF4, we then examined whether phosphorylation of BALF5 by the viral kinase BGLF4 was necessary for the Pin1-BALF5 association. To this end, EBV-negative HEK293T cells were transfected with a expression vector plasmid for BALF5, and at 24 h posttransfection, GST pulldown assays were carried out using the lysate (Fig. 2D). Because the interaction between BALF5 and Pin1 was reproduced in an overexpression system, the viral kinase is apparently not a prerequisite for the interaction, and cellular kinases, such as mitogen-activated protein kinases (MAPKs) or cyclin-dependent kinases (CDKs), may be sufficient to mediate the association (17).

Identification of the Ser/Thr-Pro motif in BALF5 required for association with Pin1. Since phospho-Ser/Thr-Pro is the binding motif for Pin1, we searched for its presence in BALF5 protein. As shown in Fig. 3A (top panel), BALF5 has seven Ser/Thr-Pro motifs. In order to map the domain in BALF5 important for the interaction, we generated a series of BALF5 truncation mutants (Fig. 3A). There are four Ser-Pro (Ser90, Ser578, Ser726, and Ser736) and three Thr-Pro (Thr35, Thr178, and Thr605) motifs in the EBV BALF5 protein. We prepared mutants designated BALF5Δ1, BALF5Δ2, BALF5Δ3, and BALF5Δ4, featuring deletion of amino acids 34 to 93, 167 to 185, 578 to 607, and 704 to 748, respectively. HEK293T cells were transfected with BALF5 wild type (WT) or its mutants, and at 24 h posttransfection whole-cell extracts were subjected to GST pulldown assays. Among the truncation mutants, BALF5Δ2 exhibited attenuated association with GST-Pin1, whereas other mutants showed comparable binding ability with the wild type (Fig. 3B). As the truncated BALF5Δ2 mutant lacks BALF5 Thr178-Pro, the motif is suggested to be a potential candidate Pin1 binding site. Pulldown of BALF5Δ1 with GST-Pin1 appeared strong, but we believe the result was just an artifact simply because the input level of the protein was also high.

Considering BALF5 T178 as the Pin1 binding motif, we next constructed an alanine substitution mutant, designated BALF5 T178A. BALF5 WT or BALF5 T178A was expressed in HEK293T cells, and then cell lysates were subjected to GST pulldown assays (Fig. 4A). Levels of copurified BALF5 T178A with GST-Pin1 were markedly lower than with the wild type, as expected (Fig. 4A). Furthermore, immunoprecipitation assays also confirmed that the T178A mutation diminished the BALF5 association with Pin1 (Fig. 4B). Therefore, we conclude that BALF5 Thr178-Pro is the Pin1 binding target.

BALF5 T178 is important for viral DNA polymerase activity. Our experiments indicated only one Pin1 binding site in the BALF5 amino acid sequence. To further extend and verify the finding, recombinant EBV with BALF5 deletion was prepared. As shown in Fig. 5A, part of the BALF5 sequence encompassing the Pin1 binding site (Thr178) was replaced with a marker cassette (for neomycin resistance and streptomycin sensitivity [Neo/st]).

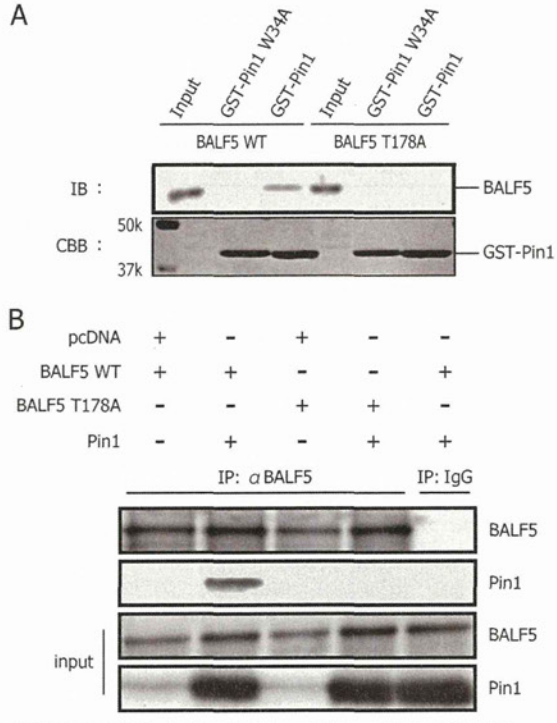

FIG 4 BALF5 T178A diminishes binding ability to Pin1. (A) Cell lysates from HEK293T cells, transfected with BALF5 wild type or T178A, were subjected to GST pulldown assays. (B) HEK293T cells were transfected with expression vectors for BALF5 wild type or T178A, with or without the expression vector for Pin1, as indicated. Cell proteins were lysed and subjected to immunoprecipitation using anti-BALF5 antibodies or normal IgG. The precipitates were then immunoblotted using anti-BALF5 or -Pin1 antibodies.

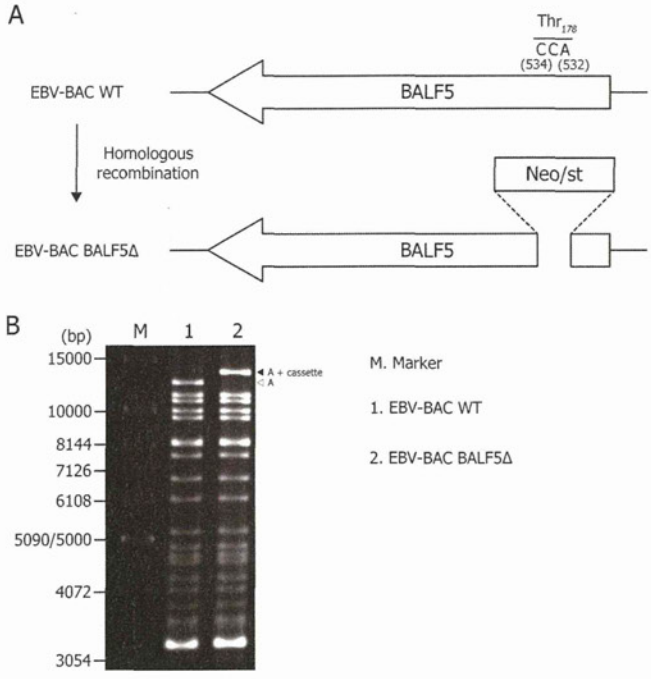

FIG 5 Scheme of EBV-BAC BALF5Δ construction. (A) Schematic arrangement of the recombination of the EBV genome using the neomycin-resistance and streptomycin-sensitivity genes (Neo/st) arranged in tandem. The sequence around the Pin1 binding site of BALF5 (Thr178) was replaced with the Neo/st cassette. (B) Electrophoresis of the recombinant viruses. The recombinant EBV genomes were digested with BamHI and separated in an agarose gel. A, the A fragment of BamHI-digested EBV-BAC.

Integrity of the BAC DNA was checked by BamHI digestion, followed by electrophoresis to confirm that the recombinant viruses did not carry obvious deletions or insertions. The BamHI-digested A fragment of EBV-BAC BALF5Δ (Fig. 5B, filled arrowhead) migrated more slowly than that of the wild type (open arrow), as expected, since the Neo/st marker cassette was inserted into the fragment (Fig. 5B).

Recombinant EBV-BAC DNA was introduced into a virus-producing cell line, HEK293, followed by hygromycin selection, to establish cell lines in which the EBV-BAC genome was maintained as an episome. More than 10 cell colonies from each recombinant virus were obtained, and viral protein expression levels in the presence and absence of BZLF1 induction were examined (data not shown). As the result, we obtained BALF5 knockout EBV-BAC cells (EBV-BAC BALF5Δ) which exhibited a typical nature, i.e., viral lytic protein expression was restricted without BZLF1 and efficiently induced by BZLF1.

In the BALF5 knockout cell line, exogenous expression of BZLF1 led to induction of early genes, such as BALF2 and BMRF1, but failed to produce BALF5, in line with expectations (Fig. 6A, BZLF1). Because of the lack of BALF5, the DNA Pol catalytic subunit, the virus could not amplify viral DNA even after induction with BZLF1 (Fig. 6C, BZLF1). Next, in order to compare the efficiencies of complementation, HEK293 EBV-BAC BALF5Δ cells were transfected with either BALF5 WT or BALF5 T178A expression vectors in addition to BZLF1. Exogenous supply of BALF5 WT restored the replication and increased viral DNA levels by 14.6-fold (Fig. 6C, BZLF1+BALF5 WT). On the other hand, the BALF5 T178A mutant increased the viral DNA only 4.54-fold (Fig. 6C, BZLF1+BALF5 T178A), when the mutant BALF5 protein expression level was equivalent to that of the wild type (Fig. 6A and B). In addition, we measured viral particles produced from the cells (Fig. 6D). Culture supernatant from the cells were collected and cocultured with naive Akata(−) cells. Since the recombinant EBVs used here encode green fluorescent protein (GFP), Akata(−) cells infected with EBV become GFP positive. When wild-type BALF5 was transfected with BZLF1, 759 infectious particles were obtained per ml of supernatant on average. Although the DNA replication levels (Fig. 6C) and viral yield (Fig. 6D) were slightly weak, we assume that this experimental condition is still physiologically relevant. The T178A mutation of BALF5 caused a slight decrease in the viral yield (Fig. 6D). These results indicate that the T178 residue of BALF5 is needed for efficient lytic replication of the EBV genome and suggest that optimal activity of the DNA polymerase is mediated through the interaction with Pin1.

To further verify the conclusion, we lastly tested if knockdown of Pin1 could influence EBV replication under this condition, too. Transfection of siRNA to HEK293 EBV-BAC BALF5Δ cells caused a considerable decrease in Pin1 levels when levels of other markers like BALF5, BZLF1, and tubulin remained unchanged (Fig. 6E). Viral replication levels this time reached 17.8-fold with BZLF1 plus BALF5 (wild type) and control siRNA, but Pin1 knockdown resulted in only a 12.2-fold increase (Fig. 6F). Although the difference in the results shown in Fig. 6F was less remarkable than the difference shown in Fig. 1B, we speculate that this reduction level by siPin1 is convincing enough because knockdown of Pin1 here

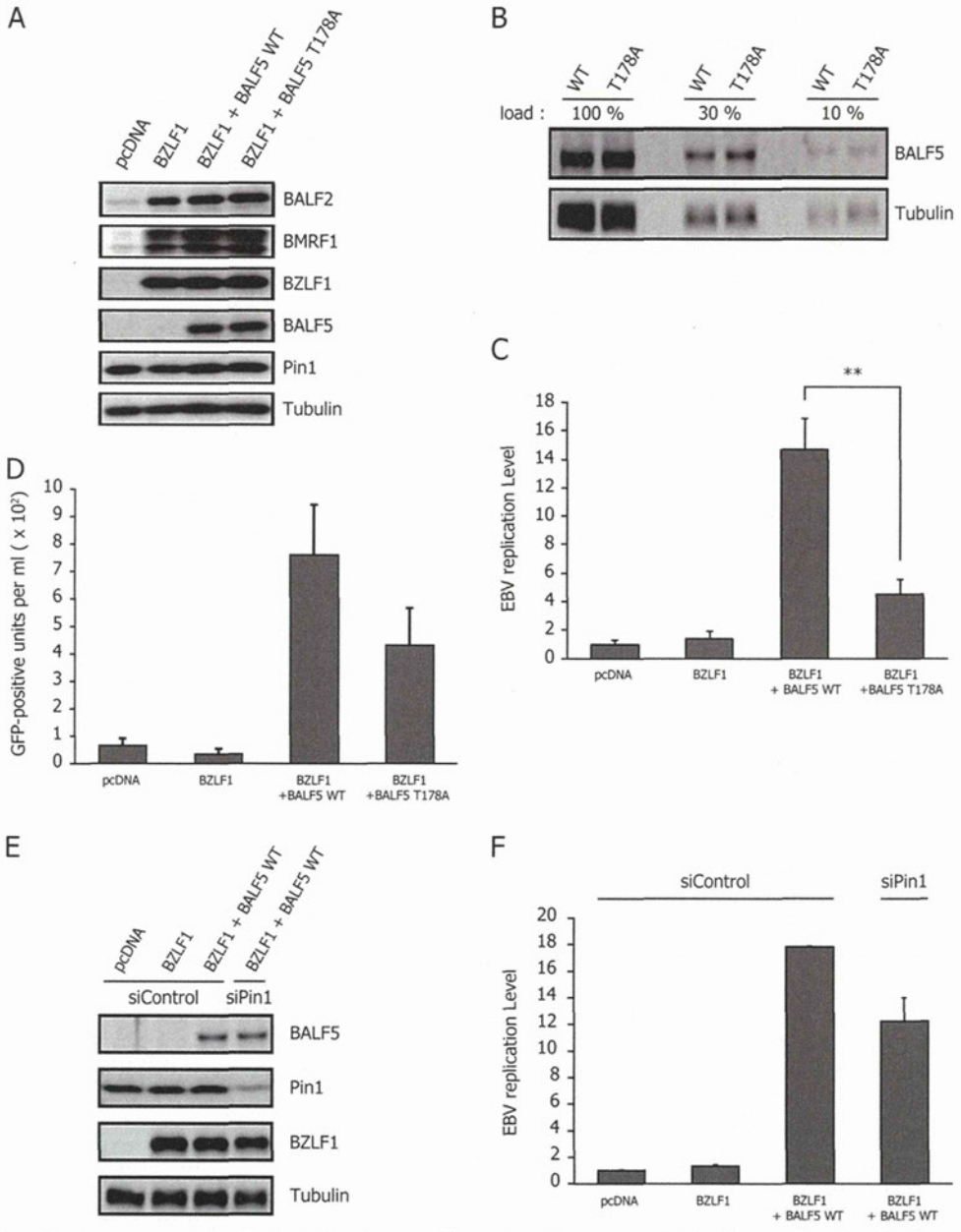

FIG 6 Significance of Pin1 binding to BALF5 Thr178 for viral replication. (A) HEK293 EBV-BAC BALF5Δ cells were transfected with 50 ng of BZLF1 and 10 ng of either BALF5 WT or BALF5 T178A expression vector using a Microporator (Digital Bio). Aliquots of cells were harvested at 24 h after transfection and subjected to immunoblotting with indicated antibodies. (B) The samples from the third and fourth lanes of the experiment shown in panel A were diluted and loaded as indicated, followed by immunoblotting with anti-BALF5 and -tubulin antibodies. The remaining cells were harvested at 36 h after transfection and subjected to qRT-PCR (C). Each bar represents the mean and standard deviation for the viral DNA level after normalization, calculated from three independent samples. **, $P < 0.005$. (D) Culture supernatants from HEK293 EBV-BAC BALF5Δ cells, transfected in the same fashion as described for panel C and followed by 3 days of incubation, were collected and used to infect naive Akata(−) cells. Viral load in the medium was determined by fluorescence-activated cell sorting analysis and is shown as the number of GFP-positive units per milliliter. (E and F) HEK293 EBV-BAC BALF5Δ cells were transfected with siRNA against Pin1 (siPin1) or a control siRNA (siControl). After 2 days, the cells were then transfected with expression vectors for BZLF1, BALF5 WT, BALF5 T178A, and/or the empty vector pcDNA, as indicated. Samples were harvested at 24 h after transfection of plasmids and subjected to immunoblotting with the indicated antibodies (E). The remaining cells were harvested at 36 h after transfection of plasmids for qRT-PCR (F).

(Fig. 6E) was not as complete as that in the experiment shown in Fig. 1A.

DISCUSSION

In this study, we obtained evidence for the first time of an interaction between the EBV lytic protein BALF5 and the cellular regulator Pin1. The results documented show clear involvement of the Pin1 protein in efficient EBV lytic replication. Initially, Pin1 was identified as a key regulator from knockdown experiments since silencing of Pin1 resulted in significant suppression of the viral replication level (Fig. 1).

We first speculated that knockdown of Pin1 might directly

influence viral lytic gene transcription because Pin1 reportedly regulates RNA polymerase II activity (32–34). Pin1 also impacts cellular signaling through factors such as Akt (35), c-Jun (36, 37), and p65/NF-κB (38). In fact, shPin1 might have slightly decreased EBV early gene expression (Fig. 1A) although we assume the levels are comparable.

Then, we found that the EBV DNA polymerase BALF5 interacted directly with Pin1, as demonstrated by GST pulldown (Fig. 2A), and that the interaction is dependent on phosphorylation of BALF5 Thr178 (Fig. 2B and 4). Results of immunoprecipitation assays also supported this conclusion (Fig. 2C and 4B). Although the association of Pin1 with BALF5 (Fig. 2 to 4) and its influence on viral DNA replication (Fig. 6) are clear, we cannot preclude the possibility that there may be other Pin1 substrates besides BALF5 that affect EBV genome amplification as Pin1 has a large number of substrates (12). Thus, the search for other Pin1 targets is still under way. In addition, it must also be noted that because there are a number of cellular target proteins that are up-/downregulated by Pin1, we cannot ignore the possibility that some of these cellular target proteins may cause even adverse effects on EBV replication. But as a whole, Pin1 clearly upregulates EBV lytic replication (Fig. 1), at least partly through the action of BALF5 (Fig. 6).

In this paper, we identified Pin1 interaction with the EBV DNA polymerase BALF5 enzyme at Thr178. The polymerase contains conserved domains, including polymerase catalytic and exonuclease domains at the C terminus, but the sequence around Thr178 is not conserved. We have no concrete idea of how Pin1 modulates BALF5 function, but it is possible that the N-terminal domain of BALF5, including Thr178, may somehow regulate its C-terminal functional domains by altering the structure of the protein in a subtle way. Further studies are required to gain an understanding of the molecular mechanism of how Pin1 regulates BALF5 enzymatic activity.

To our knowledge, EBV BALF5 is the only Pin1 target so far identified, not just in EBV but among all herpesvirus genes. Milbradt and others reported data suggesting that Pin1 may be involved in reorganization of nuclear lamin after phosphorylation by the human cytomegalovirus (HCMV)-encoded protein kinase UL97 (39). Elsewhere, Peloponese and others showed that Pin1 binds to the Tax protein of human T cell leukemia virus 1 and regulates Tax-induced NF-κB activation (40). Jeong and others demonstrated that Pin1 prolongs the Tax protein half-life by suppressing ubiquitination and proteasome-dependent degradation (41). Furthermore, Pin1 increased stability of the hepatitis B virus oncoprotein X and enhanced transactivation and cell proliferation (42). Hepatitis C virus replication is regulated by Pin1, probably through binding to NS5A/NS5B (43). Human immunodeficiency virus type 1 genome integration (44) and capsid uncoating (45) are also regulated by Pin1. Although exact roles of Pin1 in controlling herpesvirus replication remain elusive, it has already been proposed as an important modulator of viral proteins and a unique target for antiviral therapy (46).

A number of studies suggest that Pin1 has a role in tumorigenesis as it is overexpressed in a number of human cancers (47, 48). Since we determined that Pin1 is a positive regulator of EBV lytic replication, EBV-positive cancer tissue may be an efficient site for producing novel virus particles. While further studies are required to clarify the underlying mechanisms, Pin1 clearly warrants attention as a novel target for potential antiviral/cancer drug development.

ACKNOWLEDGMENTS

We thank W. Hammerschmidt, H. J. Delecluse, and S. Tsuzuki for providing the EBV-BAC system, HEK293 cells, and shRNA technology, respectively. We also express our appreciation to C. Noda and T. Gamano for preliminary experiments and technical assistance.

This work was supported by grants-in-aid for Scientific Research from the Ministry of Education, Science, Sports, Culture and Technology (numbers 23390118 and 23114512 to T.T. and numbers 22790448 and 24590566 to T.M.), the Ministry of Health, Labor and Welfare (to T.T. and H.K.), and partly by the Takeda Science Foundation (to T.T. and T.M.).

REFERENCES

1. **Tsurumi T, Fujita M, Kudoh A.** 2005. Latent and lytic Epstein-Barr virus replication strategies. Rev. Med. Virol. **15**:3–15.
2. **Feng WH, Cohen JI, Fischer S, Li L, Sneller M, Goldbach-Mansky R, Raab-Traub N, Delecluse HJ, Kenney SC.** 2004. Reactivation of latent Epstein-Barr virus by methotrexate: a potential contributor to methotrexate-associated lymphomas. J. Natl. Cancer Inst. **96**:1691–1702.
3. **Joab I, Nicolas JC, Schwaab G, de-The G, Clausse B, Perricaudet M, Zeng Y.** 1991. Detection of anti-Epstein-Barr-virus transactivator (ZEBRA) antibodies in sera from patients with nasopharyngeal carcinoma. Int. J. Cancer **48**:647–649.
4. **Daikoku T, Kudoh A, Fujita M, Sugaya Y, Isomura H, Shirata N, Tsurumi T.** 2005. Architecture of replication compartments formed during Epstein-Barr virus lytic replication. J. Virol. **79**:3409–3418.
5. **Fixman ED, Hayward GS, Hayward SD.** 1995. Replication of Epstein-Barr virus oriLyt: lack of a dedicated virally encoded origin-binding protein and dependence on Zta in cotransfection assays. J. Virol. **69**:2998–3006.
6. **Tsurumi T, Daikoku T, Nishiyama Y.** 1994. Further characterization of the interaction between the Epstein-Barr virus DNA polymerase catalytic subunit and its accessory subunit with regard to the 3′-to-5′ exonucleolytic activity and stability of initiation complex at primer terminus. J. Virol. **68**:3354–3363.
7. **Tsurumi T.** 1993. Purification and characterization of the DNA-binding activity of the Epstein-Barr virus DNA polymerase accessory protein BMRF1 gene products, as expressed in insect cells by using the baculovirus system. J. Virol. **67**:1681–1687.
8. **Bernad A, Blanco L, Lazaro JM, Martin G, Salas M.** 1989. A conserved 3′–5′ exonuclease active site in prokaryotic and eukaryotic DNA polymerases. Cell **59**:219–228.
9. **Bernad A, Zaballos A, Salas M, Blanco L.** 1987. Structural and functional relationships between prokaryotic and eukaryotic DNA polymerases. EMBO J. **6**:4219–4225.
10. **Ryo A, Nakamura M, Wulf G, Liou YC, Lu KP.** 2001. Pin1 regulates turnover and subcellular localization of beta-catenin by inhibiting its interaction with APC. Nat. Cell Biol. **3**:793–801.
11. **Lu KP, Zhou XZ.** 2007. The prolyl isomerase PIN1: a pivotal new twist in phosphorylation signalling and disease. Nat. Rev. Mol. Cell Biol. **8**:904–916.
12. **Liou YC, Zhou XZ, Lu KP.** 2011. Prolyl isomerase Pin1 as a molecular switch to determine the fate of phosphoproteins. Trends Biochem. Sci. **36**:501–514.
13. **Nakamura K, Greenwood A, Binder L, Bigio EH, Denial S, Nicholson L, Zhou XZ, Lu KP.** 2012. Proline isomer-specific antibodies reveal the early pathogenic tau conformation in Alzheimer's disease. Cell **149**:232–244.
14. **Wulf G, Finn G, Suizu F, Lu KP.** 2005. Phosphorylation-specific prolyl isomerization: is there an underlying theme? Nat. Cell Biol. **7**:435–441.
15. **Yeh ES, Means AR.** 2007. PIN1, the cell cycle and cancer. Nat. Rev. Cancer **7**:381–388.
16. **Winkler KE, Swenson KI, Kornbluth S, Means AR.** 2000. Requirement of the prolyl isomerase Pin1 for the replication checkpoint. Science **287**:1644–1647.
17. **Lu KP.** 2004. Pinning down cell signaling, cancer and Alzheimer's disease. Trends Biochem. Sci. **29**:200–209.

18. **Tun-Kyi A, Finn G, Greenwood A, Nowak M, Lee TH, Asara JM, Tsokos GC, Fitzgerald K, Israel E, Li X, Exley M, Nicholson LK, Lu KP.** 2011. Essential role for the prolyl isomerase Pin1 in Toll-like receptor signaling and type I interferon-mediated immunity. Nat. Immunol. **12**: 733–741.
19. **Ayala G, Wang D, Wulf G, Frolov A, Li R, Sowadski J, Wheeler TM, Lu KP, Bao L.** 2003. The prolyl isomerase Pin1 is a novel prognostic marker in human prostate cancer. Cancer Res. **63**:6244–6251.
20. **Bao L, Kimzey A, Sauter G, Sowadski JM, Lu KP, Wang DG.** 2004. Prevalent overexpression of prolyl isomerase Pin1 in human cancers. Am. J. Pathol. **164**:1727–1737.
21. **Lee KY, Lee JW, Nam HJ, Shim JH, Song Y, Kang KW.** 2011. PI3-kinase/p38 kinase-dependent E2F1 activation is critical for Pin1 induction in tamoxifen-resistant breast cancer cells. Mol. Cells **32**:107–111.
22. **Suizu F, Ryo A, Wulf G, Lim J, Lu KP.** 2006. Pin1 regulates centrosome duplication, and its overexpression induces centrosome amplification, chromosome instability, and oncogenesis. Mol. Cell. Biol. **26**:1463–1479.
23. **Ryo A, Liou YC, Wulf G, Nakamura M, Lee SW, Lu KP.** 2002. PIN1 is an E2F target gene essential for Neu/Ras-induced transformation of mammary epithelial cells. Mol. Cell. Biol. **22**:5281–5295.
24. **Wulf G, Garg P, Liou YC, Iglehart D, Lu KP.** 2004. Modeling breast cancer in vivo and ex vivo reveals an essential role of Pin1 in tumorigenesis. EMBO J. **23**:3397–3407.
25. **Takahashi K, Akiyama H, Shimazaki K, Uchida C, Akiyama-Okunuki H, Tomita M, Fukumoto M, Uchida T.** 2007. Ablation of a peptidyl prolyl isomerase Pin1 from p53-null mice accelerated thymic hyperplasia by increasing the level of the intracellular form of Notch1. Oncogene **26**: 3835–3845.
26. **Gershburg E, Hong K, Pagano JS.** 2004. Effects of maribavir and selected indolocarbazoles on Epstein-Barr virus protein kinase BGLF4 and on viral lytic replication. Antimicrob. Agents Chemother. **48**:1900–1903.
27. **Wang FZ, Roy D, Gershburg E, Whitehurst CB, Dittmer DP, Pagano JS.** 2009. Maribavir inhibits Epstein-Barr virus transcription in addition to viral DNA replication. J. Virol. **83**:12108–12117.
28. **Murata T, Isomura H, Yamashita Y, Toyama S, Sato Y, Nakayama S, Kudoh A, Iwahori S, Kanda T, Tsurumi T.** 2009. Efficient production of infectious viruses requires enzymatic activity of Epstein-Barr virus protein kinase. Virology **389**:75–81.
29. **Noda C, Murata T, Kanda T, Yoshiyama H, Sugimoto A, Kawashima D, Saito S, Isomura H, Tsurumi T.** 2011. Identification and characterization of CCAAT enhancer-binding protein (C/EBP) as a transcriptional activator for Epstein-Barr virus oncogene latent membrane protein 1. J. Biol. Chem. **286**:42524–42533.
30. **Delecluse HJ, Hilsendegen T, Pich D, Zeidler R, Hammerschmidt W.** 1998. Propagation and recovery of intact, infectious Epstein-Barr virus from prokaryotic to human cells. Proc. Natl. Acad. Sci. U. S. A. **95**:8245–8250.
31. **Isomura H, Stinski MF, Kudoh A, Murata T, Nakayama S, Sato Y, Iwahori S, Tsurumi T.** 2008. Noncanonical TATA sequence in the UL44 late promoter of human cytomegalovirus is required for the accumulation of late viral transcripts. J. Virol. **82**:1638–1646.
32. **Palancade B, Marshall NF, Tremeau-Bravard A, Bensaude O, Dahmus ME, Dubois MF.** 2004. Dephosphorylation of RNA polymerase II by CTD-phosphatase FCP1 is inhibited by phospho-CTD associating proteins. J. Mol. Biol. **335**:415–424.
33. **Xu YX, Hirose Y, Zhou XZ, Lu KP, Manley JL.** 2003. Pin1 modulates the structure and function of human RNA polymerase II. Genes Dev. **17**: 2765–2776.
34. **Xu YX, Manley JL.** 2007. Pin1 modulates RNA polymerase II activity during the transcription cycle. Genes Dev. **21**:2950–2962.
35. **Liao Y, Wei Y, Zhou X, Yang JY, Dai C, Chen YJ, Agarwal NK, Sarbassov D, Shi D, Yu D, Hung MC.** 2009. Peptidyl-prolyl *cis/trans* isomerase Pin1 is critical for the regulation of PKB/Akt stability and activation phosphorylation. Oncogene **28**:2436–2445.
36. **Pulikkan JA, Dengler V, Peer Zada AA, Kawasaki A, Geletu M, Pasalic Z, Bohlander SK, Ryo A, Tenen DG, Behre G.** 2010. Elevated PIN1 expression by C/EBPα-p30 blocks C/EBPα-induced granulocytic differentiation through c-Jun in AML. Leukemia **24**:914–923.
37. **Wulf GM, Ryo A, Wulf GG, Lee SW, Niu T, Petkova V, Lu KP.** 2001. Pin1 is overexpressed in breast cancer and cooperates with Ras signaling in increasing the transcriptional activity of c-Jun towards cyclin D1. EMBO J. **20**:3459–3472.
38. **Ryo A, Suizu F, Yoshida Y, Perrem K, Liou YC, Wulf G, Rottapel R, Yamaoka S, Lu KP.** 2003. Regulation of NF-κB signaling by Pin1-dependent prolyl isomerization and ubiquitin-mediated proteolysis of p65/RelA. Mol. Cell **12**:1413–1426.
39. **Milbradt J, Webel R, Auerochs S, Sticht H, Marschall M.** 2010. Novel mode of phosphorylation-triggered reorganization of the nuclear lamina during nuclear egress of human cytomegalovirus. J. Biol. Chem. **285**: 13979–13989.
40. **Peloponese JM, Jr, Yasunaga J, Kinjo T, Watashi K, Jeang KT.** 2009. Peptidylproline *cis-trans*-isomerase Pin1 interacts with human T-cell leukemia virus type 1 Tax and modulates its activation of NF-κB. J. Virol. **83**:3238–3248.
41. **Jeong SJ, Ryo A, Yamamoto N.** 2009. The prolyl isomerase Pin1 stabilizes the human T-cell leukemia virus type 1 (HTLV-1) Tax oncoprotein and promotes malignant transformation. Biochem. Biophys. Res. Commun. **381**:294–299.
42. **Pang R, Lee TK, Poon RT, Fan ST, Wong KB, Kwong YL, Tse E.** 2007. Pin1 interacts with a specific serine-proline motif of hepatitis B virus X-protein to enhance hepatocarcinogenesis. Gastroenterology **132**:1088–1103.
43. **Lim YS, Tran HT, Park SJ, Yim SA, Hwang SB.** 2011. Peptidyl-prolyl isomerase Pin1 is a cellular factor required for hepatitis C virus propagation. J. Virol. **85**:8777–8788.
44. **Manganaro L, Lusic M, Gutierrez MI, Cereseto A, Del Sal G, Giacca M.** 2010. Concerted action of cellular JNK and Pin1 restricts HIV-1 genome integration to activated $CD4^+$ T lymphocytes. Nat. Med. **16**:329–333.
45. **Misumi S, Inoue M, Dochi T, Kishimoto N, Hasegawa N, Takamune N, Shoji S.** 2010. Uncoating of human immunodeficiency virus type 1 requires prolyl isomerase Pin1. J. Biol. Chem. **285**:25185–25195.
46. **Kojima Y, Ryo A.** 2010. Pinning down viral proteins: a new prototype for virus-host cell interaction. Front. Microbiol. **1**:107. doi:10.3389/fmicb.2010.00107.
47. **Ryo A, Liou YC, Lu KP, Wulf G.** 2003. Prolyl isomerase Pin1: a catalyst for oncogenesis and a potential therapeutic target in cancer. J. Cell Sci. **116**:773–783.
48. **Ryo A, Uemura H, Ishiguro H, Saitoh T, Yamaguchi A, Perrem K, Kubota Y, Lu KP, Aoki I.** 2005. Stable suppression of tumorigenicity by Pin1-targeted RNA interference in prostate cancer. Clin. Cancer Res. **11**: 7523–7531.

Rev. Med. Virol.
Published online in Wiley Online Library
(wileyonlinelibrary.com)
DOI: 10.1002/rmv.1738

Reviews in Medical Virology

REVIEW

Genome guardian p53 and viral infections

Yoshitaka Sato[1,2,3] and Tatsuya Tsurumi[1,4*]

[1]*Division of Virology, Aichi Cancer Center Research Institute, Nagoya 464-8681, Japan*
[2]*Department of Virology, Nagoya University Graduate School of Medicine, Nagoya 466-8550, Japan*
[3]*Division of Genetics, Kobe University School of Medicine, Kobe 650-0017, Japan*
[4]*Department of Oncology, Graduate School of Pharmaceutical Sciences, Nagoya City University, Nagoya 467-8603, Japan*

SUMMARY

Because virus infections elicit various cellular responses that inhibit viral replication and growth, viruses must intervene to attenuate antiviral measures in order to thrive. The genome guardian p53 plays a central part not only in DNA damage responses, inducing cell cycle arrest or apoptosis, but also in the innate host immune control of viral infections by orchestrating diverse signaling pathways originating from many different cellular receptors and sensors. Many viruses have acquired sophisticated mechanisms to regulate p53 functions by deploying subversive proteins and modulating its post-transcriptional status. In this review, we overview the mechanisms by which DNA and RNA viruses manage p53 signaling in favor of their continued survival. Copyright © 2012 John Wiley & Sons, Ltd.

Received: 1 October 2012; Revised: 18 November 2012; Accepted: 20 November 2012

INTRODUCTION

Viruses intrinsically depend on their host cells during the course of infection. Cells infected by viruses utilize host surveillance mechanisms to ultimately block viral replication and dissemination. The coordinated genetic regulatory network in which a transcription factor p53 controls the expression of a set of diverse target genes is central to host defense. Actuary, p53-mediated apoptosis, which may be termed altruistic suicide, inhibits the further spread of infectious pathogens [1]. On the other hand, another important aspect of cellular responses is the immune system signaling elicited by infection with viruses, which usually leads to the production of type I IFN and inflammatory cytokines, resulting in elimination of the pathogens [2].

p53 is also activated in response to diverse cellular stresses such as DNA damage and oncogenic stress [3,4]. Induction of p53 triggers multiple cellular programs ranging from transient responses, such as DNA repair and cell cycle arrest, to terminal fates such as cell death and permanent cell cycle arrest, hence having central roles in tumor suppression and maintaining genomic integrity as a guardian of the genome [3,5,6].

Lane and Levine initially isolated p53 as a binding partner of SV40 LTag in 1979 [7,8]. Within a few years of its discovery, evidence of a cellular oncogene property appeared because the gene cloned from neoplastic cells could reproduce transformation [9]. Tumor-derived p53 mutants can promote cellular transformation through dominant-negative inactivation of endogenous wild-type p53, whereas wild-type p53 cannot [10]. Vogelstein and colleagues reported a common loss-of-heterozygosity at the p53 locus in human colorectal cancers [11], suggesting that p53 was actually a tumor suppressor gene rather than an oncogene. Indeed, p53 is mutated or lost in over 50% of human cancers [12], representing the most commonly mutated gene in human tumors.

In unstressed cells, p53 is kept at low levels by its negative regulator MDM2 (HDM2) through the ubiquitin-dependent proteasome pathway [13]. Upon DNA damage, p53 is phosphorylated to escape from proteasomal degradation [14], and then is stabilized and activated to function primarily as a transcription factor, consequently leading to cell

*Corresponding author: T. Tsurumi, Aichi Cancer Center Research Institute, Nagoya 464-8681, Japan.
E-mail: ttsurumi@aichi-cc.jp

Abbreviations used
Ad, adenovirus; DDRs, DNA damage responses; E6AP, E6-associated protein; HBX, hepatitis B virus X protein; HPV, human papillomaviruses; Inf., Influenza virus; KSHV, Kaposi sarcoma herpesvirus; LANA, latency-associated nuclear antigen; LTag, large T antigen; SV40, simian vacuolating virus 40; Vacc., vaccinia virus; VSV, Vesicular stomatitis virus.

cycle arrest or apoptosis through the p53-mediated gene expression cascades [4,15]. These cellular outcomes after stresses, including DNA damage, oncogene activation, hypoxia, nucleotide imbalance, and oxidative damage, are tightly linked to p53 dynamics mediated by both the levels and post-translational modifications of p53 [16,17]

Furthermore, p53 also contributes to immune responses that lead to eradication of pathogens such as viruses [18]. p53 directly transactivates the expression of several innate immunity-related genes such as IRF9, TRL3, ISG15, and MCP-1 [19–22], and interestingly, transcription of the p53 gene is induced by IFN-α/β signaling [1,23]. These findings suggest a positive feedback loop involving p53-mediated enhancement of IFN signals to boost antiviral immune responses. Viruses, in turn, have to evolve elaborate mechanisms to subvert IFN-mediated and p53-mediated host immune responses.

Viruses are grouped into two major categories: DNA and RNA viruses. Replication of viruses, especially RNA viruses, can induce type I IFNs, triggered by the production of dsRNA. On the other hand, DNA viruses activate DNA damage signaling, triggered by the production of viral DNA genomes. Viruses intervene at numerous stages in the pathways to attenuate the antiviral responses. Here, we review how viruses modulate p53 functions and its downstream signaling pathways during their propagation, the functional links between viral growth and post-translational status of p53, and the physiological importance of this interplay. The interplay between p53 and viruses is summarized in Figure 1.

DNA VIRUSES

Most DNA viruses replicate their genomes in nuclei and usually elicit DDRs, resulting in phosphorylation and stabilization of p53. Some exploit the DDR to facilitate their own genome replication, but in other cases, the DDR presents a block to viral replication, which must be overcome. Thus, DNA viruses employ a variety of strategies to inactivate or degrade p53 or sometimes to utilize p53 function for their proliferation. The prevention of p53 functions by virus, in turn, contributes to tumor progression in a certain tissue.

The high-risk HPV, which is associated with human cervical cancer, E6 protein can recruit the cellular E3 ubiquitin ligase E6AP, a prototype member of the homologous to the E6-associated protein carboxyl terminus (HECT) family, to a trimeric complex with p53 [24] that is degraded through the ubiquitin–proteasome pathway [25,26]. Degradation of p53 by the E6–E6AP complex reduces the net levels and then allows viral replication by inhibiting p53-mediated antiviral responses including DDR, apoptosis, and other stress signals.

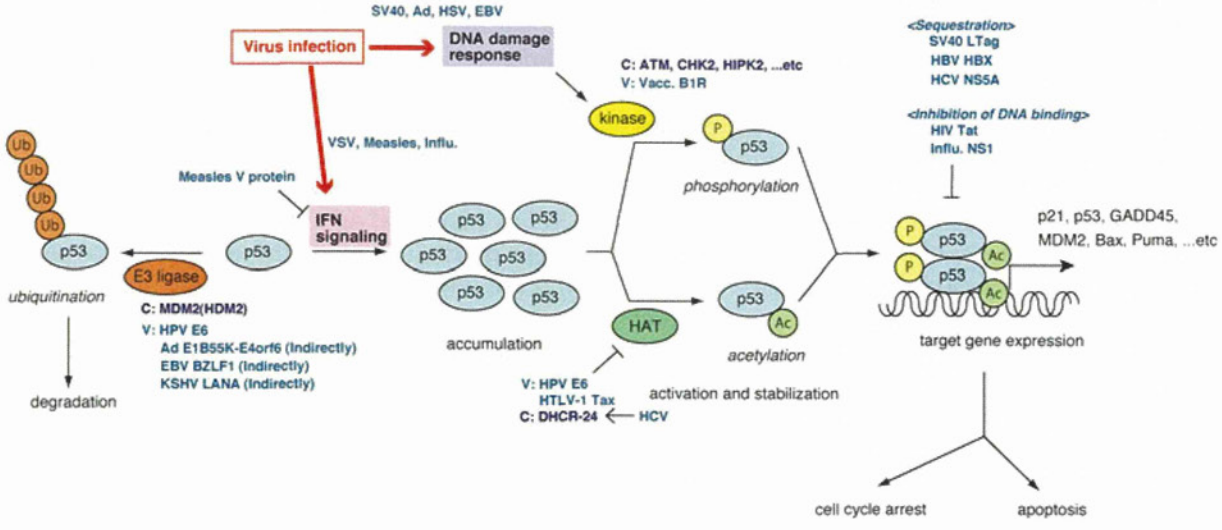

Figure 1. Intervention of viral proteins in p53-mediated antiviral responses. The viruses interfere with the p53 functions at several steps to permit a successful viral life cycle. Although infection with pathogens induces antiviral signaling pathways that stabilize and activate p53, viral factors manipulate these pathways by mimicking post-translational modifications of p53 and/or disrupting its downstream signals