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ORIGINAL RESEARCH

Classification of AIDS-related lymphoma cases between 1987 and 2012 in Japan based on the WHO classification of lymphomas, fourth edition

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AIDS-related lymphoma, antiretroviral therapy, Burkitt lymphoma, diffuse large B-cell lymphoma, Epstein–Barr virus

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Abstract

The introduction of combined antiretroviral therapy (ART) has reduced the mortality of patients with human immunodeficiency virus-1 infection worldwide. However, malignant lymphoma is a severe and frequent complication seen in patients with acquired immunodeficiency syndrome (AIDS). The diagnostic criteria for some categories of AIDS-related lymphoma were revised in the World Health Organization International Classification of Lymphoma, fourth edition. The purpose of this study was to assess the clinicopathological characteristics of Japanese patients with AIDS-related lymphoma according to the revised classification. In this retrospective study, 207 AIDS-related lymphoma cases diagnosed between 1987 and 2012 in Japan were subjected to histological subtyping and clinicopathological analyses. Diffuse large B-cell lymphoma (DLBCL) was the predominant histological subtype throughout the study period (n = 104, 50%). Among the DLBCL cases, 24% were of the germinal center (GC) type and 76% were of the non-GC type. Non-GC-type cases showed a significantly lower 1-year survival rate (43%) than the GC-type cases (82%). Cases of Burkitt lymphoma (n = 57, 28%), plasmablastic lymphoma (n = 16, 8%), primary effusion lymphoma (n = 9, 4%), Hodgkin lymphoma (n = 8, 4%), and large B-cell lymphoma arising in Kaposi sarcoma-associated herpesvirus-associated multicentric Castleman disease (n = 2, 1%) were also observed. Hodgkin lymphoma was more common in patients receiving ART (11.1%) than in ART-naïve patients (1.4%). Statistical analyses identified CD10 negativity, BCL-6

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negativity, Epstein–Barr virus positivity, and Kaposi sarcoma-associated herpesvirus positivity as risk factors for poor prognosis. This information will help in the early diagnosis of lymphoma in patients with AIDS.

Introduction

Malignant lymphoma is a severe complication in patients with acquired immunodeficiency syndrome (AIDS). The incidence of lymphoma is 60- to 200-fold higher in patients with human immunodeficiency virus-1 (HIV-1) infection than in the general, uninfected patient population [1–3]. AIDS-related lymphoma (ARL) has unique histological characteristics compared with lymphoma occurring in immunocompetent individuals. B-cell lineage lymphoma is a predominant subtype of ARL, whereas lymphomas involving other cell lineages, including T/natural killer (NK) cells, are very rare. Diffuse large B-cell lymphoma (DLBCL) is the most frequent histological subtype of ARL, and Burkitt lymphoma (BL) is another major subtype. Two oncogenic herpes viruses, Epstein–Barr virus (EBV) and Kaposi sarcoma-associated herpesvirus (KSHV or human herpesvirus 8), are also frequently associated with the pathogenesis of ARL [4]. EBV positivity is often detected in cases of DLBCL, plasmablastic lymphoma (PBL), and primary effusion lymphoma (PEL). In addition, all PEL cases are positive for KSHV. PBL and PEL generally develop in patients with AIDS; they are very rare in immunocompetent hosts.

Antiretroviral therapy (ART) successfully and drastically reduces the HIV-1 RNA copy number in serum, resulting in recovery of immune function and decreased mortality in HIV-1-infected patients [5]. ART has been shown to significantly decrease the incidence of opportunistic infections such as pneumocystis pneumonia, cytomegalovirus, and *Candida*. Although ART did not significantly reduce the incidence of lymphoma in HIV-1-infected individuals in previous studies [6–15], the clinicopathological characterization of ARL has changed because of the introduction of ART [16]. Of note, the incidence of central nervous system (CNS) lymphomas has decreased in HIV-1-infected patients undergoing ART [16]. However, the number of cases of BL and Hodgkin lymphoma (HL) have increased in patients undergoing ART [6, 17, 18]. In particular, an increased number of HL cases have been reported in ART patients with high CD4 counts [18].

Few studies have presented the clinicopathological features of a large number of ARL patients in Asian countries [17, 19, 20]. In Japan, more than half of ARL cases were categorized as EBV-associated DLBCL before the introduction of ART [17]. However, after the introduction of ART, EBV positivity has decreased among DLBCL cases, and the incidence of BL cases has increased among AIDS patients. In addition, the frequency of nodal involvement in lymphoma cases has increased in patients undergoing ART, whereas the incidence of CNS lymphomas has decreased [17]. Thus, ART induction has altered the clinicopathological characteristics of ARL in Japan.

In 2008, the World Health Organization (WHO) released the fourth edition of the classification of lymphomas based on recent accumulation of scientific evidence for these diseases [21]. Some definitions of lymphomas observed frequently in HIV-1-infected patients were altered in the revised classification [22]. For example, some variants, subgroups, and subtypes of DLBCL were redefined [23]. The definition of atypical Burkitt/Burkitt-like variant was excluded from the chapter on BL [24], and the definition of B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and BL (intermediate DLBCL/BL) was newly added [25]. PBL and PEL were explained in more detail, and the definition of another KSHV-positive lymphoma, large B-cell lymphoma arising in patients with KSHV-associated multicentric Castleman disease, was added [26, 27]. Thus, the revised WHO classification of lymphomas is likely to affect the histological classification of ARL cases. However, thus far, few reports have described the histological classification of lymphoma in HIV-1-infected patients according to this edition of the WHO classification.

In the present study, ARL cases were classified according to the fourth edition of the WHO classification of lymphomas, and alterations in the clinicopathological characteristics of ARL due to the introduction of ART were investigated. In addition, the correlations of some biomarkers with the prognosis of ARL are discussed.

Materials and Methods

Patients

Studies using human tissue were performed with the approval of the Institutional Review Boards of the National Institute of Infectious Diseases (Approval No. 344) and of five hospitals in Japan: Tokyo Metropolitan Komagome Hospital; National Center for Global Health and Medicine Hospital; the Institute of Medical Science, the University of Tokyo; Osaka Medical Center; and Nagoya Medical Center Hospital. The clinical data of 207 cases of ARL, diagnosed histologically between January 1987 and November 2012, were investigated retrospectively (Table 1). These patients were referred to one of

Table 1. Characteristics of patients diagnosed with AIDS-related lymphoma.

Factor	Total	DLBCL	BL	PBL	PEL	HL	LBL-KSHV-MCD	Other
n	207	104	57	16	9	8	2	11
Age, years (mean) [median, range]	45.4 [44, 12–76]	45.7 [44, 12–76]	43.7 [41, 25–59]	47.9 [51, 31–59]	43.5 [43, 30–59]	53.5 [56, 39–65]	48.5 [49, 39–65]	42.1 [40, 25–59]
Men (%)	198 (96)	97 (93)	55 (97)	16 (100)	9 (100)	8 (100)	2 (100)	11 (100)
CD4 (mean) [median, range]	149 [82, 0–2413]	86 [41, 0–824]	249 [216, 14–652]	85 [59, 7–394]	75 [20, 6–260]	235 [236, 22–497]	206 [206, 30–382]	303 [70, 2–2431]
ART (+) at onset (%)	26.1	22.1	31.6	18.8	11.1	75.0	50.0	18.2
EBV-positive (%)	59.9	70.3	27.3	93.8	66.7	100.0	0.0	54.5
CNS involvement (%)	29.6	43.8	20.0	9.1	0.0	0.0	0.0	20.0
LN involvement (%)	44.3	30.5	50.9	58.3	55.6	100.0	50.0	54.5
BM involvement (%)	30.1	11.4	47.2	33.3	50.0	71.4	0.0	45.5
1-year survival rate (%)	52.2	42.3	68.4	62.5	33.3	75.0	0.0	54.5

ART, antiretroviral therapy; BM, bone marrow; BL, Burkitt lymphoma; CNS, central nervous system; DLBCL, diffuse large B-cell lymphoma; EBV, Epstein–Barr virus; HL, Hodgkin lymphoma; LBL-KSHV-MCD, large B-cell lymphoma arising in Kaposi sarcoma-associated herpesvirus (KSHV)-associated multicentric Castleman disease; LN, lymph node; PBL, plasmablastic lymphoma; PEL, primary effusion lymphoma.

the five hospitals mentioned above. As 325 cases of ARL were reported in all of Japan during the study period, according to a national survey on the clinical manifestation of patients with HIV-1 infection (Yasuoka A. 2012 Annual report of the Health and Labor Sciences Research Grants for AIDS from the Ministry of Health, Labor and Welfare Japan, Japanese), we could safely assume that approximately two-thirds of all ARL cases in Japan were covered in the present study. The clinical data, such as age, gender, risk factors, CD4 cell count, use of ART, and prognosis, were collected from the medical records of the various hospitals. The CD4 cell counts at the time of lymphoma diagnosis were considered. In this study, ART was defined as the prescription of at least one antiretroviral drug, including a protease inhibitor or a nonnucleoside reverse transcriptase inhibitor. In Japan, ART was introduced in 1996, and the first lymphoma case of patient on ART appeared in 1997. Thus, we divided the patients into two groups based on their date of diagnosis: the pre-ART era group (n = 43), including those diagnosed from 1987 to before the first lymphoma case of the ART (+) patient in 1997, and the ART era group (n = 164), including all other cases diagnosed from 1997 to 2012. ART status was not available for seven patients in the ART era group. CNS and lymph node (LN) involvement of the lymphoma were determined according to autopsy records or clinical records.

Immunohistochemistry and in situ hybridization

The cell lineage of each case was determined using immunohistochemistry, as described previously [28]. CD3, CD10, CD20, CD30, CD38, CD45RO, CD79a, CD138, BCL-2, BCL-6, IRF4/MUM1, cIgM, immunogloblin light-chain lambda, kappa, Ki67 (MIB-1), LMP-1, and EBNA-2 antibodies were used as primary antibodies. The presence of EBV was examined using in situ hybridization for EBV-encoded small RNAs (EBER), as described previously [29]. KSHV was detected by immunohistochemistry using an antibody against KSHV-encoded latency-associated nuclear antigen 1 (LANA-1) [30]. *MYC* rearrangement was investigated using fluorescent in situ hybridization on paraffin sections, as described previously [31].

Subtyping of lymphomas

The histological subtyping of lymphomas was based on the fourth edition of the WHO classification [21]. All cases were reviewed by five pathologists (YO, TH, MM, YK, and HK) and classified according to a flowchart (Fig. 1). The diagnosis of BL was based on histological,

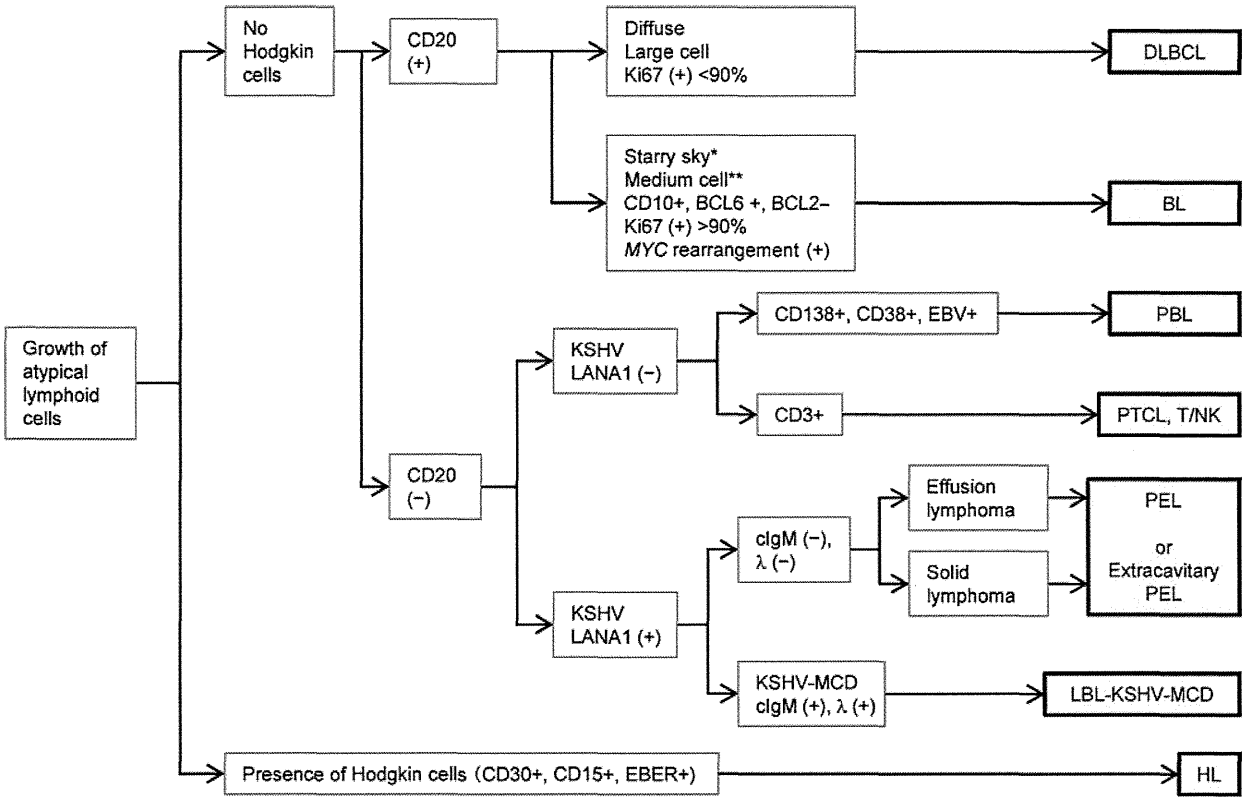

Figure 1. Diagnostic flowchart for AIDS-related lymphoma. CD20-positive cases were categorized as diffuse large B-cell lymphoma or Burkitt lymphoma (BL) according to their morphology, immunophenotype, and *MYC* rearrangement. Some BL cases did not show the typical morphology of BL, such as the starry sky pattern (*) and medium-sized cells (**). For the CD20-negative group, if positive for Kaposi sarcoma-associated herpesvirus (KSHV)-encoded latency-associated nuclear antigen 1 (LANA-1), the case was categorized as primary effusion lymphoma or large B-cell lymphoma arising in KSHV-associated multicentric Castleman disease. KSHV-negative cases were examined using immunohistochemistry for CD3, CD138, CD38, and in situ hybridization for EBV to determine its subtype (see Table 1 for abbreviations).

immunohistochemical, and chromosomal data, as recommended in the revised WHO classification system. DLBCL was subclassified into the germinal center (GC) type and the non-GC type, according to the algorithm reported previously by Hans et al. [32].

Statistical analyses

Analyses of statistical significance were performed using the chi-square test for bivariate tabular analysis and using the Mann–Whitney test for comparison of two independent groups of sampled data, such as the CD4 cell count.

Results

Histological classification of ARL

The clinical characteristics of the 207 ARL cases are summarized in Table 1 and Figure 2. The study group included 198 men and nine women, with a mean age of 45.4 years (range, 12–74 years). The HIV-1 transmission route was homosexual contact in 154 cases (74.4%) and heterosexual contact in 38 cases (18.4%). The remaining 10 patients (4.8%) were hemophiliacs and intravenous drug users. DLBCL was the predominant histological subtype diagnosed throughout the study period. Of all the DLBCL cases, 72 could be further subclassified as GC and non-GC types (Table 2). Seventeen cases of DLBCL were of the GC type (23.6%), and this number was lower than that reported in a study conducted in the US [33]. Of 40 DLBCL cases that were subjected to CD5 testing, including 29 non-GC-type cases, none were positive for CD5, suggesting that CD5-positive DLBCL was rare among patients with ARL [34]. Two cases of GC-type DLBCL showed *MYC* rearrangement. However, these cases were clearly distinguished from BL because of their morphological features, which included extremely large cells with severe pleomorphism and no starry sky pattern (Fig. 3). BL was the second most common subtype. Approximately 40% of BL cases did not show the typical morphology of

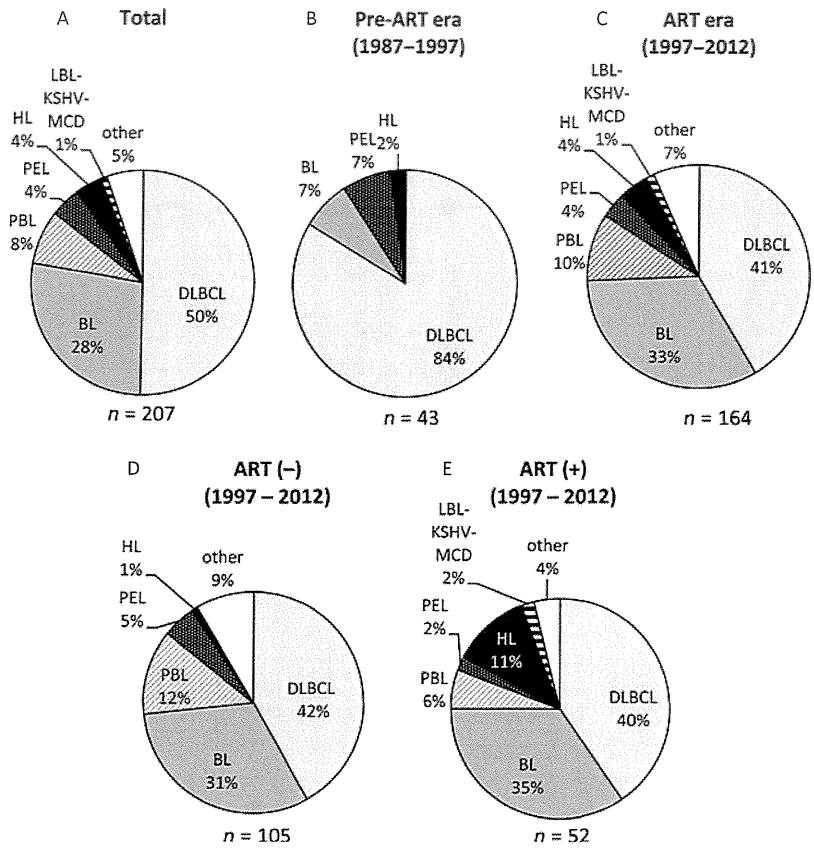

Figure 2. Pie charts for the histological subtype of AIDS-related lymphomas. The histological subtypes of AIDS-related lymphomas (ARLs) during the entire study period (1987–2012, panel A), the preantiretroviral therapy (pre-ART) era (1987–1997, panel B), and the ART era (1997–2012, panel C) are shown. In addition, the characteristics of ART-naïve patients (D) and patients who received ART at the onset of lymphoma (E) in the ART era are shown. The numbers of cases are presented under each pie (see Table 1 for abbreviations).

BL, that is, medium-sized cells and a starry sky pattern (Fig. 3). These cells were larger and showed greater nuclear pleomorphism. However, these cells were $CD10^+$, $CD20^+$, BCL-6^+, and BCL-2^-; had a Ki67 index of >90%; and showed *MYC* rearrangement. These cases, initially classified as atypical BL according to the third edition of the WHO classification, were now categorized as BL according to the fourth edition [24, 35]. All cases of KSHV positivity were detected in homosexual patients. Among the nine PEL cases, six were of solid lymphomas (extracavitary PEL), two were of effusion lymphomas alone, and one was of both effusion and solid lymphomas. The HL cases showed the following subtypes: five were of the mixed cellularity type, two were of the nodular sclerosing type, and one was of the lymphocyte-depleted type. The other rare types of lymphomas included extranodal NK/T-cell lymphoma, nasal-type (two cases); angioimmunoblastic T-cell lymphoma (two cases); anaplastic large-cell lymphoma (ALK-negative) (one case); peripheral T-cell lymphoma, not otherwise specified (one case); adult T-cell lymphoma (one case); follicular lymphoma (one case); undefined DLBCL or BL (one case); EBV-associated lymphoproliferative disorder (one case); and undefined disease (one case).

CD4 counts and prognosis of each lymphoma subtype

CD4 counts differed among the lymphoma subtypes (Table 1). The mean CD4 counts of patients with BL and HL (249 and 235 cells/μL, respectively) were significantly higher than those of patients with DLBCL, PBL, and PEL (86, 85, and 75 cells/μL, respectively; $P < 0.05$, Mann–Whitney test). CD4 counts were also associated with EBV positivity in lymphomas. The mean CD4 count in patients with EBV-positive lymphoma (83.3 cells/μL) was significantly lower than that in patients with EBV-negative lymphoma (246.2 cells/μL; $P < 0.01$, Mann–Whitney test). Even among the patients with DLBCL, the mean CD4 count in patients with EBV-positive DLBCL (45.7 cells/μL) was lower than that in patients with EBV-negative DLBCL (182.6 cells/μL; $P < 0.01$, Mann–Whitney

Table 2. Comparison of germinal center (GC) and non-GC types of DLBCL.

Factor	GC type	Non-GC type	*P* value
n	17	55	–
Age, years (mean) [median, range]	51 [52, 31–76]	45 [44, 26–68]	0.12 (MW)
Men (%)	16 (94)	51 (93)	0.73 (CY)
CD4 (mean) [median, range]	197 [175, 17–824]	69 [31, 0–444]	**<0.01 (MW)**
ART (+) at onset (%)	47	19	**0.046 (CY)**
EBV-positive (%)	18	82	**<0.01 (C)**
CNS involvement (%)	7	59	**<0.01 (C)**
LN involvement (%)	64	28	**<0.01 (C)**
BM involvement (%)	13	8	0.51 (C)
1-year survival rate (%)	82	43	**<0.01 (C)**

ART, antiretroviral therapy; BM, bone marrow;C, chi-square test; CNS, central nervous system; CY, chi-square test with Yates' correction; DLBCL, diffuse large B-cell lymphoma;GC, germinal center; EBV, Epstein–Barr virus; LN, lymph node; MW, Mann–Whitney test

P values were calculated using the chi-square test (C), chi-square test with Yates' correction (CY), and Mann–Whitney test (MW). *P* values < 0.05 are presented in bold.

test). During the entire study period, 60.8% (66/121) of patients with EBV-positive lymphomas and 42.6% (31/79) of patients with EBV-negative lymphomas died within the first year of diagnosis; this indicated a better prognosis for EBV-negative cases than for EBV-positive cases, including HL cases ($P < 0.001$, chi-square test). However, in the ART era (after 1997), the survival rates of EBV-positive and EBV-negative cases of non-Hodgkin lymphoma were 56.9% (41/72) and 63.0% (46/73), respectively, and this difference was not significant ($P = 0.455$, chi-square test).

Effect of ART on the onset of ARL

In total, 54 patients with ARL received ART at the onset of lymphoma. To determine whether ART introduction affected the onset of lymphoma, the clinicopathological characteristics of ARL occurring in patients receiving ART were compared with those occurring in ART-naïve patients (Fig. 2B–E, and Table 3). The 1-year survival rate was 65% in patients on ART, which was greater than that in ART-naïve patients (45%) ($P = 0.012$, chi-square test), suggesting a better prognosis for patients receiving ART than for ART-naïve patients. The histological differences between cases in the pre-ART era (1987–1997) and those in the ART era (1997–2012) are shown in Figure 2B and C. In the pre-ART era, 84% of ARL cases were of DLBCL, whereas the incidence of BL increased and the incidence of DLBCL decreased in the ART era. Considering the use of ART, the frequencies of BL and DLBCL did not differ significantly between patients receiving ART and ART-naïve patients (Table 3). However, the GC type of DLBCL was observed more frequently in patients receiving ART than in ART-naïve patients (Table 3). In addition, HL was observed more frequently in patients receiving ART (11.1%) than in ART-naïve patients (1.4%) during the entire study period. EBV positivity decreased and the 1-year survival rate was significantly improved in patients on ART. In addition, we analyzed 157 cases in the ART era to reveal the effect of ART in patients receiving this treatment (Fig. 2D and E). The incidence of HL increased from 1.0% in ART-naïve patients to 11.5% in patients receiving ART, even in the ART era ($P < 0.01$, chi-square test with Yates' correction). However, no significant differences were found in EBV positivity and LN involvement between patients receiving ART and ART-naïve patients in the ART era (Appendix Table A1).

Correlation of biological markers with the prognosis of ARLs

The correlation of certain biological markers with the prognosis of ARL was also investigated (Table 4). The cases positive for two markers of GC-type B cells, CD10 and BCL-6, showed a significantly higher 1-year survival rate than did those negative for CD10 and BCL-6. However, the expression of CD20, CD138, BCL-2, IRF4/MUM1, or CD30 did not correlate with the 1-year survival rate. Infection with EBV and/or KSHV significantly reduced the 1-year survival rate. Thus, in ARL, the following conditions were associated with a poor prognosis: CD10 negativity, BCL-6 negativity, EBV positivity, and KSHV positivity.

Discussion

In this study, we classified Japanese ARL cases according to histopathology. To the best of our knowledge, this is the first report to classify lymphomas histologically in a large number of HIV-1-infected patients according to the fourth edition of the WHO classification of lymphomas. Throughout the study period, DLBCL was the predominant histological subtype of ARL, followed by BL. EBV infection was present in 60% of all ARL cases. Although receipt of ART at the onset of lymphoma improved the 1-year survival rate of ARL, ART induction also resulted in an increase in the frequency of HL. In Figure 1, we present a flowchart for the diagnosis of ARL; the presence/absence of CD10, BCL-6, KSHV-LANA-1, and EBER were correlated with the prognosis of patients with ARL.

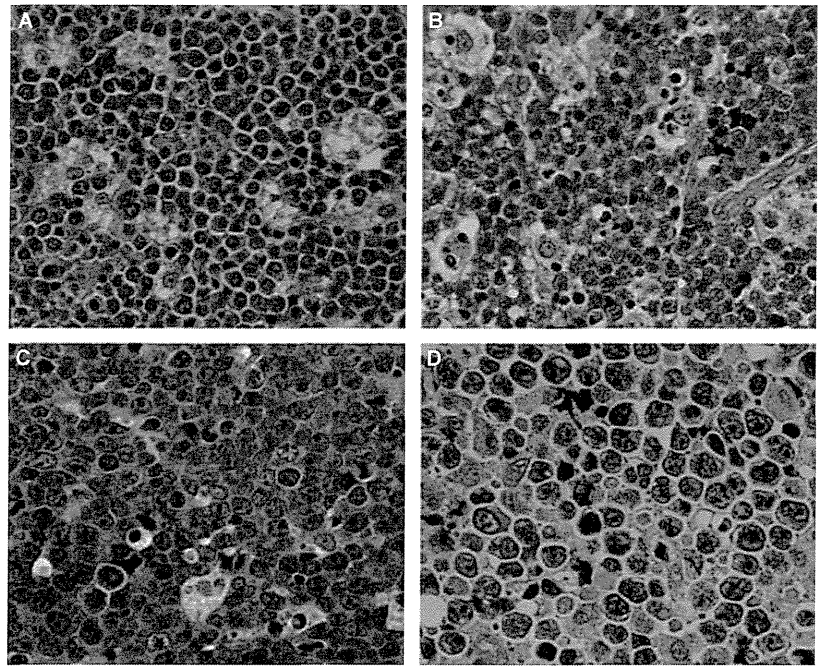

Figure 3. Differential diagnosis of diffuse large B-cell lymphoma and Burkitt lymphoma. (A) Burkitt lymphoma (BL). Each cell has a slight pleomorphism compared with a typical BL case. (B) BL. Although a starry sky pattern is shown, cells are large and pleomorphic. However, these cells are $CD10^+$, $CD20^+$, BCL-6^+, and BCL-2^-, with a Ki67 index of >90% and *MYC* rearrangement. (C) BL. The starry sky pattern is not clear, but some tingible body macrophages are observed. Cells have greater nuclear pleomorphism than those in typical BL. These cells are also $CD10^+$, $CD20^+$, BCL-6^+, and BCL-2^-, with a Ki67 index of >90% and *MYC* rearrangement, indicating the BL phenotype. (D) Diffuse large B-cell lymphoma (DLBCL) with *MYC* rearrangement. The cells in this case were $CD10^+$, $CD20^+$, BCL-6^+, and BCL-2^-, with *MYC* rearrangement and a Ki67 index of >90%. Extremely large cell morphology and severe nuclear pleomorphism without the starry sky pattern indicates DLBCL.

This information will help in the early diagnosis of lymphomas in patients with AIDS.

Among HIV-1-infected patients, BL was sometimes composed of larger cells, compared to the typically observed medium-sized cells with plasmacytoid differentiation, and did not show the typical starry sky pattern. Such cases with atypical morphology were positive for CD10, BCL-6, and *MYC* rearrangement. As microarray studies demonstrated that these cases with atypical morphology shared a gene expression profile with BL [36, 37], cases defined as atypical BL according to the third edition of the WHO classification were then categorized as BL according to the fourth edition, suggesting that the morphological spectrum of BL is very wide [24, 35]. In the present study, such atypical BL cases with typical BL phenotypes and atypical morphology were categorized as BL according to the fourth edition of the WHO classification [24]. Although the morphology of atypical BL varied among cases, approximately 40% of BL cases in the present study showed atypical morphology, suggesting that atypical BL is frequent among AIDS patients with BL. DLBCL with *MYC* rearrangement should be distinguished from atypical BL. Two cases of lymphoma with CD20, CD10, and BCL-6 positivity and the *MYC* rearrangement with large cell morphology did not show any histological features of BL. Therefore, we categorized these cases as GC-type DLBCL with *MYC* rearrangement. Intermediate DLBCL/BL was newly defined in the fourth edition of the WHO classification [25]. However, intermediate DLBCL/BL is a temporary category for high-grade B-cell lymphomas with a poor clinical outcome and is used mainly for cases with double- and triple-hit translocations [38]. In addition, the fourth edition of WHO classification criteria did not include intermediate DLBCL/BL as an ARL [22]. We did not encounter any cases of intermediate DLBCL/BL in the present study. However, as we could not perform a full chromosome analysis in all cases of BL and DLBCL, some cases in the present study might be categorized into this group. Further studies including a complete chromosome analysis will be required to clarify the presence of intermediate DLBCL/BL in ARL cases.

In the present study, we identified certain effects of ART introduction at the onset of lymphoma on the clinicopathological characteristics of ARL. An increasing number of HL cases in patients receiving ART have been reported in the United States and Japan [6, 18, 39]. The mean CD4 count in patients with HL was higher than that in patients with other lymphomas, implying that the

Table 3. Effect of ART on the onset of AIDS-related lymphoma.

Factors	ART (−)	ART (+)	*P* value
n	146	53	—
Histology			
DLBCL	78 (53.4%)	23 (42.6%)	0.211(C)
(non-GC/GC)	(43/9)	(10/8)	**0.046 (CY)**
BL	36 (24.7%)	18 (33.3%)	0.192 (C)
PBL	13 (8.9%)	3 (5.6%)	0.653 (CY)
PEL	8 (5.5%)	1 (1.9%)	0.489 (CY)
HL	2 (1.4%)	6 (11.1%)	**<0.01 (CY)**
LBL-KSHV-MCD	0 (0%)	1 (1.9%)	0.596 (CY)
Other	9 (6.2%)	2 (3.7%)	0.763 (CY)
Age, years (mean) [median, range]	44 [42, 12–76]	49 [49, 29–75]	**<0.01 (MW)**
Men (%)	96	96	0.848 (CY)
CD4 (mean) [median, range]	104 [50, 0–560]	269 [176, 4–2431]	**<0.01 (MW)**
EBV-positive (%)	66	44	**0.010 (C)**
CNS involvement (%)	35	22	0.132 (C)
LN involvement (%)	61	58	0.701 (C)
BM involvement (%)	27	35	0.311 (C)
1-year survival rate (%)	45	65	**0.012 (C)**

ART, antiretroviral therapy; DLBCL, diffuse large B-cell lymphoma; GC, germinal center; BL, Burkitt lymphoma; PBL, plasmablastic lymphoma; PEL, primary effusion lymphoma; HL, Hodgkin lymphoma; LBL-KSHV-MCD, large B-cell lymphoma arising in Kaposi sarcoma-associated herpes virus-related multicentric Castleman disease; EBV, Epstein–Barr virus; CNS, central nervous system; LN, lymph node; BM, bone marrow; C, chi-square test; CY, chi-square test with Yates' correction; MW, Mann–Whitney test.

ART (−): patients who did not receive ART at the onset of lymphoma; ART (+): patients who received ART at the onset of lymphoma. *P* values < 0.05 are presented in bold.

Table 4. Prognostic significance of biological markers.

Markers	Result	1-year survival		Survival rate (%)	*P* value
		Live	Death		
CD20	+	77	78	49.7	0.863
	−	22	21	51.2	
CD10	+	51	22	69.9	**<0.01**
	−	32	44	42.1	
BCL-6	+	50	24	67.6	**<0.01**
	−	29	40	42.0	
CD138	+	11	8	57.9	0.659
	−	40	23	63.5	
BCL-2	+	30	18	62.5	0.986
	−	52	31	62.7	
IRF4/MUM1	+	32	30	51.6	0.778
	−	25	21	54.3	
CD30	+	29	20	59.2	0.931
	−	36	24	60.0	
EBER	+	49	66	42.6	**<0.01**
	−	48	31	60.8	
KSHV	+	3	8	27.3	**0.034***
	−	50	26	65.8	

EBER, Epstein–Barr virus encoded small RNAs; KSHV, Kaposi sarcoma-associated herpesvirus.

P-values were calculated using the chi-square test or chi-square test with Yates' correction (*). *P* values < 0.05 are presented in bold.

frequency of HL is associated with ART and immune reconstitution syndrome in HIV-1-infected patients. The proportion of BL cases did not increase significantly in patients on ART compared with ART-naïve patients (Table 3 and Fig. 2). As we reported in 2006, BL and PBL cases increased in the ART era compared with the pre-ART era [17]. The present study also showed that the incidence of DLBCL drastically decreased from 84% among patients in the pre-ART era to 42% among ART-naïve patients in the ART era, whereas the BL incidence increased from 7% in the pre-ART era to 31% in the ART era (Fig. 2B and D). However, the frequencies of these subtypes did not differ significantly between patients receiving ART and ART-naïve patients in the ART era (Fig. 2D and E), suggesting that these changes were not associated with the introduction of ART. In the present study, the mean CD4 count of ART-naïve patients in the pre-ART era (62.2 cells/μL) was significantly lower than that of ART-naïve patients in the ART era (121.5 cells/μL; $P < 0.01$, Mann–Whitney test). Since 1998, the importance of HIV-1 testing is well recognized in the general population of Japan, and many persons with risk factors have visited clinics for HIV-1 testing, resulting in earlier detection of HIV-1 in the ART era than in the pre-ART era. Such early detection of HIV-1 in patients might be associated with histological differences between patients in the pre-ART era and the ART era. In contrast, the introduction of ART was associated with lower EBV positivity and higher 1-year survival rates in patients receiving ART than in ART-naïve patients.

Despite the increasing number of HL cases after the introduction of ART, DLBCL is still the most frequent subtype of ARL. In Japan, 23.6% of DLBCL cases were of the GC type. This rate is lower than that reported in the United States [33]. Non-GC-type DLBCL was more frequent among HIV-1-negative cases in the United States. [32]. In the present study, non-GC-type DLBCL showed higher rates of EBV infection, poorer prognosis, and more frequent CNS involvement than the GC type. In addition, the GC/non-GC ratio was higher in patients receiving ART than in ART-naïve patients. EBV infection was found to be less frequent among GC-type cases, and this may be associated with the high CD4 count among GC-type cases. Although a large clinical study reported

no significant difference between the survival rates of patients with GC-type and non-GC-type DLBCL in the United States [33], another study showed that the non-GC type was associated with a poorer outcome [28]. To estimate the prognosis of these groups, further clinical studies on a large set of DLBCL cases are warranted.

A diagnostic flowchart for ARL diagnosis, as used in the present study (Fig. 1), will be useful for the routine pathological diagnosis of ARL. We propose the use of CD3, CD20, CD10, BCL-6, IRF4/MUM1, BCL-2, Ki67, EBER, KSHV-LANA-1, CD138, CD30, and CD15 as markers for the classification of ARL. This set of biological markers can help distinguish between DLBCL, BL, PEL, HL, PBL, and T-cell lymphomas. GC-type DLBCL can also be distinguished from non-GC-type DLBCL by using this set of markers. *MYC* rearrangement should be analyzed to confirm the diagnoses of BL and DLBCL. Immunohistochemistry of CD20 is important for the differential diagnoses of PBL and KSHV-associated lymphomas because these lymphomas are usually negative for CD20 and have a poor prognosis. Statistical analyses identified CD10 negativity, BCL-6 negativity, EBV positivity, and KSHV positivity as risk factors for poor prognosis. The CD10 negativity, BCL-6 negativity, and EBV positivity may be associated with the poor prognosis of non-GC-type DLBCL, while the KSHV positivity was associated with the extremely low survival rates of 11 patients with KSHV-associated lymphoma (Table 1). EBV-positive cases had a poorer prognosis than did EBV-negative cases; however, EBV positivity was reduced with ART introduction. As DLBCL and BL are groups with biological heterogeneity and are not disease entities caused by a single genetic alteration, information regarding associated biomarkers may help predict their clinical outcomes.

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Conflict of Interest

None declared.

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Appendix

Table A1. Effect of ART on the onset of ARL in the ART era.

Factors	ART (–)	ART (+)	*P*
n	105	52	–
Histology			
DLBCL	44 (41.9%)	21 (40.4%)	0.856 (C)
(non-GC/GC)	(26/9)	(10/8)	0.1665 (C)
BL	33 (31.4%)	18 (34.6%)	0.688 (C)
PBL	13 (12.4%)	3 (5.8%)	0.197 (C)
PEL	5 (4.8%)	1 (1.9%)	0.667 (CY)
HL	1 (1.0%)	6 (11.5%)	**<0.01 (CY)**
LBL-KSHV-MCD	0 (0%)	1 (1.9%)	0.719 (CY)
Other	9 (8.6%)	2 (3.8%)	0.448 (C)
Age, years (mean) [median, range]	45.1 [42.6, 25–76]	49.2 [48.5, 30–75]	**<0.01 (MW)**
Men (%)	97	96	0.880 (CY)
CD4 (mean) [median, range]	121.5 [76, 0–560]	280 [184, 6–2431]	**<0.01 (MW)**
EBV-positive (%)	59	44	0.076 (C)
CNS involvement (%)	20	20	0.981 (C)
LN involvement (%)	49	59	0.275 (C)
BM involvement (%)	30	35	0.556 (C)
1-year survival rate (%)	60	66	0.504 (C)

157 cases in the ART era (1997-) were analyzed. *P* values were calculated using the Chi-square test (C), Chi-square test with Yates correction (CY), and Mann–Whitney test (MW). See Table 1 for abbreviations. *P* values < 0.05 are presented in bold.

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HIV protease inhibitor Lopinavir induces apoptosis of primary effusion lymphoma cells via suppression of NF-κB pathway

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ABSTRACT

Primary effusion lymphoma (PEL) is a non-Hodgkin lymphoma that occurs predominantly in patients with advanced AIDS. In this study, we examined the effect of HIV protease inhibitors, Lopinavir (LPV), Ritonavir (RTV) and Darunavir (DRV) on PEL cell lines *in vitro* and *in vivo*. LPV and RTV, but not DRV induced caspase-dependent apoptosis and suppressed NF-κB activity by inhibiting IKK phosphorylation in PEL cells. In a PEL xenograft mouse model, LPV significantly inhibited the growth and invasion of PEL cells. These results suggest that LPV may have promise for the treatment and prevention of PEL, which occurs in HIV/AIDS patients.

1. Introduction

Primary effusion lymphoma (PEL) is a subtype of non-Hodgkin B cell lymphoma that mainly presents in patients with advanced AIDS, but is sometimes also found in immunosuppressed patients, such as those who have undergo organ transplantation [1,2]. Among AIDS-related NHLs, PEL generally has an extremely aggressive clinical course with a median survival of only 6 months [2,3]. PEL usually presents as a lymphomatous effusion in body cavities and is caused by Kaposi sarcoma-associated herpes virus (KSVH/HHV-8) [1]. A number of constitutively activated signaling pathways play critical roles in the survival and growth of PEL cells. These include nuclear factor (NF)-κB, JAK/STAT and PI3 kinase [4–6]. KSHV/HHV-8 encodes a virus Fas-associated death domain-like interleukin-1β-converting enzyme (FLICE) inhibitory protein (vFLIP) that has the ability to activate the NF-κB pathway [7–9]. vFLIP has been shown to bind to the IKK complex to induce constitutive kinase activation [10] and, as a result, PEL cells have high levels of nuclear NF-κB activity, whereas inhibition of NF-κB induces apoptosis in PEL cells [5,11]. These studies support the idea that vFLIP-mediated NF-κB activation is necessary for the survival of PEL cells and that this pathway represents a target for molecular therapy for this disease.

HIV-1 protease inhibitors (HIV-PIs) have been successfully used in the treatment of HIV-1 infection. Incorporation of HIV-PIs in combination antiretroviral therapy (cART) has significantly reduced morbidity and mortality and prolonged the lifespan of patients with HIV infection. However, HIV-PIs have been shown to directly affect cell metabolism, interfere with host proteases and induce metabolic abnormalities such as insulin resistance, lipodystrophy, and hyperlipidemia, even though they were designed to selectively interfere with the catalytic site of HIV protease. Recently, HIV-PIs have become a focus of attention for having anti-tumor effects [12]. HIV-PIs have been shown to block angiogenesis, tumor cell invasion and tumor cell growth, and to induce endothelial reticulum stress, autophagy and tumor cell apoptosis both *in vivo* and *in vitro* [13–15]. Interestingly, the mechanisms of these anti-tumor effects are different with each HIV-PI, indicating that, although classified together, HIV-PIs are quite distinct compounds [16].

Ritonavir (RTV) has been shown to inhibit the chymotrypsin-like activity of the 20S proteasome and to activate the chymotrypsin-like activity of the 26S proteasome conversely [17–19]. RTV also has been reported to inhibit the transactivation of NF-κB induced by activators such as TNFα, HIV-1 Tat protein and the human herpesvirus 8 protein ORF74 [20]. It is possible that inhibition of NF-κB activation by RTV is linked to additional pathways other than proteasome inhibition. HIV-PIs also have been shown to have direct antiangiogenic and antitumor activity [12]. Recently, it was reported that RTV inhibits the growth and infiltration of ATL cells through targeting NF-κB [14,21]. Lopinavir (LPV) is a frequently used HIV-PI, but only a few antitumor effects have been reported [22]. Recently, a second generation HIV-PI,

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Darunavir (DRV), was developed. DRV has high activity against HIV protease and, as a result, DRV does not lead to tolerance induction [23–25], and since DRV is designed to specifically bind to HIV protease, the adverse effects are less frequent compared with other HIV-PIs.

In the present study, we investigated the antitumor activity of HIV-PIs against human PEL cell lines *in vitro* and *in vivo*. We found that RTV and LPV inhibit constitutively active NF-κB, leading to PEL apoptosis. In contrast, a second generation HIV-PI, DRV, has little effect against PEL cells. Our findings provide the experimental basis for utilizing HIV-PIs against tumors in HIV-1-infected individuals.

2. Materials and methods

2.1. Cell lines and reagents

The human PEL cell lines, BCBL-1 (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH) [26], BC-1 [27], BC-3 [28], (obtained through ATCC International Essentials of Life Science Research, USA) and TY-1 [29] were maintained in RPMI1640 supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) in a humidified incubator at 37 °C and 5% CO_2. Ritonavir (RTV), Lopinavir (LPV) (Abbott Labs, North Chicago, IL), Darnavir (DRV) (Pfizer, NY) and LY294002 (Cell Signaling Technology, Danvers, MA) were dissolved in DMSO. DHMEQ (a kind gift from Dr. K. Umezawa, Keio University, Japan) is a NF-κB inhibitor that acts at the level of the nuclear translocation of NF-κB [30].

2.2. Tetrazolium dye methylthiotetrazole (MTT) assay

The antiproliferative effects of HIV-PIs against PEL cell lines were measured by the methylthiotetrazole (MTT) method (Sigma, St. Louis, MO). Briefly, 2×10^4 cells were incubated in triplicate in a 96-well microculture plate in the presence of different concentrations of HIV-PIs in a final volume of 0.1 ml for 24 h at 37 °C. Subsequently, MTT (0.5 mg/ml final concentration) was added to each well. After 3 h of additional incubation, 100 μl of a solution of 0.04 N HCl were added to dissolve the crystal. The absorption values at 570 nm were determined with an automatic enzyme-linked immunosorbent assay (ELISA) plate reader (Multiskan, Thermo ElectronVantaa, Finland). Values are normalized to the untreated (control) samples.

2.3. Cell cycle analysis

For cell cycle analysis, after PEL cells were treated with HIV-PIs (25 μM) for various hours, the cells were incubated in 70% ethanol at 4 °C overnight, treated with RNase A and stained with propidium iodide (PI, 50 μg/ml). The DNA content in each cell was analyzed on LSR II flow cytometer (BD Bioscience, San Jose, CA). Data were analyzed on FlowJo software (Tree Star, San Carlos, CA).

2.4. Annexin V assay

Apoptosis was quantified using the Annexin V: FITC apoptosis MEL MEBCYTO apoptosis kit (MBL, Nagoya, Japan) [31]. Briefly, after treatment with various concentration of HIV-PIs for 12 h, cells were harvested, washed with Annexin binding buffer and then incubated with Annexin V-FITC for 15 min in the dark, and PI added before being analyzed on a LSR II cytometer.

2.5. Caspase activity measurements with flow cytometry

Active caspase 3, 8 and 9 activities were measured using APOPCYTO (MBL) according to the manufacturer's instructions. Briefly, various concentrations of HIV-PI- treated or -untreated cells (200 μl) were incubated with 2 μL substrate, FITC-DEVD-FMK was added to each well, and incubated for 60 min at 37 °C in CO_2 incubator. After incubation, cells were washed with washing buffer, 2 μg/ml PI added and analyzed by LSR II. Data were analyzed on FlowJo software for the expression of active caspase 3-, 8- and 9-positive cellular events among PI-negative (living) cells.

2.6. Western blot analysis

BCBL-1 cells with or without treatment of 40 μM LPV for 1, 3 and 6 h were collected and washed in cold PBS before the addition of 300 μl cold buffer A (10 mM HEPES KOH pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.1% NP-40, 0.5 mM DTT, 0.5 mM PMSF, 2 μg/ml pepstatin A, 2 μg/ml aprotinin and 2 μg/ml leupeptin). After incubation on ice for 10 min, the samples were vortexed for 10 s and centrifuged at 5000 rpm for 1 min and supernatant collected as a cytoplasmic sample. Nuclei were pelleted by centrifugation and washed once with buffer A. Then, 100 μl buffer C (50 mM HEPES-KOH pH 7.9, 10% glycerol, 420 mM NaCl, 5 mM MgCl2, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 2 μg/ml pepstatin A, 2 μg/ml aprotinin and 2 μg/ml leupeptin) were added to the nuclei, sonicated for 10 s 10 times and incubated on ice for 3 h. Nuclear extracts were obtained by centrifugation at 15,000 rpm for 15 min. Then the cytoplasmic protein and nuclear extracts (40 μg protein) were separated by 10% SDS–PAGE and blotted onto a PVDF membrane (GE Healthcare, Buckinghamshire, UK). Detection was performed using the ECL Plus Western Blotting Detection System (ECL; GE Healthcare). Primary antibodies used were as follows: anti-p65 (F-6), anti-I-κBα (C-21), anti-IKKαβ (H-479), anti-phospho-IKKαβ (Thr23), anti-actin (C-2), anti-γ tubulin (C-20) (Santa Cruz Biotechnology, Santa Cruz, CA), and and anti-Akt (4691), anti-phospho (Thr308)-Akt (2965), anti phospho (Ser473)-Akt (4060), anti-phospho (Ser241)-PDK1 (3438), anti-PI3 kinase (4257), anti-phospho p85 (Tyr458)/ p55 (Tyr199)-PI3 kinase (4228), anti-phospho-I-κBα (Ser32/36) (Cell Signaling Technology, Danvers, MA). Western blots were quantified using the ImageQuant LAS 4000 system (GE Healthcare). Relative density was evaluated and normalized with actin or γ tubulin.

2.7. Transient transfection and NF-κB p65 reporter gene assay

BCBL-1 was transfected with the NF-κB-LUC plasmid (Stratagene, La Jolla, CA) using the Neon transfection system (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions and incubated at 37 °C for 24 h. Cells were treated with RTV, LPV, DRV and DHMEQ for 1, 3 and 6 h, and protein was extracted and subjected to determination of luciferase activity by the Dual Luciferase Assay system (TOYO INK Corporation, Tokyo, Japan) according to the manufacturer's instructions. Firefly luciferase activity was standardized using luciferase activity by Renilla luciferase activity.

2.8. RT-PCR

Total RNA was extracted from the cells using Trizol (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesized from RNA using a PrimeScript RT-PCR kit (Takara Bio, Otsu, Japan) with random primers. The PCR products were analyzed by 1.5% agarose gel electrophoresis and ethidiumbromide staining. Primer sequences were as follows: ORFK13 (v-FLIP): 5′-ATTGACATTAGGGCATCC-3′ and 5′-AAAGGAGGA GGGCAGGTT, ORF72 (v-cyclin): 5′-GATAATAGAGGCGGGCAATG-3′ and 5′-TAA AGCAGGTGTCCAAAGAA-3′, ORF73 (LANA): 5′-GAAGTGGATTACC CTGTTGT TAGC-3′ and 5′-TTGGATCTCGTCTTCCATCC-3′, ORF50 (RTA): 5′-GCC CTCTGC CTTTTGGTT- 3′ and 5′-GATGATGCTGACGGGTGTG-3′, GAPDH: 5′-CGGGAAG CTTGTGATCA ATGG-3′ and 5′-GGCAGTGATGGCATG GACTG-3′[32,33]

2.9. Xenograft mouse model

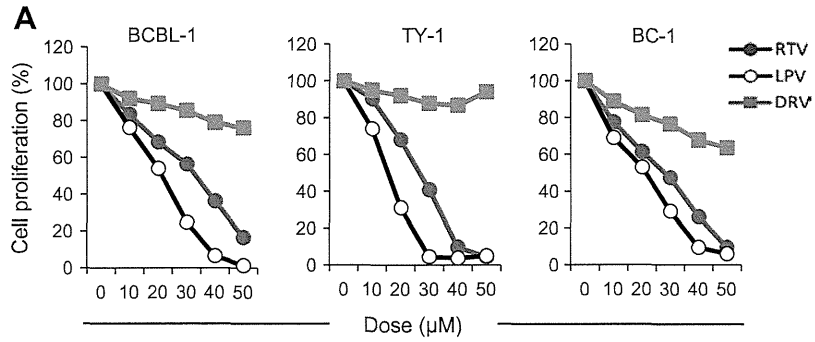
NOD/Scid/Jak3-deficient (NOJ) mice were established by backcrossing Jak3-deficient mice [34] with the NOD.Cg.-Prkdcscid strain for 10 generations [35]. NOJ male mice of 8 to 10 weeks old were housed and monitored in our animal research facility according to the institutional guidelines. All experimental procedures and protocols were approved by the Institutional Animal Care and Use Committee at Kumamoto University. NOJ mice were intraperitoneally inoculated with 1×10^7 BCBL-1 cells suspended in 100 μl PBS. The mice were then treated with intraperitoneal injections of PBS or HIV-PIs (40 μmol/kg per day). Tumor burdens were evaluated by measuring the volume of ascites.

2.10. Immunohistochemistry

To investigate the expression of KSHV/HHV-8 ORF73 (LANA) protein, tissue samples were fixed with 10% neutral-buffered formalin, embedded in paraffin and cut into 4 μm sections. The sections were deparaffinized by sequential immersion in xylene and ethanol and rehydrated in distilled water. They were then irradiated for 15 min in a microwave oven for antigen retrieval. Endogenous peroxidase activity was blocked by immersing the sections in methanol/0.6% H_2O_2 for 30 min at room temperature. Affinity-purified PA1-73N antibody [20], diluted 1:3,000 in PBS/5% bovine serum albumin (BSA), was then applied, and the sections were incubated overnight at 4 °C. After washing in PBS twice, the second and third reactions and the amplification procedure were performed using kits according to the manufacturer's instructions (catalyzed signal amplification system kit; DAKO, Copenhagen, Denmark). The signal was visualized using 0.2 mg/ml diaminobenzidine and 0.015% H_2O_2 in 0.05 mol/l Tris–HCl, pH 7.6.

2.11. Statistical analysis

All assays were performed at least in triplicate and expressed as mean values ± SD. The statistical significance of the differences observed between experimental groups was determined using Student's *t* test. $P < 0.05$ was considered significant.



B

CC50	BCBL-1	TY-1	BC-1	BC-3
RTV	35μM	27μM	28μM	32μM
LPV	22μM	16μM	21μM	20μM
DRV	>50μM	>50μM	>50μM	>50μM

Fig. 1. HIV protease inhibitors inhibit the proliferation of PEL cells. (A) PEL cell lines (BCBL-1, TY-1 BC-1 and BC-3) were incubated with 10, 20, 30, 40, 50 μM RTV, LPV DRV for 24 h. A cell proliferation assay was carried out using MTT as described in Section 2. A representative result from 3 independent experiments is shown. (B) Show the IC50 value of each HIV protease inhibitors.

3. Results

3.1. RTV and LPV cause dose-dependent inhibition of the proliferation and apoptosis of PEL cell lines

We initially sought to determine whether HIV-PI treatment leads to the inhibition of PEL cell proliferation. Four PEL cell lines (BCBL-1, TY-1, BC-1 and BC-3) were cultured in the presence of 10, 20, 30, 40, and 50 μM HIV-PIs for 24 h, and proliferation was analyzed by MTT assays. Fig. 1A shows that as the dose of RTV and LPV increased from 10 to 50 μM, cell growth inhibition increased in a dose-dependent fashion in all PEL cell lines (Fig. 1A). On the other hand, marked anti-proliferative effects were not observed by DRV at these concentrations. Fig. 1B shows the IC50 value of each HIV-PI individual. In subsequent experiments, we determined whether the observed suppressive effects of RTV and LPV in the MTT assay were due to the induction of cell cycle arrest or apoptosis. As shown in Fig. 2, 25 μM RTV and LPV treatment for 12 h induced cell cycle arrest. Next we used Annexin V and propidium iodide dual staining to detect apoptosis. Annexin-positive propidium iodide-negative fraction represents the early phase of apoptosis whereas Annexin-positive propidium iodide-positive fraction represents the late phase of apoptosis and necrosis [31]. As shown in Fig. 3A, 40 μM RTV and LPV treatment for 12 h caused apoptosis in all cell lines tested, but DRV did not induce apoptosis in these cell lines. Next, we measured the activation of caspase 3, 8 and 9 to further confirm that RTV and LPV induced apoptosis in PEL cells. As shown in Fig. 3B, RTV and LPV treatment of PEL cells induced the activation of caspase 3, 8 and 9, a hallmark of cells undergoing apoptosis.

3.2. LPV efficiently blocks the constitutive NF-κB activity of PEL cell lines

As several reports have suggested that NF-κB can act as a survival factor and is required for the proliferation of PEL cells, and PEL cells are known to induce apoptosis with inhibition of the NF-κB pathway, [5,11,36] we examined whether LPV inhibits NF-κB activation. When PEL cell lines were treated with 40 μM LPV for 1, 3 and 6hr, the amount of phosphorylated I-κBα protein was severely reduced; however, the amount of I-κBα protein was almost the same, indicating that LPV suppresses NF-κB activity by suppressing the activation of I-κBα phosphorylation (Fig. 4A). Suppression of I-κB phosphorylation blocked the nuclear translocation of NF-κB p65 and led to the accumulation of NF-κB p65 protein (Fig. 4A). Next, we fractioned nuclear protein and analyzed the expression of p65 by Western blotting (Fig. 4B) to confirm NF-κB p65 suppression by LPV. When PEL cell lines were treated with 40 μM LPV for 6 h, the amount of nuclear NF-κB p65 protein was reduced as expected, indicating that LPV suppresses NF-κB activity. To confirm that LPV could inhibit NF-κB transcriptional activity in PEL cell lines, we performed the NF-κB promoter assay. Treatment with RTV and LPV suppressed the transcriptional activity of NF-κB in all cell lines tested (Fig. 4C); however, DRV did not inhibit NF-κB activity. These results revealed that LPV blocks the constitutive NF-κB activity of PEL cells.

3.3. Inhibition of NF-κB activity is not depend on Akt pathway

Akt pathway regulates NF-κB activity and it was reported that LPV inhibits Akt pathway[37]. Therefore, we examined whether LPV suppresses IKK phosphorylation through inhibition of Akt pathway. When PEL cell lines were treated with 40 μM LPV for 1, 3 and 6 h, the amount of phosphorylated Akt protein was severely reduced. However amount of phosphorylated PI3 kinase which is upstream of Akt pathway was almost same indicated that LPV suppressed Akt phosphorylation (Fig. 5A) Furthermore, we examined whether inhibition of Akt pathway suppresses IKK phosphorylation in PEL cells. LY294002 (PI3 kinase inhibitor) treatment suppressed Akt phosphorylation but could not suppressed IKK phosphorylation (Fig. 5B), indicated that inhibition of Akt pathway cannot suppress IKK phosphorylation in PEL cells. These results indicate that suppression of IKK phosphorylation by LPV is not due to the inhibition of Akt pathway.

3.4. LPV dose not induce HHV-8 reactivation

It is well known that v-FLIP has the ability to activate the NF-κB pathway through binding to the IKK complex to induce

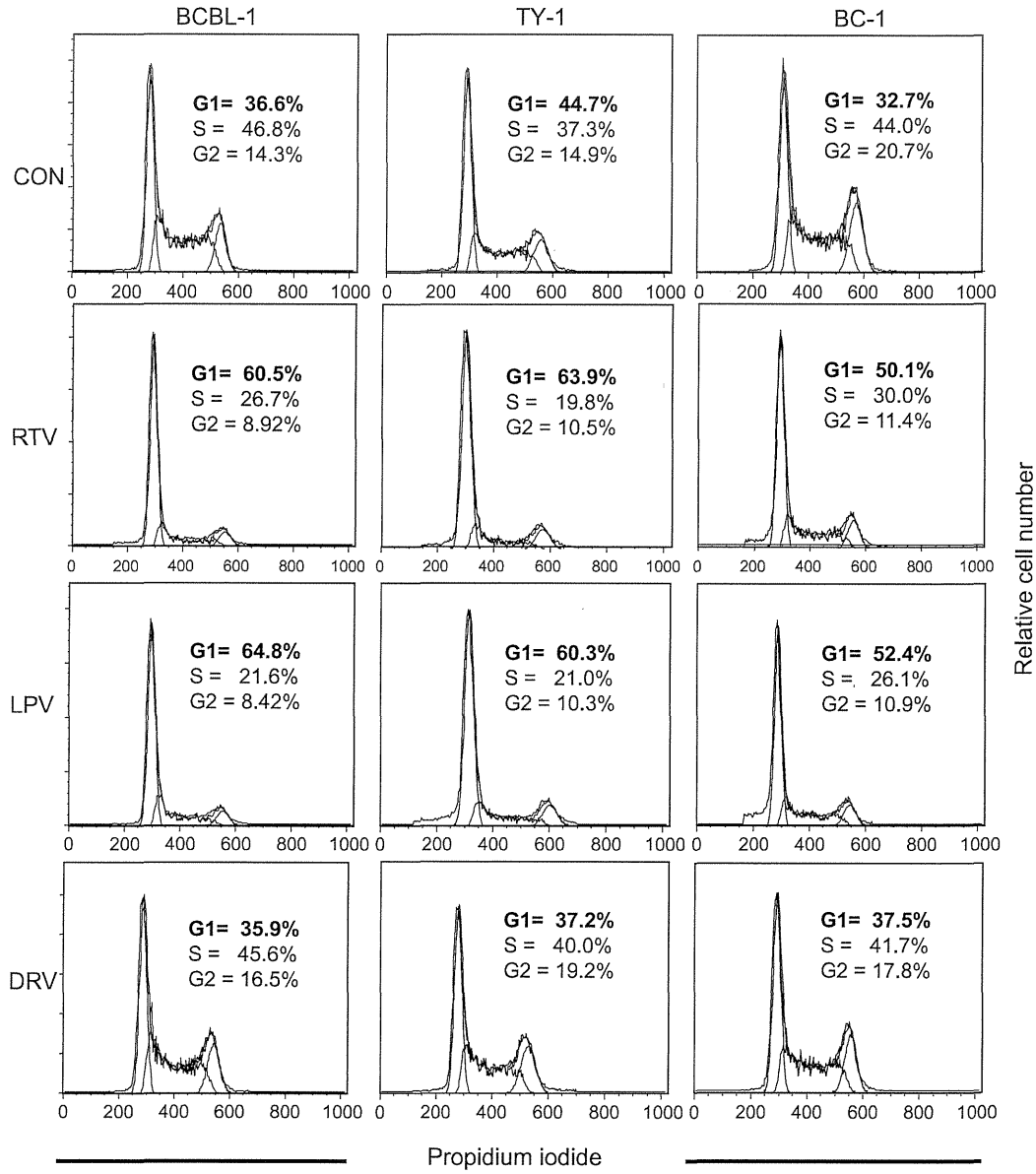


Fig. 2. HIV protease inhibitors cause cell cycle arrest of PEL cells. PEL cell lines BCBL-1, TY-1 and BC-1 were treated with RTV, LPV, DRV (25 μM) for 12 h, and DNA histograms were determined and the cell cycle was analyzed using FlowJo software. A representative result from 3 independent experiments is shown.

constitutive kinase activation [7,36,38]. Therefore, RT-PCR was performed to examine the effects of LPV on viral gene expression including v-FLIP. LPV treatment did not change both of lytic (RTA) and latent gene (v-FLIP, LANA and v-cyclin) expression (Fig. 5C), indicated that LPV have no direct effect on viral gene expression including v-FLIP.

3.5. Antitumor effect of LPV in PEL-inoculated mice

As the above results suggested the efficacy of RTV and LPV for the treatment of PEL patients, we next examined the *in vivo* effects of LPV in a PEL-inoculated mouse model. Severely immunodeficient, NOD/Scid/Jak3-deficient mice (NOJ mice) [35] were inoculated intraperitoneally with 1×10^7 BCBL-1 cells. BCBL-1 produced massive ascites within 4 weeks of inoculation (Fig. 6A). As PEL is characterized by lymphomatous effusion of serous cavities and rarely presents with a definable tumor mass [1,2], these mice are a clinically relevant PEL model. A dose of 40 μmol/kg/day LPV and DRV or PBS was administrated via intraperitoneal injection on day 3 after cell inoculation and every day thereafter for 28 days. LPV-treated mice appeared to be healthy and had a significantly lower volume of ascites than DRV or PBS-treated mouse ascites (0.47 ± 0.35 ml vs. 5.08 ± 1.52 ml, $n = 10$ each, $p < 0.001$) (Fig. 6B). Organ infiltration by tumor cells was analyzed and evaluated by hematoxylin–eosin staining and LANA immunostaining (Fig. 6C). We found that mice inoculated intraperitoneally with BCBL-1 exhibited infiltration into the lung, liver, and spleen without macroscopic lymphoma formation. The number of LANA-positive cells in LPV-treated mice was significantly reduced (0–1 cells per field magnification, 340) compared to non-treated, DRV mouse (10–20) cells per field magnification, 340) (Fig. 6D). These data indicate that LPV significantly inhibits the growth and infiltration of PEL cells *in vivo*.

4. Discussion

In the present study, we investigated the effects of three different HIV PIs, RTV, LPV and DRV, on PEL cells both *in vitro* and *in vivo*.

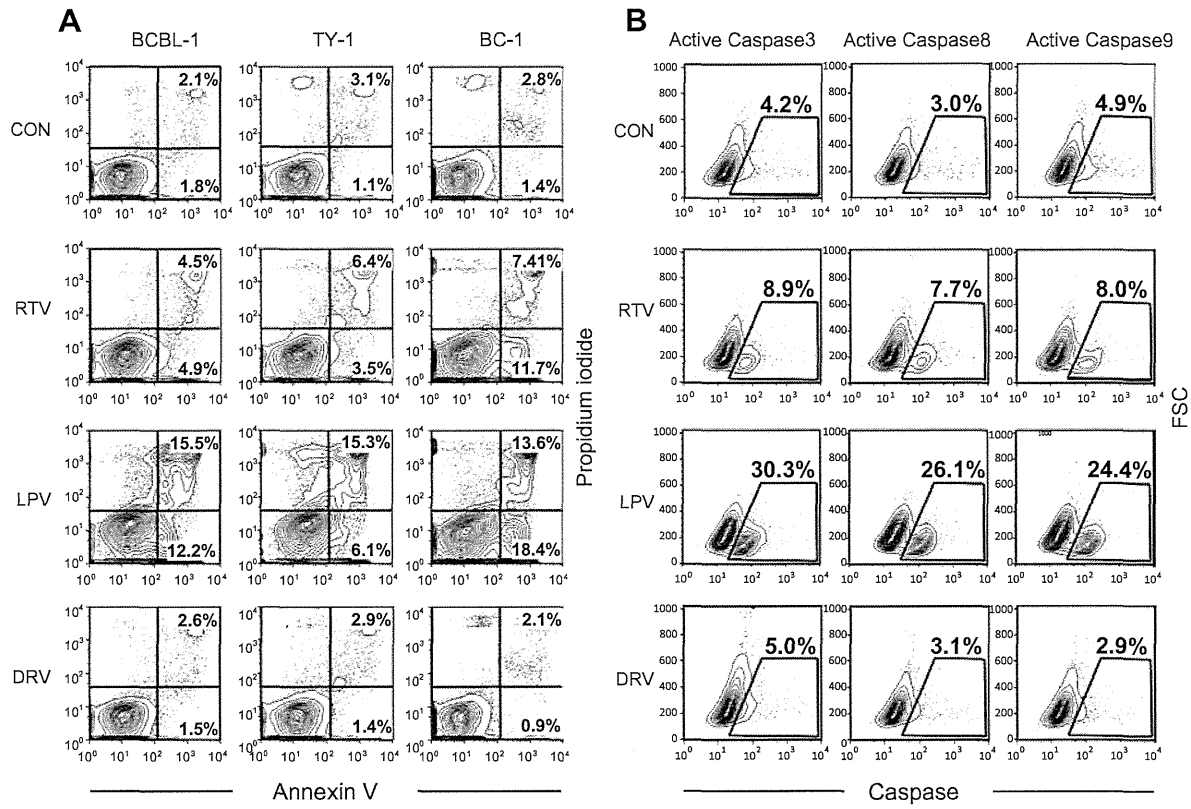


Fig. 3. HIV protease inhibitors cause apoptosis of PEL cells. (A) HIV protease inhibitors induced apoptosis as detected by Annexin V and propidium iodide dual staining. PEL cell lines BCBL-1, TY-1 and BC-1 were treated with 40 μM of RTV, LPV and DRV for 12 h and were subsequently stained with Annexin-FITC and propidium iodide before being analyzed by flow cytometry. (B) HIV protease inhibitor induces apoptosis of PEL cells via caspase-3-, 8- and 9-dependent pathway. The PEL cell line BCBL-1 was treated with RTV LPV DRV (40 μM) for 12 h and was subsequently stained with caspase-3, 8, 9 before being analyzed by flow cytometry. A representative result from 3 independent experiments is shown.

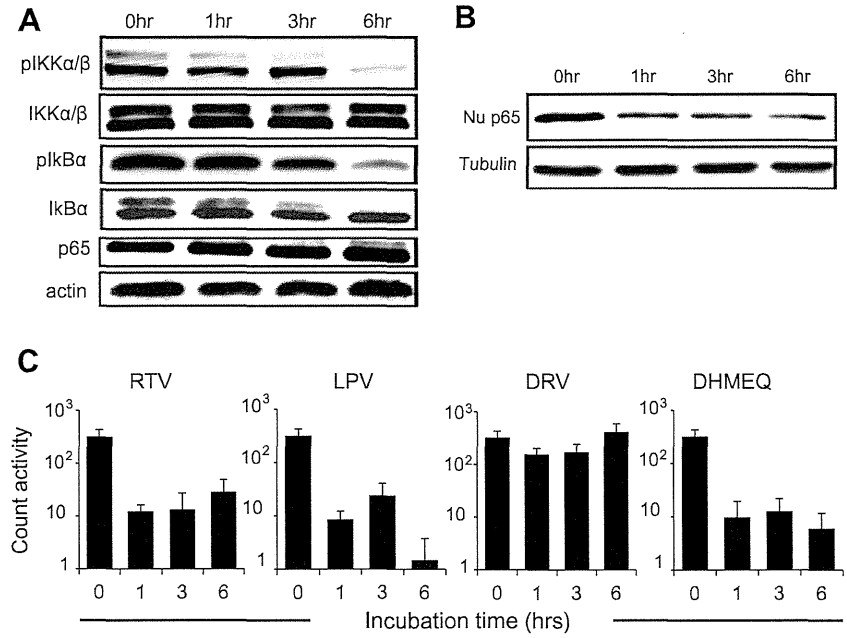


Fig. 4. Inhibitory effects of HIV protease inhibitors on the expression of NF-κB pathways. (A) The PEL cell line BCBL-1 was treated with LPV (40 μM) for 1, 3 and 6hr and cytoplasmic proteins were extracted and Western blot was performed. The numbers indicate the relative expression of each protein level normalized with actin. (B) The PEL cell line BCBL-1 was treated with LPV (40 μM) for 1, 3 and 6 h and nuclear proteins were extracted and Western blot was performed to detect NF-κB p65. The numbers indicate the relative expression of p65 normalized with γ tubulin. A representative result from 3 independent experiments is shown. (C) BCBL-1 was transfected with NF-κB-LUC plasmid and 24 h later cells were treated with 40 μM of RTV, LPV, DRV and DHMEQ for 1, 3, 6 h and protein was extracted, and firefly and Renilla luciferase were measured on a luminometer. Firefly luciferase activity was normalized to Renilla luciferase expression.

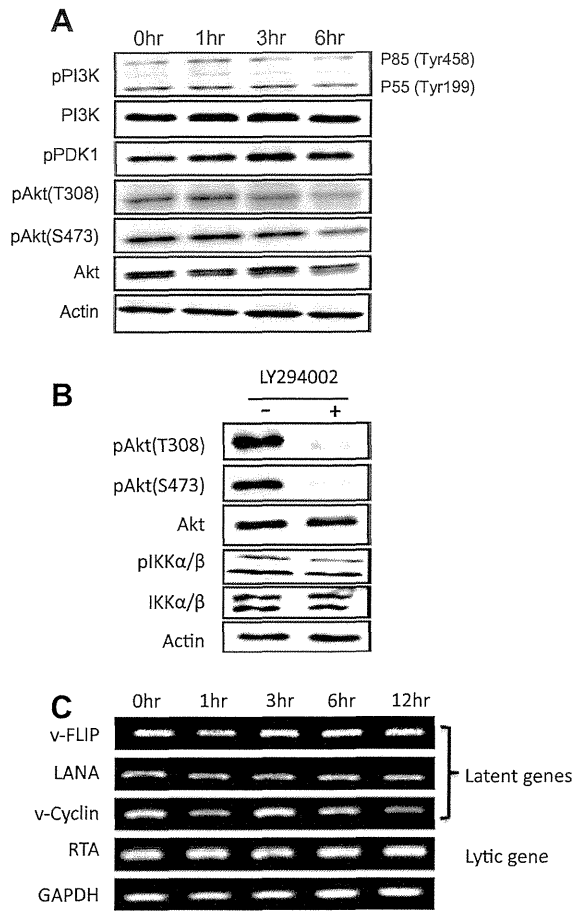


Fig. 5. Effects of LPV against upstream of IKKα and IKKβ. (A) The PEL cell line BCBL-1 was treated with LPV (40 μM) for 1, 3 and 6hr and cytoplasmic proteins were extracted and Western blot was performed. (B) The PEL cell line BCBL-1 was treated with LY294002 (50 μM) for 3hr and cytoplasmic proteins were extracted and Western blot was performed. (C) Viral gene expression after treatment with LPV (40 μM) for 1, 3 and 6hr was examined by RT-PCR.

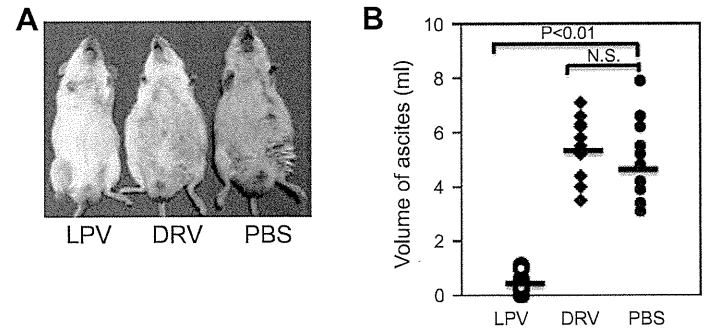
Our results showed that RTV and LPV but not DRV exhibited potent pro-apoptotic effects on PEL cells and provided evidence that such apoptosis occurs via then inhibition of NF-κB activity. These direct anti-tumor effects were shown in animal models free of viruses and immunocompetent cells such as T cells and NK cells, and in which direct anti-tumor effects of HIV-PIs that were independent of drug-mediated HIV suppression and immune reconstitution could be detected.

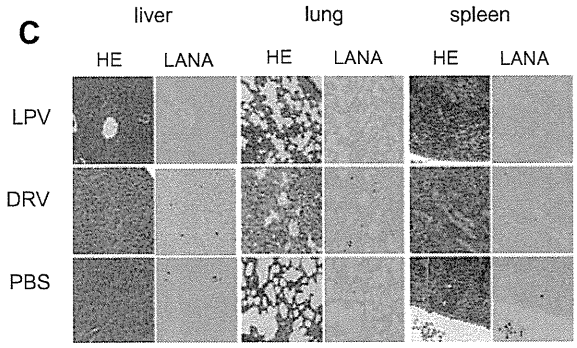
PEL is an incurable, aggressive B-cell malignancy and most patients that suffer from it respond poorly to traditional chemotherapy and develop chemoresistance. A novel strategy for treatment is needed for this aggressive lymphoma. Several agents have been tested in the search for a more effective treatment for PEL. It is now postulated that the mechanisms of lymphomagenesis involve the deregulation of several signaling pathways that may act either independently or crosstalk with each other. These include NF-κB, JAK/STAT and PI3 kinase pathways [4–6] in the case of PEL. PEL is associated with KSHV/HHV-8 infection and KSHV/HHV-8 contains a homologue of the cellular FLIP protein vFLIP, which has the ability to activate the NF-κB pathway through binding to the IKK complex to induce constitutive kinase activation. [7,36,38] Moreover, inhibition of NF-κB activity leads to the apoptosis of KSHV-infected PEL cells [5,11]. These results suggest that the NF-κB pathway is an effective target for the treatment of PEL. Activation of NF-κB is involved in various kinds of cancer development and progression [39–41] as well as in virus-associated lymphomas, indicating that NF-κB is a good molecular target for cancer treatment. HIV-PIs, such as RTV, LPV and DRV, have been successfully used in clinical treatments of HIV infection, with patients exhibiting a marked decrease in HIV viral load and a subsequent increase in CD4+ T-Cell counts [42–45]. Antitumor effects of HIV-PIs are expected, since patients treated with HAART but failing in CD4 T cell recovery still show a significantly lower risk of AIDS-related malignancies [46,47]. In fact, antitumor effects and the induction of apoptosis by HIV-PIs have been reported in the last 10 years [14,19,21]. In our study, we demonstrated that RTV and LPV are able to suppress the growth of PEL cells and induce apoptosis via the inhibition of NF-κB activity, especially by blocking the phosphorylation of I-κBα (Fig. 4A).

It was previously reported that HIV-PIs inhibit proteasome activity [21,48], and proteasome inhibition leads to the accumulation of I-κBα resulting in inhibition of NF-κB pathway. However, we could not detect any I-κBα protein accumulation in our study (Fig. 4A), indicating that LPV induced NF-κB inhibition is not due to the proteasome inhibition. In the present study, we showed that LPV inhibited IKKα and IKKβ phosphorylation (Fig. 4A). IKKα and IKKβ have been pursued by many groups as targets for the development of therapeutic agents to be used for the treatment of cancer, as well as inflammatory and metabolic diseases [49]. Akt and TAK1 have been identified as upstream kinase for IKKα and IKKβ and it was already reported that several HIV-PIs including LPV inhibit Akt pathway [37]. In present study, we also detected that LPV suppressed Akt pathway (Fig. 5A). However, specific inhibitor of Akt phosphorylation (LY294002) could not suppress IKKα and IKKβ phosphorylation in PEL line (Fig. 5B). It was also reported that TAK1 specific inhibitor cannot inhibit v-FLIP induced IKK kinase activation [50]. Taken together, Akt and TAK1 are not important molecule for v-FLIP mediated IKK phosphorylation in PEL cells. Actually, v-FLIP has the ability to activate NF-κB pathway thorough the direct binding to IKKγ [7,36,38]. If LPV induced HHV-8 lytic activation, v-FLIP expression is reduced and suppress NF-κB pathway. However, lytic activation of HHV-8 was not observed with the addition of LPV(Fig. 5C). v-FLIP is not a kinase that activates the IKK complex by inducing its phosphorylation. Instead, v-FLIP activates the complex by direct interaction via a mechanism believed to involve a conformational change of IKK complex [51]. Our findings emphasize the implication of v-FLIP for PEL survival and LPV might inhibit IKK phosphorylation by blocking of the v-FLIP and IKKγ binding at least independent of Akt.

Although designed to target only the HIV protease, HIV protease inhibitors are known to cause toxicity in patients, such as insulin resistance and lipodystrophy, suggesting that HIV protease inhibitors have other targets in mammalian cells. In fact, one of the most potent HIV-PIs, Nelfinavir, exerted pleiotropic biochemical and cellular effects on cancer cells that included the induction of endoplasmic reticulum (ER) stress, autophagy, and apoptosis *in vitro* and *in vivo* [52]. These preclinical studies suggested the idea that HIV-PIs can reduce the risk of cancer among HIV-1-infected persons. As HIV-1-infected patients have a higher risk of cancers than the uninfected population, and the complication of cancer is becoming one of the most important life-threatening events, if HIV-PIs can reduce the risk of cancer, this is a benefit for HIV-1-infected patients. Until now, only one cohort study has been performed and the risk of cancer was the same among those using HIV-PI or HIV-PI-sparing regimens [53]. Additional studies using large registries and post-marketing surveillance are needed to provide further data on the possible relationship between HIV-PIs and cancer inhibition among HIV-infected persons.

DRV was designed to form robust interactions with the protease enzyme from many strains of HIV-1, including strains from patients with multiple resistance mutations to HIV-PIs. DRV did not show these antitumor effects or inhibitory effects on the NF-κB





D

No.	drug	spleen	liver	lung
4096	LPV	-	-	+/-
4097	LPV	-	-	-
4098	DRV	++	++	++
4099	DRV	++	++	++
4100	PBS	+	+	+
4101	PBS	++	+	+

++: 1 area(x100)>10 points
+: 1 area (x100) 1-9 points
-: no points

Fig. 6. Treatment of NOD/Scid/Jak3-deficient mice with HIV protease inhibitor LPV suppresses the development and metastasis of KSHV-associated lymphoma *in vivo*. (A) A photograph of LPV, DRV-treated and non-treated ascites-bearing mice 4 weeks after being inoculated with BCBL-1 intraperitoneally. (B) Quantization of volume of ascites. (C) Hematoxylin-eosin staining and immunohistochemical staining using anti-LANA (PA1–73 N antibody) was performed to detect BCBL-1 in liver, lungs and spleen. (D) Quantification of BCBL-1 metastasis.

pathway in our study. DRV has very high activity against HIV-1 protease [23–25], but not against mammalian proteases, indicating the lack of anti-tumor effects as well as few adverse effects. In fact, clinical studies have demonstrated that DRV has more potent antiviral efficacy with more favorable lipid profiles that other HIV-PIs[54]. Thus, appropriate and creative use of HIV-PIs is required based on their actions and adverse effects.

In conclusion, we have shown the ability of HIV-PI, RTV and LPV to induce cell death through blocking the NF-κB pathway in PEL cells. Our study provides a rationale for a clinical trial of HIV-PIs in patients with PEL and other NF-κB-activated tumors. Further investigations aimed at determining the efficacy of HIV-PIs are warranted and may lead to the development of new effective therapies for this intractable lymphoma.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgements

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 The Journal of AIDS Research

活動報告

活用状況を考慮した「拠点病院診療案内」のあり方についての検討
——拠点病院診療案内の活用に関するアンケート調査結果より——

Investigation of Usefulness of AIDS Core Hospital List
——Findings of Questionnaire Survey about the Utilization of AIDS Core Hospital List——

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はじめに

厚生労働省エイズ対策研究事業「HIV感染症の医療体制の整備に関する研究」班では，冊子「拠点病院診療案内」（以下，「診療案内」）を全国のエイズ治療拠点病院（以下，拠点病院）の協力により，2001年度より作成し，関係機関に配布している。「診療案内」は全国拠点病院のHIV/エイズ診療に関する情報を中心に病院情報を掲載し，拠点病院や保健所等の施設間連携の促進を目的として，ブロック拠点病院情報担当職員を中心に毎年編纂を行い，拠点病院の最新情報を提供してきた。今回，今後の「診療案内」，ならびにそのWebサイト化に向けて情報内容を充実・向上させることを目的として「拠点病院診療案内の活用に関するアンケート」を行った。

対象および方法

2010年5月から8月に「診療案内2009-2010」を配布した1,127施設（全国拠点病院378施設，行政機関747施設，その他2施設）のHIV診療や関連業務に関わるスタッフを対象とした。「拠点病院診療案内の活用に関するアンケート」調査票を送付し，FAXで回答を得た。

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結　果

622施設から934件の回答を得た（回答率55%）。回答者は行政関係者59%，病院関係者41%と行政関係者の回答率が高かった。回答者職種比率は保健師が36%，医師22%，看護師11%で，薬剤師6%，技師6%，ソーシャルワーカー5%，事務4%，心理職3%であった。

「診療案内」の利用有無は，「ある」が全体で50%であり，機関別では行政関係が52%，病院関係が50%であった。

利用用途は，最も多かったのが行政関係では「患者・HIV検査受検者への情報提供」，病院関係では「患者転居にともなう拠点病院検索のため」であった（図1）。

利用目的は達せられたかの問いには，「十分」が77%，「必要な情報の掲載が不十分であったが目的は達した」が21%で，98%が目的は達したと回答した。「必要な情報の掲載が不十分で目的は果たせなかった」が2%あった。

掲載が不十分であったとされた必要な情報は，「新しい情報」，「担当課，担当医師（記載が無い）」，「具体的な受診手続き」，「実際の診療実績」，「出産可能かどうか」，「地域の情報」，「土曜日，夜間の受診について」，「通訳の有無」などであった。

「診療案内」に施設情報を掲載するにあたり，担当者の明確化など，院内で確認したことがあったかの問いでは，「全ての掲載事項は掲載依頼前に決まっていた」219件（72%），「一部の掲載事項は掲載依頼前に決まっていたが，