TABLE II. Seroprevalence to HHV-8 Among Amerindian Populations According to Age

All tribes	Tested	Positive	APP[a]
≤12	58	12	20.6 (12/58)
13–24	38	3	15.6 (15/96)
25–36	34	9	18.5 (24/130)
37–49	9	5	20.9 (29/139)
≥50	21	12	25.6 (41/160)
Total	160	41	

[a]Accumulated positive percentage.

Epidemiologic information was available for 419 HIV-1-infected subjects. A simple regression analysis (Table VI) showed that there was no significant association of seropositivity to HHV-8 with gender ($P = 0.1902$), age ($P = 0.2761$), or $CD4^+$ T lymphocyte count ($P = 0.7909$), but it was statistically associated to their sexual preference ($P = 0.0032$).

Molecular Characterization

Seven samples (out of 111) had the ORF26 region amplified using a nested PCR producing a 233 bp segment. Three samples were from HIV-1-infected males (Bel72, age 40, bisexual; Bel11312, age 48, bisexual; Bel11050, age 52, homosexual). Amerindian samples were originated from three females, one from the Tiriyó (Tir8715, age unknown), one from the Arara Laranjal (Ara8832, 35 years old), and one from the Zo'e (Zoe9317, age unknown) and one male from the Zo'e (Zoe9326, age unknown).

The seven amplified samples were submitted to a nested PCR in order to amplify the VR1 region of the *K1* gene. Four (two from HIV-1-infected patients, one from the Tiriyo, and one from the Zo'e) were successfully amplified producing a 435 bp segment.

Phylogenetic Tree

The phylogenetic tree compared the nucleotide sequences of the four samples isolated in the present study with 22 other samples available in the GenBank (Fig. 2). Two samples (HIV-1-infected patients) grouped within subtype B (bootstrap of 100%), one (Zo'e) with suptype C (bootstrap of 97%) and one (Tiriyo) with subtype E (bootstrap 96%).

DISCUSSION

In the present study, four native Indian communities of three different linguistic groups were tested for the presence of antibodies to HHV-8 using an EIA, yielding a total prevalence of 29.9%. The Tiriyo showed the highest seroprevalence (42.8%). There was a marked difference of the seroprevalence between females (37.6%) and males (16.4%) which was statistically significant ($P = 0.0261$). In the urban setting, the seroprevalence among HIV-1 positive subjects was lower (15.5%) and the gender prevalence distribution was higher among males (17.6%) than females (12.7%) but it was not statistically significant. The prevalence of HHV-8 among the Amerindians was significantly higher ($P < 0.0001$) than in the urban setting.

A previous study using long-term stored serum samples of Brazilian Amerindians and an immunofluorescence assay for the detection of latent antibodies (LANA) detected a total seroprevalence of 53% [Biggar et al., 2000], but the prevalence rates were calculated using very low numbers in each group examined; similar prevalence rates were found for the Tiriyo (57.4%) and the Wayampi (55.7%) [Cunha et al., 2005]. The data are not in agreement with the present information, in which several levels of prevalence rates were described in agreement with the data found among Amerindians in a neighboring geographic area [Kazanji et al., 2005]. High prevalence rates were found in Uganda, Nigeria [Lennette et al., 1996], and Cameroon [Gessain et al., 1999] where HHV-8 infection is endemic and the presence of KS is high. There was no clinical evidence of KS among the groups of the present study.

The seroprevalence among HIV-1-infected subjects was the first information describing a co-infection of HHV-8 in the North region of the country and was similar to the prevalence rates described among the general population of Belem (16.3%; Freitas et al., 2002, using an EIA), the origin of most of the subjects examined; it was not different from other prevalence studies from the French Guiana (13.2%) and Italy (11.5%), both of which used an immunofluorescence assay [Perna et al., 2000; Plancoulaine et al., 2000]. Other studies among blood donors and the general population in Brazil ranged from 1 to 18.8% [Caterino-de-Araujo et al., 1999; Pierrotti et al., 2000; Zago et al., 2000; Souza et al., 2004] as well as in patients without KS in Brazil [18.5%; Keller et al., 2001], but quite different from the prevalence described among men who have sex with men (32.6%) and in AIDS patients (39.2% for non-KS patients and 98.7% for AIDS patients with a diagnosis of KS; Souza et al., 2004).

The finding of antibodies in 20.7% of children under 12 years old and in three HIV-1-infected children is consistent with the results obtained with children from the general population which ranges from 3.7 to 15%

TABLE III. Prevalence of Antibodies (ELISA) to HHV-8, According to Gender, Among HIV-1 Infected and/or With AIDS, in Belém, Brazil

	Total		Male		Female	
Urban population	Tested	Positive (%)	Tested	Positive (%)	Tested	Positive (%)
HIV-1 infected and/or with AIDS	477	74 (15.5)	273	48 (17.6)	204	26 (12.7)

TABLE IV. Prevalence of Antibodies (ELISA) to HHV-8, According to Age, Among HIV-1 Infected and/or With AIDS

Age (years)	Tested	Positive	APP[a]
≤12	21	3	14.3 (3/21)
13–24	47	9	17.6 (12/68)
25–36	209	30	15.2 (42/277)
37–49	142	17	14.1 (59/419)
≥50	45	13	15.5 (72/464)
Total	464	72	

[a]Accumulated positive percentage.

[Lennette et al., 1996; Mayama et al., 1998; Gessain et al., 1999; Plancoulaine et al., 2000; Cattani et al., 2003]. Although HHV-8 is also transmitted through sexual intercourse [Simpson et al., 1996], the high prevalence of antibodies among children reinforces the possibility of other routes of dissemination [Lennette et al., 1996; Biggar et al., 2000; Plancoulaine et al., 2000; Machado et al., 2005].

The high level of antibodies in Amerindians could be due possibly to vertical transmission of a virus which usually persists within the human host. There was a steady but constant rise in the prevalence of antibodies from the second age group considered (13–24 years of age) to persons aged 50 years or older, showing that virus transmission from this age range onward occurs via sexual contact and is maintained through the natural persistence and its vertical transmission from the female host. This was not the case for the HIV-1-infected group, which showed an apparently different pattern of infection distribution. Seroprevalence was approximately the same for all ages indicating that the virus is acquired equally throughout the host life as a possible consequence of sexual transmission alone.

The presence of antibodies to HHV-8 was detected in 25.8% (25/97) of HIV-1-infected male homosexuals. This is in agreement with results found elsewhere (12–90%), suggesting the sexual transmission as an important route for the transmission of the virus [Lennette et al., 1996; Simpson et al., 1996; Regamey et al., 1998; Caterino-de-Araujo et al., 1999, 2003; Nascimento et al., 2005].

The only positive association found in the present study among HIV-1-infected subjects involved the presence of antibodies to HHV-8 and male sexual behavior. There was no statistical association of the prevalence of antibodies related to gender, age, or $CD4^+$ T lymphocyte count. The association of homosexuality and $CD4^+$ T lymphocyte count was described among males, HIV-1 infected or not, residing in San Francisco, USA [Martin et al., 1998], but no association was described according to age, race, or education. Likewise, the prevalence of antibodies among black South African cancer patients was not associated with gender or place of birth, but it declined with the rise of the number of years of education and rose with the number of sexual partners [Sitas et al., 1999].

The ORF26 region was amplified in 8% (4/50) of the seroreactive Amerindians and in 4.9% (3/61) of the HIV-1-infected subjects (one homosexual and two bisexual males). The small number of amplified products is a common event when compared to serologic prevalence rates both among native Indians (3/19 described by Biggar et al. [2000] and 3/95 described by Cunha et al. [2003]), and among HIV-1-infected subjects in Brazil [Keller et al., 2001], Italy [Bigoni et al., 1996], and France [Dupon et al., 1997]. Virus nucleic acid is present in low levels in peripheral blood and amplification is not a sensitive method, in comparison to the amplification of HHV-8 genome in cases of tumor lesions where it reaches positivity in 100% [Sitas et al., 1999; Spira et al., 2000].

The sequencing of the VR1 region (gene *K1*) yielded a multiplicity of molecular subtypes of HHV-8. Subtype B was described among the HIV-1-infected subjects, subtype C among the Zo'e, and subtype E among the Tiriyo. Zong et al. [1999] found subtype B almost exclusively among African patients with KS, similar to the results obtained by Cook et al. [1999] in samples from biopsies, peripheral blood, semen, and HHV-8 cell lines originated from African countries. Its presence among HIV-1-infected subjects from the urban area of Belem is not surprising, when the composition of the genetic background of the Brazilian population in the North region of the country is taken into account [Santos and Guerreiro, 1995; Rodrigues, 1999]. Considering that the subjects have never left the country nor had any relation whatsoever with subjects of African origin, it is reasonable to believe that the virus was brought in with the African slaves during the colonization period of the country.

The presence of subtype E was described previously in two Brazilian Indians, one Arawete and one Assurini [Biggar et al., 2000], in a Wayampi from the French

TABLE V. Simple Logistic Regression Analysis Correlating Serorreactivity to HHV-8, Gender, and Age Among Amerindian Populations

All tribes[a]	Tested	Positive (%)	*P* Value	Odds ratio	IC 95%
Gender					
Male	72	11 (6.9)	0.0261	0.4208	0.196–0.902
Female	88	30 (18.7)			
Age					
Children (≤12 years)	58	12 (7.5)	0.4273	1.3572	0.638–2.885
Adults	102	29 (18.1)			

[a]Karararo, Arara Laranjal, Tiriyo.

TABLE VI. Simple Logistic Regression Analysis Correlating Seroprevalence to HHV-8, Gender, Age, $CD4^+$ T Lymphocyte Counts, and Sexual Preference Among HIV-1 Infected and/or With AIDS

HIV-1 infected	Tested	Positive (%)	*P* Value	Odds ratio	IC 95%
Gender					
Male	232	42 (10.3)	0.1902	1.4324	0.837–2452
Female	187	25 (5.7)			
Age (years)					
≤24	41	09 (2.15)	0.2761	0.6444	0.292–1.421
≥24	378	58 (13.8)			
$CD4^+$ T Lymphocytes					
≤350 cells/mm^3	250	39 (9.3)	0.7909	0.9308	0.548–1.582
≥350 cells/mm^3	169	28 (6.7)			
Sexual preference					
Heterosexual	322	42 (10.0)	0.0032	0.4320	0.247–0.755
Homo/bisexual	97	25 (6.0)			

Guiana [Kazanji et al., 2005], and in Equador [Whitby et al., 2004]; together with the present information of subtype E found within the Tiriyo, confirms not only its presence but also its distribution as a unique molecular subtype among Amerindians from different geographic locations and different linguistic groups. Furthermore, subtype C, which is commonly associated to patients with classical KS, iatrogenic, and KS associated with AIDS in Middle East and Asia [Zong et al., 1999] was described for the first time among Amerindians, the Zo'e, an epidemiologically closed community of the Tupi linguistic group.

The seroprevalence of HHV-8 shows an endemic infection among Amerindians which was significantly higher than in the urban setting, both in the general population and among HIV-1 carriers. It is plausible to suggest that HHV-8 is an ancient virus of those native Indian populations as they are epidemiologically closed groups with a very recent history of limited contact with urban communities. The suggestion is strengthened by the description of a high diversity of molecular subtypes (three out of five), as well as a unique one, found, so far, only within the boundaries of the Amazon region of Brazil and Equador. The three reasons above described

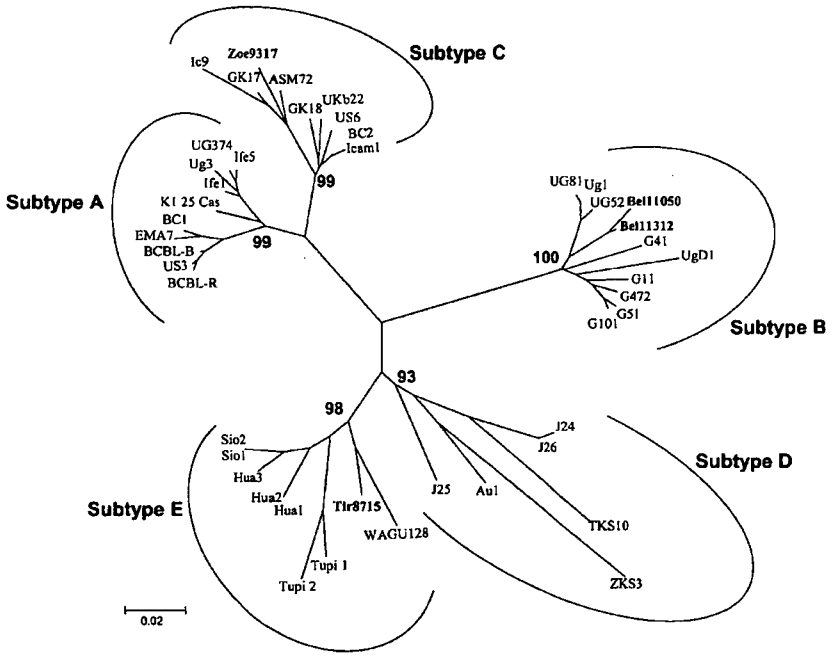

Fig. 2. Unrooted phylogenetic tree showing the relationship among HHV-8 samples available in the Genebank and samples of the present study: Zo'e 9317, HIV-1-infected patients (Bel11050 and Bel11312), Tiriyo (Tir8715). The tree was constructed using the Neighbor-Joining (NJ) method using segments of 400 nucleotides of the VR1 region of the *K1* gene. The statistical support was applied using 1,000 bootstrap replicate.

form a body of a preliminary strong evidence which will be further pursued.

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Original article

Integration of HIV-1 caused STAT3-associated B cell lymphoma in an AIDS patient

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Abstract

Signal transducer and activator of transcription 3 (STAT3) is a DNA-binding transcription factor activated by multiple cytokines and interferons. High expression of STAT3 has also been implicated in cancer and lymphoma. Here, we show a case of B cell lymphoma in which a defective human immunodeficiency virus 1 (HIV-1) integrated upstream of the first STAT3 coding exon. The lymphoma cells with anaplastic large cell morphology formed multiple nodular lesions in the lung of an acquired immunodeficiency syndrome (AIDS) patient with Kaposi's sarcoma. The provirus had a 5′ long terminal repeat (LTR) deletion, but the 3′ LTR had stronger promoter activity than the STAT3 promoter in reporter assays. Immunohistochemistry showed increased expression of STAT3 in the nuclei of lymphoma cells. Transfection of STAT3 resulted in transient cell proliferation in primary B cells in vitro. Although this is a very rare case of HIV-1-integrated lymphoma, these data suggest that up-regulation of STAT3 caused by HIV-1 integration resulted in the development of B cell lymphoma in this special case.

Keywords: HIV-1; Integration; AIDS-related lymphoma; STAT3

1. Introduction

Malignant lymphoma is an important complication of patients with acquired immunodeficiency syndrome (AIDS). A large part of AIDS-related lymphomas are of B cell linage, and positive for Epstein–Barr virus (EBV) or Kaposi's sarcoma-associated herpesvirus (KSHV) [1–4]. Since human immunodeficiency virus 1 (HIV-1) is not usually detected in AIDS-related lymphoma cells, HIV-1 infection plays an indirect role in lymphomagenesis by impairing host immune surveillance. However, proviral DNA can either disrupt expression of tumor suppressor genes or enhance expression of cellular oncogenes. Alternatively, retroviral promoters can integrate into the host genome in such a manner that expression of a nearby oncogene is enhanced by a strong promoter within the proviral 3′-long terminal repeat (3′LTR). In humans, abnormal T cell proliferation following gene therapy for severe combined immunodeficiency resulted from retroviral integration into the intron of the *LMO2* proto-oncogene [5].

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In AIDS patients, some cases of lymphomas had HIV-1 integration within the *fur* gene, just upstream from the *c-fes/fps* proto-oncogene [6]. That report, however, did not investigate the functional effect of this integration event. These observations suggest that HIV-1 may contribute directly to lymphomagenesis by inserting an active promoter into a cellular oncogene [6]. In the present study, we report a case of AIDS-related lymphoma in which HIV-1 integrated upstream of the STAT3 gene. The association of HIV-integration and lymphomagenesis was investigated.

2. Materials and methods

2.1. Samples

Lymphoma tissues in the lung of a patient with HIV-1 infection were obtained at autopsy. Formalin-fixed pathological samples of lymphoma, including nine unrelated cases of AIDS-related lymphoma and 15 cases of non-Hodgkin lymphoma in HIV-1-uninfected individuals, were studied. All samples were obtained with informed consent according to the Declaration of Helsinki. The study protocol was approved by the institutional review board of National Institute of Infectious Diseases (Approval No. 93).

2.2. Immunohistochemistry and in situ hybridization

Immunohistochemistry was performed as described before [7,8]. Primary antibodies were: anti-CD3 (Dako, Copenhagen, Denmark), CD20 (Dako), CD30 (Dako), CD45 (Dako), CD45RO (Dako), CD79a (Dako), CD138 (Serotec, Oxford, UK), and $p80^{NPM/ALK}$ (Nichirei, Tokyo, Japan), STAT3 (sc8019, Santa Cruz Biotechnology, Santa Cruz, CA), pSTAT3 (sc8059, Santa Cruz), KSHV-encoded LANA [8], and vIL-6 [7] antibodies. In situ hybridization for EBERs was performed as described before [9].

2.3. PCR and DNA sequences

PCR detection for KSHV-encoded open reading frame (ORF) 26, EBV W region, HIV-1 V3, and β-globin gene was performed as described previously [9,10]. For PCR amplification of HIV-1 3′LTR and STAT3 junction, HIV3LTR-F (5′-TCTGAGCCTGGGAGCTCTCT-3′, 9561–9580 in GenBank K03455) and Stat3intron-R (5′-AGTGCATGGCACATAACAGA-3′, 41131–41150 in GenBank AY572796) were used. For amplification of HIV-1 5′LTR and STAT3 junction, 6 reverse primers of 5′LTR (55R 5′-TCAGGGAAGTAGCCTTGTGTGTGGT-3′, 78R 5′-GCCCTGGTGTGTAGTTCTGTCAATC-3′, 348R 5′-GAAAGTCCCCAGTGGAAAGTCCCTT-3′, 495R 5′-GCAGTGGGTTCCCTAGTTAGCC-3′, 563R 5′-TTACCAGAGTCACACAACAGACGGG-3′, and 612R 5′-CACTGCTAGAGATTTTCCACACTGAC-3′), and a reverse primer positioning between 5′LTR and gag (676R 5′-CGAGTCCTGCGTCGAGAGATCTCCT-3′) were used with a forward primer of Stat3-intronF2 (5′-CATTTTTCTTTCCTTCTCTGTTGTC-3′, 40881–40905 in GenBank AY572796). These primers for HIV-1 were designed based on the sequence of HIV-1 IIIB (GenBank K03455).

2.4. Cloning of HIV-1 integration sites

The methods used were essentially as described for the Gene Walker Kit (BD Clontech, Palo Alto, CA). Lung tumor DNA was cleaved with four different blunt cutting enzymes (*Dra*I, *Eco*RV, *Pvu*II and *Ssp*I). Gene specific primers for HIV-1 LTR were 5′-ACCACACACAAGGCTACTTCCCTGA-3′ (GSP-1) and 5′-AAGGGACTTTCCACTGGGGACTTTC-3′ (GSP-2).

2.5. Real-time PCR

Copy numbers of HIV-1 integration site and STAT3 gene were measured with real time PCR as described previously [11]. Two probe and primer sets were used (Set 1: forward primer: 5′-CTAGAGATCCCTCAGACCATTTTAGTC-3′, reverse: 5′-AAAAGTATAAATGAGGATCCAGGAAGAT-3′, probe: 5′-6FAM-TGTGGAAAATCTCTAGCAGAATCTCAGG-TAMRA-3′; Set 2: forward primer: 5′-GCAGCTTGACACACGGTACCT-3′, reverse: 5′-AAACTGCCGCAGCTCCATT-3′, probe: 5′-6FAM-AGCAGCTCCATCAGCTCTACAGTGACAGC-TAMRA-3′).

2.6. Plasmids

For the promoter assay, genes of the HIV-1 3′LTR, STAT3-intron (40951–41959 of GenBank AY572796), and STAT3-promoter (1–1998 of GenBank AY572796) were amplified from DNA of the HIV-1-integrated lymphoma using the LTR-*Mlu*I-F, 5′-GAG*ACGCGT*TGGAAGGGCTAATTCACTCCC-3′ and LTR-*Xho*I-R, 5′-GTG*CTCGAG*TGCTAGAGATTTTCCACACT-3′, the Intron-*Mlu*I-F, 5′-GAG*ACGCGT*GAATCTCAGGCAGATCTTCC-3′ and Intron-*Xho*I-R, 5′-CAC*CTCGAG*CCTGCTAAAATCAGGGGTCCC-3′, and, the Stat3prom-*Mlu*F1, 5′-GAG*ACGCGT*ACCCATAGTCGCAGAGGTAGA-3′ and Stat3prom-*Xho*R1, 5′-GAG*CTCGAG*CGCTGAATTACAGCCCCTTCA-3′, respectively. Enzyme sites are indicated in italics. A fragment of the HIV-1 3′LTR was amplified also from HIV-1 pNL4-3 (GenBank AF324493). The PCR product was subcloned into *Mlu*I-*Xho*I site of pGL3-basic vector (Promega, Madison, WI). For the STAT3-expression plasmid, STAT3 cDNA was amplified from the mammalian gene collection-human (MGC-1607, American type culture collection, Manassas, VA) using forward primer (STAT3-HpaI-F10 5′-CACCGTTAACGGATCCTGGACAGGCACCC-3′) and reverse primer (STAT3-R24 5′-CATGTCAAAGGTGAGGGACTCAAA-3′). The PCR product was TA cloned using pcDNA 3.1 Directional TOPO Expression kit (Invitrogen, Carlsbad, CA). For cell proliferation experiment, the STAT3 expression vector was digested with *Hin*dIII and *Eco*RV and ligated into *Bsm*BI and *Eco*RV sites of pMACS 4-IRES.II vector, which is a bicistronic expression vector containing multiple cloning site followed by an internal ribosome entry site (IRES) element

from encephalomyocarditis virus and the truncated (non-functional) CD4 cDNA (Miltenyl Biotec, Auburn CA).

2.7. Promoter assay

Plasmids were transiently transfected into HeLa cells with a renilla reporter gene construct using Lipofectamine Plus (Invitrogen). Luciferase activity was measured with a dual luciferase assay system (Promega). In the HIV-1-Tat (+) group, an HIV-1-Tat expression vector, kindly provided by Dr. Kenzo Tokunaga, National Institute of Infectious Diseases, Tokyo, Japan, was cotransfected.

2.8. DNA methylation analysis

Methylation of the cytosine residue of the CpG site was analyzed by the bisulfite genomic sequencing method, as described previously [12]. The primer pair for selective analysis was as follows: sense primer, 5′-TATAAACCAGCATGG GATGGATGA-3′; antisense primer, 5′-CCCAGGCTCGGA TCTGGTCTAACC-3′.

2.9. Cell proliferation assay for primary lymphocytes

Primary B cells were negatively selected from whole blood of healthy volunteers using RosetteSep B cell enrichment (StemCell Technology, Vancouver, BC, Canada) [13]. Cell proliferation assay was performed using BrdU Cell proliferation ELISA kit (Roche Molecular Biochemicals, Indianapolis, IN).

3. Results

3.1. HIV-1 was concentrated in lymphoma cells in a case of AIDS-related lymphoma

A 59-year-old, homosexual, HIV-1-positive male with a CD4 cell count of 6/mm^3 showed high fever and multiple KS skin lesions. Computed tomography scanning revealed multiple nodules in the lung (Fig. 1A). Despite treatments with antibiotics and combined chemotherapy, with intensive care, he died 30 days after admission. The clinical course of the patient was also reported previously [14]. At autopsy, multiple nodules were present in the lung (Fig. 1B). Histologically, these nodules were composed of large atypical cells with anaplastic large cell morphology infiltrating into interstitial and alveolar areas in the lung tissue (Fig. 1C). Immunohistochemistry demonstrated that the tumor cells were CD3$^-$, CD20$^-$, CD30$^+$, CD45$^+$, CD45RO$^+$, CD79a$^-$, CD138$^-$, and p80$^{NPM/ALK-}$, suggesting that the lung tumor was composed of lymphoma cells (Fig. 1D and data not shown) [14]. Southern blot hybridization of DNA extracted from the lung tumor with an immunoglobulin junction hinge (JH) probe demonstrated immunoglobulin gene rearrangement, confirming a B

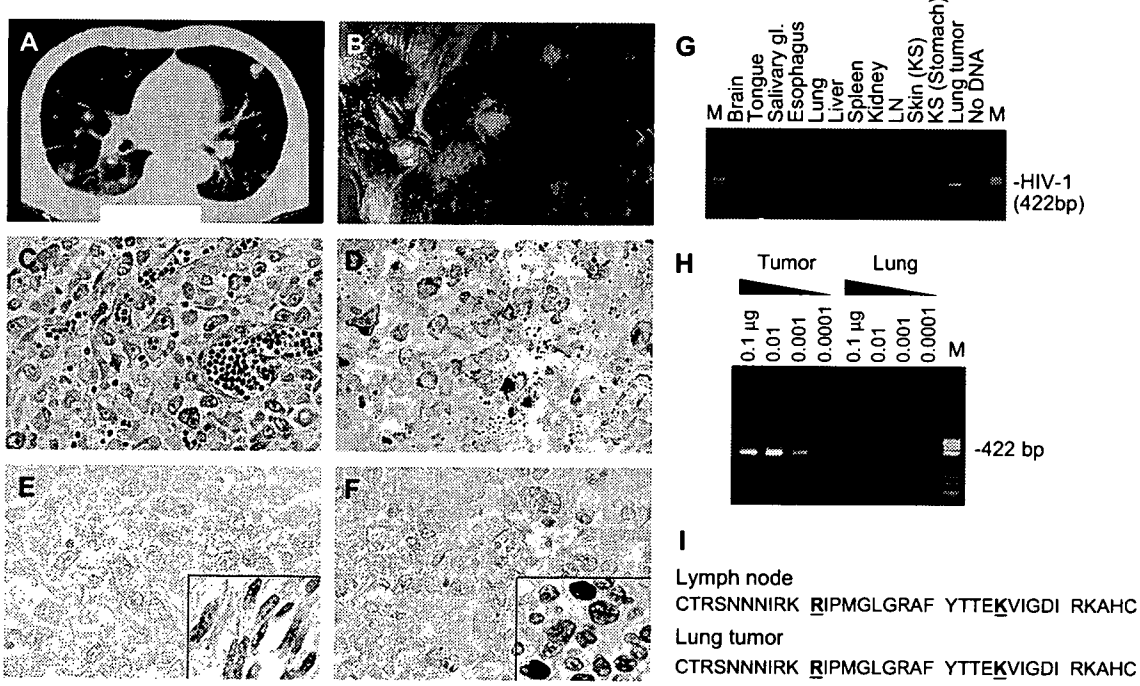

Fig. 1. Pathological findings of tumors in the lung of a patient with AIDS. CT scan (A), macroscopic view (B) and Hematoxylin and eosin staining (C) of the lung tumor. (D) Immunohistochemistry of CD45RO. (E) Immunohistochemistry for KSHV-LANA in the lung tumor cells. Inset shows gastric KS cells from the patient. (F) In situ hybridization for EBV-EBER in the lung tumor cells. Inset shows a positive control of EBV-positive lymphoma from an unrelated patient. (G) PCR detection for HIV-1 V3 region in various organs of the patient. LN, lymph node; M, DNA molecular weight marker (pBR322/*Hae*III). (H) Semi-quantitative PCR for HIV-1. DNA quantities are indicated at the top of the panel. DNA extracted from the lung tumor and surrounding lung tissues was tested. (I) Predicted amino acid sequence of HIV-1 gp120 V3 loop of HIV-1 amplified from the lymph node and lung tumor by PCR. Positions 11 and 25 are indicated by bold letters with underlines. DNA sequences are deposited in GenBank under accession numbers DQ116951 to DQ116954 (HIV-1 envelope from LN and lung tumor).

cell linage (data not shown). Since KS lesions were found in the oral cavity, stomach, sole and some lymph nodes at autopsy, we examined KSHV positivity in the lymphoma (lung tumor). KSHV-encoded ORF26 was amplified in both gastric KS lesions and lung tumor by PCR (data not shown). However, immunohistochemistry demonstrated that expression of KSHV LANA was very weak or absent in the lymphoma cells, whereas KS cells in the stomach strongly expressed LANA (Fig. 1E). Immunohistochemistry also demonstrated that the lung tumor cells were negative for KSHV-encoded vIL-6 (data not shown). The lymphoma cells were positive for EBV by PCR (data not shown), but in situ hybridization failed to detect EBERs (Fig. 1F). Thus, these data suggest that KSHV and EBV were present in the lymphoma at low copy numbers. Surprisingly, HIV-1 DNA was detected in the lymphoma cells by PCR, but not in other organs besides the lymph nodes (Fig. 1G). Semi-quantitative PCR revealed that there was a 100-fold higher copy number of HIV-1 DNA from the lymphoma than from surrounding lung tissue (Fig. 1H). PCR products of HIV-1 V3 region were TA-cloned and each 10 clones were sequenced. Although two (clones L2 and T3) and three (clones T1, T3, and T6) kinds of sequences were obtained from the lymph nodes and lymphoma, respectively, all sequences coded the same amino acid sequence in the V3 loop (net charge = + 7). Basic amino acids at positions 11 and 25 of the gp120 V3 loop and a high positive net charge strongly suggest that fusogenic X4 viruses were detected in the lymphoma cells and lymph nodes (Fig. 1I) [15].

3.2. HIV-1 integration in the STAT3 gene

A high copy number of HIV-1 in the lymphoma suggested integration of HIV-1 into the genome of lymphoma cells. Genome walking PCR produced a 400 bp fragment which contained a 300 bp fragment with >99% identity with the HIV-1 IIIB 3′LTR sequence (GenBank K03455) and A 40 bp genomic segment just before the first coding exon of STAT3 (Fig. 2A). PCR using primers in HIV-1 3′LTR and STAT3-intron yielded an independent amplicon with HIV-1 3′LTR and the predicted STAT3 genomic sequences from DNA of the lymphoma cells (Fig. 2B). These data confirmed that HIV-1 had integrated into the intervening sequence just before the first coding exon of STAT3. PCR using a primer pair binding to the STAT3 intron and upstream of HIV-1 gag demonstrates that the 5′LTR of the integrated HIV-1 was truncated (Fig. 2C). The sequence analysis revealed that the integrated HIV-1 lacked a fragment at the position of 1–587 in the 5′LTR (Fig. 2A,D, GenBank AF538307). Compared with the sequence of the 3′ integration site, HIV-1 integration resulted in duplication of the cellular 5 bp (GAATC) and addition of a dinucleotide at the integration site by HIV-1 integrase, which is commonly seen among retrovirus integrases [16,17]. Consequently, the integration event was produced by a defective virus (Fig. 2A,D). The absence of p24-staining of the tumor is consistent with this conclusion (data not shown).

3.3. Copy number of the integrated HIV-1 in the lymphoma tissue

Generally, pathological tissues obtained from lymphoma lesions contain not only lymphoma cells, but also surrounding CD4-positive T cells or alveolar macrophages. Although immunohistochemistry demonstrated no or rare CD4-positive cells in the lymphoma tissue, we tried to determine a copy number of the integrated HIV-1 in the lymphoma tissue by a real time PCR targeting genes near the integration site to deny the possibility that HIV-1 integration was originated in the contaminated CD4-positive cells (Fig. 3). A fragment of HIV-1-integration site was amplified at 12,570 copies/100 ng of DNA by the real time PCR, whereas exon 1 of STAT3 gene was amplified at 121,597 copies/100 ng. Since each cell has two copies of STAT3 gene on two alleles, these data suggest that HIV-1 integration occurred about 20% of the population that the DNA was extracted from. As shown in Fig. 1C, the lymphoma tissue contained many cells other than lymphoma cells, such as alveolar epithelial cells, macrophages, and endothelial cells. However, CD4-positive T cells were rare in the tissue, and the HIV-1 was X4 virus. Therefore, these data suggest that the HIV-1 might be detected from lymphoma cells, not from contaminated T cells or macrophages, and integrate into more than 20% of the lymphoma cells.

3.4. Promoter activity and methylation of HIV-1 3′LTR

LTRs of HIV-1 usually have a promoter activity in HIV-1-infected T cells and macrophages [18]. To investigate if the HIV-1 3′LTR contained a functional promoter, we constructed a plasmid containing the patient's HIV-1 3′LTR or upstream intron sequence of STAT3 before a luciferase reporter gene. Transfection of the plasmid to HeLa cells revealed that the sequence of 3′LTR derived from the patient had significant promoter activity at a similar level to that of 3′LTR in HIV-1 NL4-3, but the upstream intron sequence of STAT3 did not (Fig. 4A). 3′LTR was a stronger promoter than the STAT3 promoter derived from the patient. Cotransfection with a plasmid expressing HIV-1-Tat enhanced the activity of the patient's 3′LTR 31-fold, whereas the activity of the STAT3 promoter was not enhanced. These data suggest that the HIV-1 3′LTR contains promoter activity. It is known that DNA CpG methylation inactivates retroviral promoter including HIV-1 LTR [12,19]. However, a bisulfite genomic sequence revealed that the fragment of HIV-1 3′LTR did not have any CpG or non-CpG methylation in the DNA extracted from the lymphoma (Fig. 4B,C). These data suggest that methylation might not reduce or inhibit the transcriptional activity of HIV-1 3′LTR in the HIV-1-integrated lymphoma cells.

3.5. Expression of STAT3 in the HIV-1-integrated lymphoma

We investigated expression of STAT3 in the case of HIV-1-integrated lymphoma. Immunohistochemistry demonstrated a high level of STAT3 expression predominantly in the nuclei

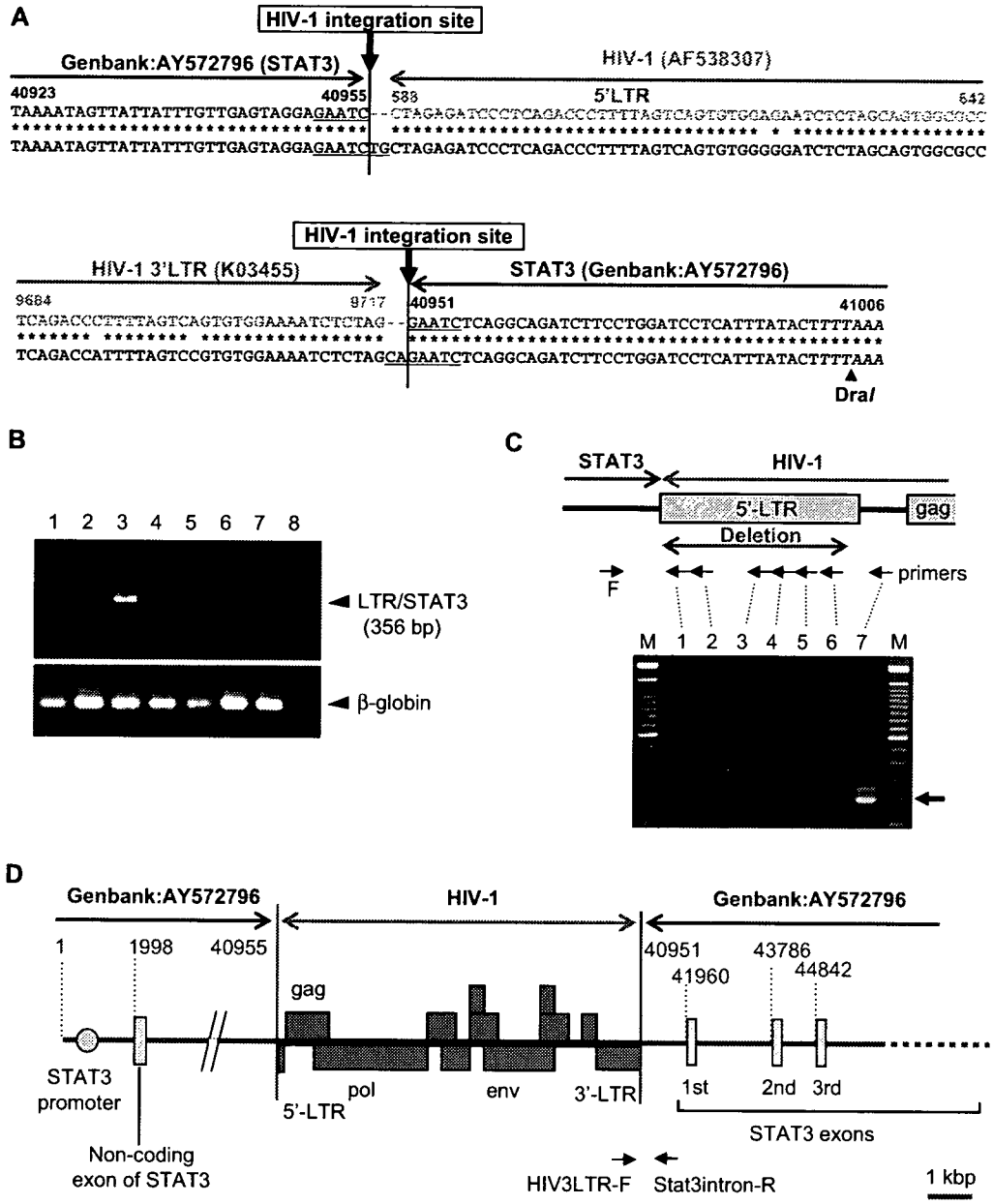

Fig. 2. Identification of HIV-1 integration site in the lymphoma cells with genome walking. (A) Sequence of the HIV-1 5′-LTR (upper panel) and 3′-LTR (lower panel) insertion site in the lymphoma genome. Whole sequences of PCR products are registered as GenBank DQ355432 (5′-LTR, 190 bp) and DQ117603 (3′-LTR, 1.5 kbp), respectively. The sequence of the lymphoma genome is shown in the lower line in black letters. The upper colored line indicates the HIV-1 LTR sequence (blue, GenBank K03455 or AF538307) and STAT3 genomic sequence (violet, GenBank AY572796). HIV-1 intervening sequence between 5′LTR and gag is indicated by green. Duplication of the cellular 5 bp (GAATC) and additional dinucleotides (TG in 5′-LTR and CA in 3′-LTR) by HIV-1 integrase are underlined. *DraI* site is indicated by italics. (B) PCR for the junction region of 3′LTR and STAT3 gene using HIV3LTR-F and Stat3intron-R primers (see Fig. 3D). 1, PBMCs from a healthy donor; 2, HIV-1-positive Molt4 cell line; 3, lymphoma cells with HIV-1integration; 4, KS lesion from the patient; 5, AIDS-related lymphoma from an unrelated patient; 6, lymphoma from a non-HIV-1infected patient; 7, BCBL-1 (KSHV-positive B cell line); 8, No DNA. The lower panel shows the results of an internal control (β-globin gene). (C) PCR of genomic DNA with a STAT3-intron forward primer (F in this figure, Stat3-intronF2) in combination with 5′ LTR reverse primers (lanes 1–6, 55R, 78R, 348R, 495R, 563R and 612R), and a reverse primer positioning between 5′LTR and gag (lane 7, 676R). The upper panel shows the positions of these primers. A 188 bp product was identified when the 676R primer was used with the STAT3 intron primer (lane 7). If the 5′LTR was intact, the predicted size of this amplicon would have been 777 bp. (D) Map of the defective HIV-1 insertion site in the STAT3 gene. Violet numbers indicate the number in GenBank AY572796 (STAT3). Blue boxes are HIV-1 genomes.

of the HIV-1-integrated lymphoma cells (Fig. 4D). To know the phosphorylation status of STAT3, we immunostained the slide using an anti-pSTAT3 (Tyr-705) antibody as a primary antibody. However, any signal was not found in the lymphoma cells (data not shown). We also examined STAT3 expression in 24 cases of lymphoma, including nine cases of AIDS-related lymphoma and 15 of non-AIDS-related lymphoma, normal tonsilar tissues and lymph nodes derived from unrelated patients. The nine cases of AIDS-related lymphoma contained seven of EBV-positive diffuse large B cell lymphoma

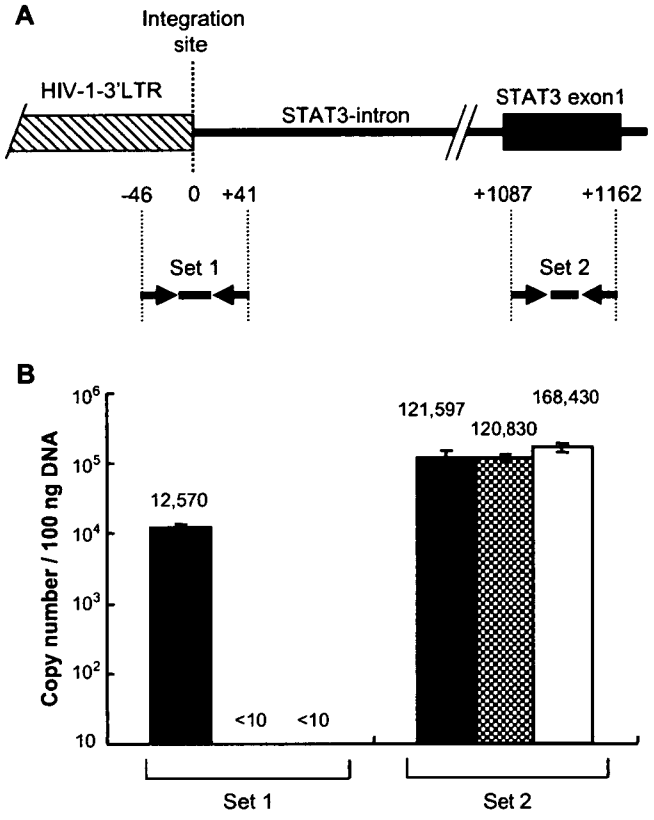

Fig. 3. Quantitative analysis of genes for HIV-1 integration site. (A) Probe-primer sets for real time PCR. The top line with boxes is a genome map around HIV-1 integration site of HIV-1 3'LTR. Numbers with plus and minus under the genome map indicate distances (bp) from the integration site. Arrows and heavy lines are probe-primer sets of real time PCR. (B) Copy numbers of HIV-1 integration site and STAT3 gene. Black, gray and white bars indicate mean copy number per 100 ng DNA of this case, HIV-1-positive Molt4 cell line, and TY-1 (HIV-1-negative, KSHV-positive B cell line), respectively. Copy numbers per 100 ng DNA are indicated on the top of each bar. Error bars indicate standard errors of triplicate samples.

(DLBCL), and two cases of Hodgkin's disease. The 15 cases of non-AIDS-related lymphoma contained 12 EBV-positive or EBV-negative DLBCL and three cases of Hodgkin's disease. Immunohistochemistry revealed that several cases of AIDS-related lymphoma and one of HIV-unrelated lymphoma expressed STAT3 predominantly in the cytoplasm (Fig. 4E); however, no case expressed STAT3 predominantly in the nucleus (Table 1). STAT3 expression was not found, or was weak, in other cases examined (Fig. 4F). These data suggest that the integration of HIV-1 induced high expression of STAT3 in the lymphoma cells of the patient.

3.6. Transfection of STAT3 expression plasmid to primary B cells in vitro

To investigate if expression of STAT3 induces cell growth, we constructed an expression plasmid for STAT3 and transfected the plasmid to B cells. At first, to confirm expression of STAT3 by Nucleofector transfection, His-tagged STAT3 was expressed in TY-1, a KSHV-positive B cell line. Immunofluorescence assay using anti-STAT3 and anti-6x His antibodies revealed that transfection efficiency to lymphocytes was 30–40% in this experiment (Fig. 5A). Addition of IL-6 to culture medium of transfected TY-1 altered the localization of STAT3 from the cytoplasm to the nucleus, suggesting that the transfected STAT3 reacted with IL-6 stimulation (Fig. 5B). Then, we investigated the proliferation of STAT3-transfected primary B cells. Cell proliferation assay after 48 h transfection showed that the proliferation of STAT3-transfected primary B cells were slightly higher than that of vector-transfected primary B cells (Fig. 5C, Mann–Whitney test, $p < 0.01$). However, 4 days after transfection, the difference was not statistically significant (data not shown). The transfection of STAT3 to B cells was repeated 4 times with similar results. These data suggested that transfection of STAT3 might induce a transient proliferation in the primary B cells in vitro.

4. Discussion

In the present study, we present a case of AIDS-related B cell lymphoma with HIV-1 integration. HIV-1 with defective 5'LTR integrated into the upstream region of the first STAT3 coding exon. The 3' LTR had strong promoter activity, resulting in increased expression of STAT3 in the nuclei of lymphoma cells. This is the first case report describing dysregulation of STAT3 by HIV-1 integration, resulting in B cell lymphoma development.

STAT3 is an important molecule for IL-6-type cytokines that signal and stimulate proliferation and terminal differentiation of B cells [20]. STAT3 also plays some oncogenic roles. Activated and phosphorylated STAT3 has been observed in a variety of experimental and numerous human malignancies [21–23]. A recent study reveals that high expression of unphosphorylated STAT3 results in up-regulation of oncogenes, suggesting that overexpression of either form of STAT3, phosphorylated and unphosphorylated, might induce cancer [24]. Although we failed to detect phosphorylated STAT3, high expression of STAT3 in the nucleus implies that activated STAT3 may bind to DNA and activate some genes constitutively. Alternatively, it implies that overexpression of unphosphorylated STAT3 in the nucleus might induce various oncogenes such as cdc2, cyclin B1 and mras [24]. However, our transfection study of STAT3 resulted in transient cell proliferation in the primary B cells (Fig. 5), suggesting that additional factors other than STAT3 expression might be required for complete transformation of primary B cells. HIV-1 integrated into *c-fes/fps* in other reported cases of AIDS-related lymphoma [6], and it has been demonstrated that *c-fes* activates STAT3 [25]. Thus, STAT3 may play some roles in the lymphomagenesis in the cases of HIV-1-integrated lymphoma.

This case was B cell lymphoma. HIV-1 usually infects and integrates into T cells or macrophages, and it is uncommon for HIV-1 to infect B cells. In the report by other group, HIV-1 provirus was frequently detected in macrophages infiltrating lymphomas, not in lymphoma cells [6]. However, in our case, we concluded that the HIV-1 integration occurred in the lymphoma cells, not in T cells or macrophages infiltrating

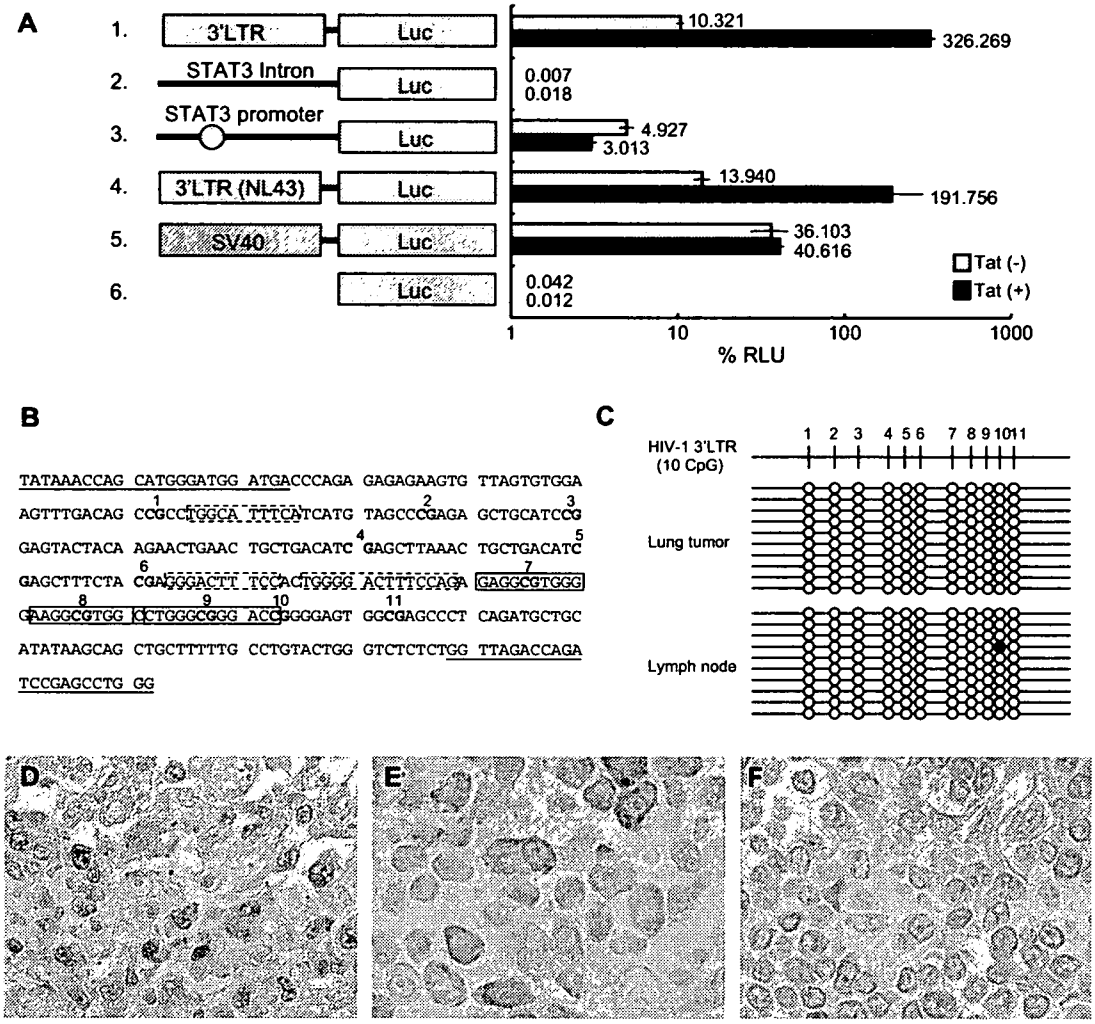

Fig. 4. Promoter activity of HIV-1 3′LTR and STAT3 expression in the lymphoma. (A) Promoter activity of HIV-1 3′LTR by reporter assay. Schematic representation of promoter constructs used in transient transfection assays is shown on the left. Forty-eight hours after transfection, cells were collected and the luciferase activity was measured. The percentage relative luminescence units (RLU) were calculated by dividing firefly activity by renilla activity. Horizontal bars indicate standard deviations of three independent experiments. (B and C) No methylation in a promoter enhancer region of HIV-1 3′LTR in the HIV-1-integrated lymphoma. (B) CpG sites in the promoter enhancer region of 3′LTR of the HIV-1 provirus in the patient with HIV-1-integrated lymphoma (218–529 in GenBank DQ117603). CpG sites are in boldface and numbered from the 5′ end of the LTR (1–11). Nuclear factor-κB and Sp1 sites identified with Motif Search (Kyoto University Bioinfomatics center, Kyoto, Japan, http://motif.genome.jp/) at a 75% cut-off value are indicated by boxes with broken and solid lines, respectively. Sequences used for primers are indicated by underlining. (C) Levels of CpG methylation of the promoter enhancer region of HIV-1 3′LTR in the HIV-1-integrated lymphoma and lymph nodes in the patient. Results of bisulfite genomic sequencing coupled with TA cloning are shown. The methylation status of 10 clones for each sample is presented; methylation of each CpG site is expressed as a filled circle, and unmethylated sites are shown as open circles. Top, schematic description of CpG sites in the 3′LTR of (B). (D–F) Immunohistochemistry of STAT3. The HIV-1-integrated lymphoma cells expressed STAT3 predominantly in the nucleus (D), however, signals of STAT3 were weak and localized in the cytoplasm in the other case of KSHV-positive, AIDS-related lymphoma (E), and were very weak in a case of EBV-positive, AIDS-related lymphoma (F). Original magnification is ×400.

in the lymphoma, because of following reasons: (1) there were few T cells in the lymphoma tissue by immunohistochemistry for CD3 (data not shown); (2) HIV-1 DNA was detected in the lymphoma at a high copy number, that is very rare or none in AIDS-related lymphoma [26]; (3) HIV-1 sequences suggested the possibility of X4 viruses, which leads the integrated HIV-1 sequences are usually not found in the macrophages; (4) some different HIV-1 V3 sequences were identified between the lymphoma and lymph node; and (5) the titer of HIV-1 DNA in the lymphoma were higher than that in the lymph node (Fig. 1G). Then, how did HIV-1 infect B cells in the patient? Although detail mechanism of HIV-1 infection to B cells in this case was still unknown, we presume that KSHV played an important role in HIV-1 infection to B cells. This case of lymphoma was positive for KSHV and EBV by PCR, however, KSHV and EBV did not play a direct role in the oncogenesis of the lymphoma because of the low or absent expression of

Table 1
STAT3 expression in AIDS-related and unrelated lymphoma

STAT3 expression	Nucleus	Cytoplasm	No expression	Total
AIDS-related lymphoma	1*	7	2	10
Non-AIDS-related lymphoma	0	1	14	15

*HIV-integrated lymphoma reported in the present study.

LANA and EBERs. It is possible that KSHV infection might increase susceptibility of B cells expressing CD4 and CXCR4 to infection with the X4 genotype of the HIV-1 [27]. Moreover, it is demonstrated that KSHV-encoded ORF50 protein increases susceptibility of B cells to infection with HIV-1 [28]. Although ORF50, CD4 and CXCR4 were not detected in the lymphoma cells by immunohistochemistry (data not shown), it is possible that KSHV-infected B cells might be infected and integrated by HIV-1 in the early stage of lymphoma development.

Although an intensive study revealed that there were many hot spots of HIV-1 integration [29], the STAT3 gene was not included in the list of hot spots. Thus, the STAT3 gene is a novel target of HIV-1 integration. Since HIV-1 DNA has not been detectable in DNAs extracted from AIDS-related lymphoma cases by Southern blot hybridization, so far [26], HIV-1 integration should be rare in AIDS-related lymphoma. A recent study demonstrates a decrease in EBV-positive lymphoma among patients with AIDS because of introduction of highly active antiretroviral therapy (HAART) [30]. Therefore, novel mechanisms other than oncogenesis by EBV or KSHV may have been involved in the lymphomagenesis of AIDS-related lymphoma recently. There is no report describing a frequency of HIV-1 integration among AIDS related lymphoma. HIV-1 usually infects T cells or macrophages in AIDS patients, however, T cell lymphoma is still rare among AIDS-related lymphoma in the HAART era [30]. In addition, HIV-1 infection to B cells would occur in a very special condition, such as under KSHV infection. Taken together, although the case we described in the present study contained an important scientific phenomenon on STAT3, HIV-1-integrated lymphoma should be very rare among AIDS-related lymphoma.

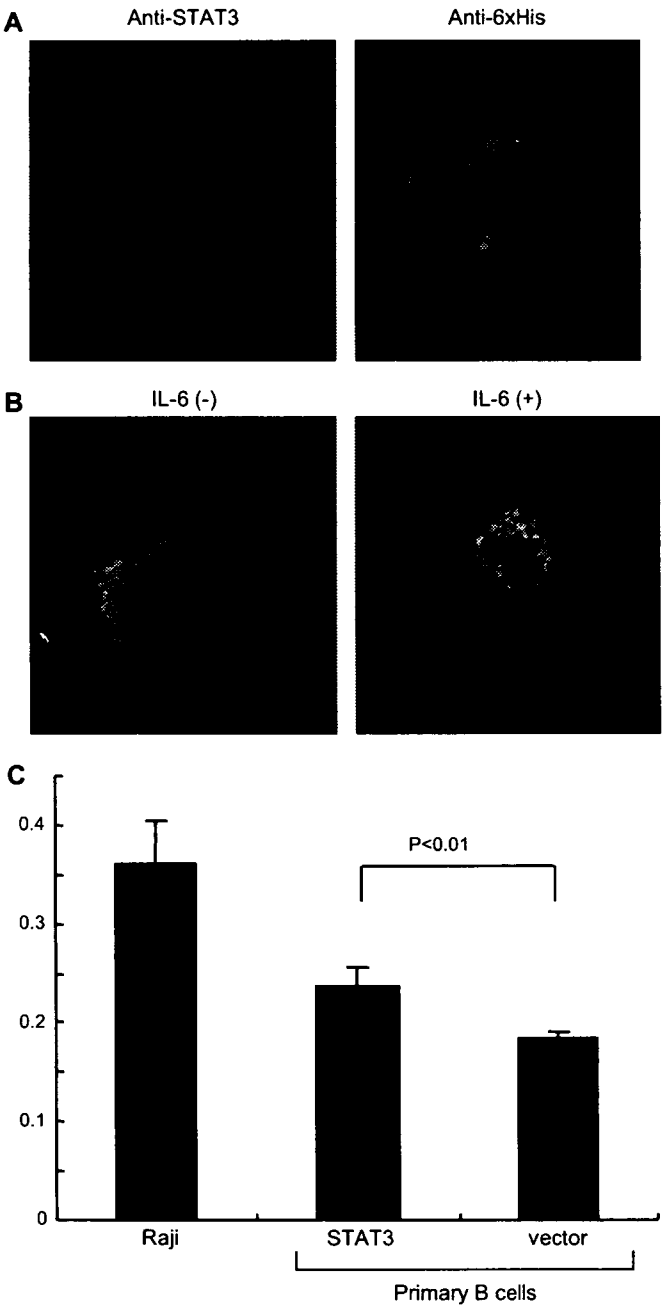

Fig. 5. Transfection of STAT3 into B cells in vitro. (A) STAT3 expression in the STAT3-transfected TY-1, a KSHV-positive B cell line. The cells were transfected with STAT3 expression vector by Nucleofector (Amaxa, Cologne, Germany) using O-06 program. STAT3 expression was detected by anti-STAT3 mouse monoclonal antibody (green in left panel) and anti-6xHis antibody, followed by Alexa 488-conjugated anti-mouse IgG antibody (molecular probe, green in right panel). Red color indicates nuclear counterstaining of propidium iodide. (B) Localization of transfected STAT3 in TY-1. His-tagged STAT3 was detected by anti-6x His antibody in the cytoplasm of B cells (left panel). In the presence of IL-6 (Peprotech, Rocky Hill, NJ, 0.1 ng/ml), transfected STAT3 localizes in the nucleus predominantly (right panel). (C) Cell proliferation assay for STAT3-transfected primary B lymphocytes. Primary B cells were isolated from PBMC. The purity of B cell (CD19+) was >95%. The cells were transfected with STAT3 expression vector expressing STAT3 and CD4 by Nucleofector using U-15 program. Transfection efficiency to primary B cells was around 20%. To increase the proportion of transfected cells, the transfected B cells were separated with CD4 microbeads after 16 h of the transfection (Miltenyl Biotec, Auburn, CA). 48 h after transfection of STAT3 or vector to primary B cells, the proliferation rate was measured with BrdU ELISA (Roche). Raji is an EBV-positive Burkitt lymphoma cell line (untransfected). Numbers in Y-axis indicates absorbance in ELISA. Error bars indicate standard errors of 8 independent experiments.

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Rapid detection of human herpesvirus 8 DNA using loop-mediated isothermal amplification

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Abstract

The reliability of a loop-mediated isothermal amplification (LAMP) method for the detection of human herpesvirus 8 (HHV-8) DNA was evaluated. Although LAMP products were produced with the DNA sample extracted from BCP-1 cells, LAMP products were not produced with the DNAs from seven other human herpesviruses. The detection limit of the HHV-8 LAMP method was 100 copies of target sequence/tube. To determine whether the HHV-8 LAMP method could be used to quantify viral DNA, threshold times, which are defined as the time (in s) it takes to reach the threshold turbidity level (0.1), were measured for the amplification of serial dilutions of a DNA plasmid containing the target sequence. The standard curve possessed a correlation coefficient of 0.9428 with a slope of −84.079 and *y*-intercept value of 1936.2. Additionally, an attempt was made to detect viral DNA in 17 specimens collected from Kaposi's sarcomas and two cell lines obtained from primary effusion lymphomas. HHV-8 DNA was detected in 14 of the 17 Kaposi's sarcoma tissue samples and both of the primary effusion lymphoma cell lines. Viral DNA was not detected in HHV-8 LAMP-negative samples using the real-time PCR method.

Keywords: HHV-8; Real-time PCR; LAMP

1. Introduction

Human herpesvirus 8 (HHV-8), which is also called Kaposi's sarcoma-associated herpesvirus, is a member of the subfamily Gammaherpesvirinae, which has been associated with all forms of Kaposi's sarcoma, primary effusion lymphoma, and multicentric Castleman's disease (Boshoff and Chang, 2001; Cesarman et al., 1995; Soulier et al., 1995). In contrast to other human herpesviruses, epidemiological surveys indicate that HHV-8 is not a ubiquitous virus. Areas with a high prevalence of this virus are usually highly endemic for classic or endemic Kaposi's sarcoma. For example, HHV-8 infection is highly endemic in Central, East, and South Africa areas, in which the viral seroprevalence can reach up to 50% in the general population (Dukers and Rezza, 2003). Following the expansion of the human immunodeficiency virus (HIV) type 1 epidemic in Africa, Kaposi's sarcoma is becoming one cancer, which is diagnosed frequently in several African countries (Dedicoat and Newton, 2003). Therefore, an easy and rapid diagnostic procedure for HHV-8 infection would be a valuable tool in these countries. Additionally, it has been reported that HHV-8 can be transmitted during organ transplantations and blood transfusions (De Paoli, 2004) and the risk for the development of Kaposi's sarcoma is high in immunosuppressed organ transplant recipients, monitoring patients for HHV-8 infections is also important in developed countries.

Although a variety of serological assays for the diagnosis of HHV-8 infections have been described, most of them are not sufficiently sensitive or specific. A combination of several assays can be used to obtain adequate degrees of sensitivity and specificity (Chandran et al., 1998; Schatz et al., 2001; Spira et al., 2000). Detection of viral DNA in clinical samples, such as tissues that are thought to be Kaposi's sarcoma or lymphoma cells, is considered to be an appropriate method for determining

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whether HHV-8 infection is involved in the pathogenesis of the disease. In addition, because monitoring the viral load can be useful for patient management, real-time PCR is the most valuable method for the diagnosis of HHV-8 infections (Lallemand et al., 2000; Tedeschi et al., 2001; Broccolo et al., 2002a). This method, however, has not yet become a common procedure in hospital laboratories particularly in developing countries, primarily because an expensive thermal cycler is required.

Notomi et al. (2000) described a novel nucleic acid amplification method, termed loop-mediated isothermal amplification (LAMP), which amplifies DNA targets with high specificity, efficiency, and speed. The most significant advantage of LAMP is the ability to amplify specific sequences of DNA under isothermal conditions. Thus, LAMP requires only simple, cost-effective equipment that can be made available easily in hospital laboratories. This is the major advantage for using the method in developing countries, which are highly endemic regions of HHV-8. Because the LAMP protocol exhibits high specificity and high amplification efficiency, this method can be a valuable tool for the rapid diagnosis of infectious diseases (Enosawa et al., 2003; Iwamoto et al., 2003; Kuboki et al., 2003; Ihira et al., 2004; Parida et al., 2004; Yoshikawa et al., 2004; Okamoto et al., 2004; Enomoto et al., 2005; Sugiyama et al., 2005; Kimura et al., 2005; Suzuki et al., 2006) in both commercial and hospital laboratories. The aim of the study was to establish a LAMP-based HHV-8 DNA amplification technique, and its reliability examined for the detection of viral DNA from clinical specimens.

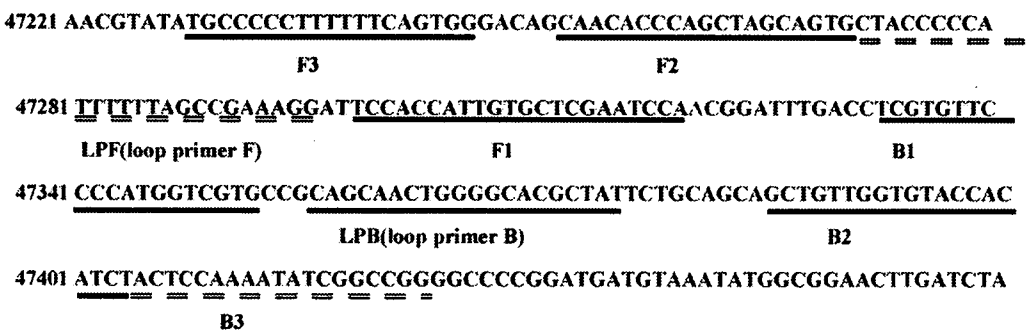
2. Materials and methods

2.1. Study design

HHV-8 DNA extracted from BCP-1 cells was used as a positive control to determine the appropriate conditions for the HHV-8 LAMP protocol, as well as the specificity and baseline sensitivity of this method. DNA from herpes simplex virus (HSV)-1 (KOS strain), HSV-2 (186 strain), varicella-zoster virus (VZV) (Oka-vaccine strain), Epstein-Barr virus (EBV) (peripheral blood mononuclear cells (PBMCs) containing a high copy number of EBV DNA as determined using real-time PCR analysis), human cytomegalovirus (HCMV) (AD-169 strain), human herpesvirus type 6 (HHV-6) A (U1102 strain), HHV-6B (Z29 strain), and human herpesvirus 7 (HHV-7) (RK strain) was used to determine the specificity of the HHV-8 LAMP method. A plasmid containing the HHV-8 target sequence was used to determine the sensitivity of the assay.

In order to determine the reliability of the HHV-8 LAMP method for the detection of viral DNA in clinical samples, two different primary effusion lymphoma cell lines and 17 Kaposi's sarcoma tissue samples (10 of the samples were frozen, whereas the other and seven samples were paraffin-embedded), which

A. Location and name of each target sequence as a primer in HHV-8 ORF26 gene



B. Sequence of each primer

Name of primers	Sequence
H8orf26BIP	5'-TCGTGTTCCCCATGGTCGTG AGATGTGGTACACCAACAGC-3' (B1-B2c)
H8orf26FIP	5'-TGGATTCGAGCACAATGGTGGA CAACACCCAGCTAGCAGTG-3' (F1c-F2)
H8orf26B3	5'-CCGGCCGATATTTTGGAGT-3' (B3c)
H8orf26F3	5'-TGCCCCCTTTTTTCAGTGG-3' (F3)
H8orf26LPB	5'-CAGCAACTGGGGCACGCTAT-3' (LPB)
H8orf26LPF	5'-CCTTTCGGCTAAAAAATGGGGGTAG-3' (LPFc)

Fig. 1. (A) Locations and names of the target sequences used as primers for the LAMP of ORF 26 from HHV-8. (B) Names and sequences of the primers for the HHV-8 LAMP method. B2c, sequence complementary to B2; F1c, sequence complementary to F1.

were stored at the Department of Pathology (National Institute of Infectious Diseases, Tokyo, Japan) were used. The results of the HHV-8 LAMP analysis were compared with the results obtained with a previously established HHV-8 real-time PCR assay to assess the reliability of LAMP as a rapid diagnostic tool for detecting HHV-8 infections.

2.2. DNA extraction

For the initial development of the HHV-8 LAMP assay, viral DNA was extracted from BCP-1 cells, PBMCs containing a high copy number of EBV DNA collected from the typical infectious mononucleosis patient caused by primary EBV infection, and HSV-1-, HSV-2-, VZV-, HCMV-, HHV-6A-, HHV-6B-, and HHV-7-infected cells using a QIAamp Blood Mini kit (Qiagen, Chatsworth, CA). This DNA extraction kit was also used to extract DNA from the clinical specimens. After extraction, DNA was eluted in 100 μl of buffer and stored at −20 °C.

2.3. HHV-8 LAMP

The LAMP reaction was conducted according to the descriptions from Notomi et al. (2000) and Nagamine et al. (2002). The LAMP method requires a set of four primers (B3, F3, BIP, and FIP) that recognize a total of six distinct sequences (B1, B2, B3, F1, F2, and F3) in the target DNA. Primers for the HHV-8 LAMP reactions were designed from the sequence of the HHV-8 open reading frame (ORF) 26, which encodes the minor capsid protein, using Primer Explorer V software (FUJITSU, Tokyo, Japan) (Fig. 1). The BIP primer for ORF 26 of HHV-8 (H8orf26BIP) consisted of the sequence of B1 (20 nucleotides; nt) and the sequence complementary to B2 (20 nt). The FIP primer for ORF 26 of HHV-8 (H8orf26FIP) consisted of the sequence complementary to F1 (22 nt) and the sequence of F2 (19 nt). The B3 (H8orf26B3) and F3 (H8orf26F3) primers for ORF 26 of HHV-8 corresponded to the F2-B2 regions. Because it has been demonstrated that additional loop primers increase the amplification efficiency (Nagamine et al., 2002), loop primers for ORF 26 of HHV-8 (H8orf26LPB and H8orf26LPF) were also synthesized. H8orf26LPB consisted of the LPB sequence, and H8orf26LPF consisted of the sequence complementary to LPF. The LAMP reaction was performed with a Loopamp DNA amplification kit (Eiken Chemical, Tochigi, Japan). Each 25-μl reaction mixture contained 2.4 μM H8orf26FIP, 2.4 μM H8orf26BIP, 0.4 μM of each outer primer (H8orf26F3 and H8orf26B3), 1.2 μM of each loop primer (H8orf26LPB and H8orf26LPF), 2× reaction mixture (12.5 μl), *Bst* DNA polymerase (1 μl), and 5 μl of the sample. Reaction mixtures were incubated at 63 °C for 45 min. A LA-200 turbidimeter (Teramecs, Kyoto, Japan) was used to measure turbidity during the LAMP reaction (Mori et al., 2001). The turbidity cutoff value used to distinguish negative samples from positive samples was fixed at 0.1. After turbidity measurements, LAMP products were subjected to electrophoresis on a 1.5% agarose gel. Gels were visualized under UV light after ethidium bromide staining. Great care was taken to avoid contamination between samples; different rooms were used for DNA extraction, LAMP set up, and gel analysis. In addition, pipette tips with filters for aerosol protection were used to minimize contamination.

2.4. Real-time PCR for the detection of HHV-8

Real-time PCR was used to measure the quantity of HHV-8 DNA in each sample. The sequences of the primers and probes have been described by Lallemand et al. (2000). PCR was performed using the TaqMan PCR kit (PE Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. A standard curve for measuring the amount of HHV-8 DNA was constructed using the C_T values obtained from a serially diluted plasmid containing the ORF 73 target sequence. The C_T value from each sample was plotted on a standard curve, allowing copy numbers to be automatically calculated using Sequence Detector v.1.6 software (PE Applied Biosystems).

2.5. Cloning of HHV-8 DNA

In order to determine the sensitivity of the HHV-8 LAMP method, a plasmid containing the target HHV-8 DNA sequence was constructed. First, upstream (H8S1; AACGTATATGCCCCCTTTTT) and downstream (H8S2; TCCGCCATATTTACATCATCC) primers spanning the sequence between the F3 and B3 primers were synthesized. HHV-8 DNA obtained from BCP-1 cells was amplified with these two primers using a conventional PCR. The PCR product was cloned into a pGEM-T vector using pGEM-T Vector System II (Promega, Madison, WI) according to the manufacturer's instructions. The resulting plasmid (pGEMH8S12) was used to make standard dilutions for the evaluation of the lower detection limit of the LAMP protocol.

3. Results

In order to develop an effective assay for the rapid detection of HHV-8 DNA, the specificity of the HHV-8 primers was evaluated. HHV-8 LAMP reactions were performed with DNA extracted from BCP-1 cells, PBMCs containing a high copy number of EBV DNA, and HSV-1-, HSV-2-, VZV-, HCMV-, HHV-6A-, HHV-6B-, and HHV-7-infected cells. Because the LAMP products consisted of several inverted-repeat structures, positive samples result in a number of bands of different sizes following electrophoresis on agarose gels. Although amplified HHV-8 DNA resulted in typical ladder patterns as shown in Fig. 2, no LAMP products were detected in the reactions performed with DNA from other human herpesviruses. The specificity of the primers was tested by using a turbidity assay. The use of HHV-8 specific primers only elevated the turbidity in the HHV-8 DNA-containing samples.

After confirmation of the specificity of the LAMP assay, the sensitivity of this method was determined. Serial dilutions of the pGEMH8S12 plasmid were used to determine the detection limit of the method. Both agarose gel electrophoresis and turbidity assays determined that the detection limit of the HHV-8 LAMP method was 100 copies/tube (Fig. 3). In order to deter-

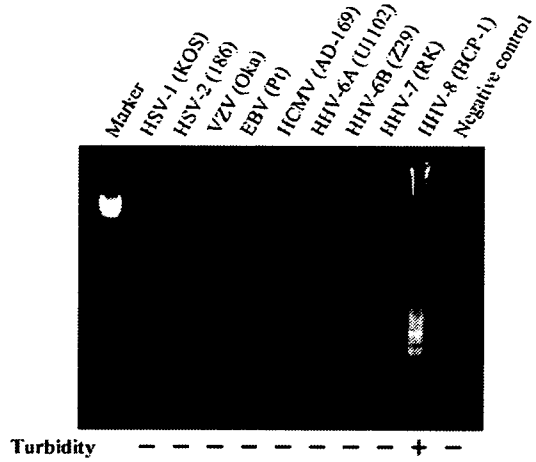


Fig. 2. DNA extracted from cells infected with one of the nine human herpesviruses was amplified using the HHV-8 LAMP protocol to determine the specificity of the method. Marker, 123-base-pair DNA ladder.

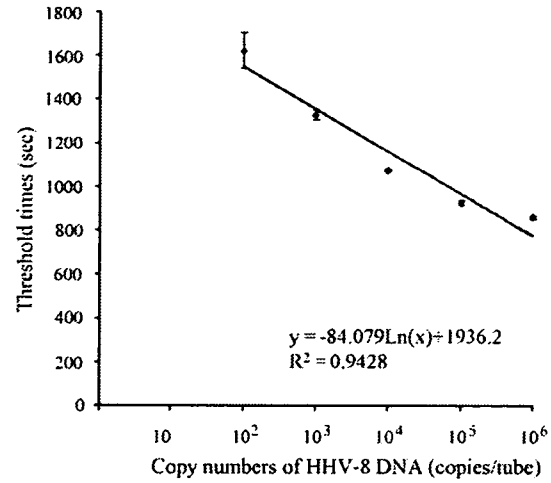


Fig. 4. Correlation between the threshold time (in s) and the copy number of the DNA plasmid (pGEMH8S12) containing the target sequence. Values on the *x*-axis are the threshold times, which are defined as the time it takes to reach the threshold level of turbidity (0.1).

mine whether the HHV-8 LAMP protocol could be used to quantify viral DNA, threshold times, which are defined as the time (in s) it takes to reach the threshold turbidity level (0.1), were measured for the amplification of the serial dilutions of the plasmid DNA. The standard curve possessed a correlation coefficient of 0.9428 with a slope of −84.079 and *y*-intercept value of 1936.2 (Fig. 4) for samples with between 100 and 1,000,000 copies/tube.

After these initial validation studies, the reliability of the HHV-8 LAMP protocol as a rapid method for viral DNA detection from clinical specimens was evaluated. As shown in Table 1, HHV-8 DNA was detected in sample numbers 4–19 using HHV-8 real-time PCR analysis (copy numbers ranging between 138 and 16,594,820 copies/tube). HHV-8 LAMP products were detected using turbidity assays in samples numbers 4–19. Four of the seven DNA samples extracted from the paraffin-embedded Kaposi's sarcoma tissues were determined to be HHV-8 positive using the HHV-8 LAMP assay. In order to determine whether the HHV-8 LAMP could be used to quantify the amount of viral DNA in clinical samples, the correlation between the copy numbers of viral DNA determined using real-time PCR and the threshold times measured with HHV-8 LAMP assay was examined. As shown in Fig. 5, weak association between these two parameters was observed.

4. Discussion

The HHV-8 LAMP method specifically amplified HHV-8 DNA, and did not amplify the DNA from seven other human herpesviruses, including EBV, another member of the *Gammaherpesvirinae* subfamily (Fig. 2). This specificity was confirmed by agarose gel electrophoresis and turbidity assays. The detection limit of the HHV-8 LAMP method was 100 copies/tube, as determined by both agarose gel electrophoresis and turbidity assays. These findings demonstrate that the HHV-8 LAMP method specifically and efficiently amplifies viral DNA. Although the turbidity assay is generally less sensitive than visualizing the ethidium bromide stained products on agarose gels, the sensitivities of these two detection methods were not

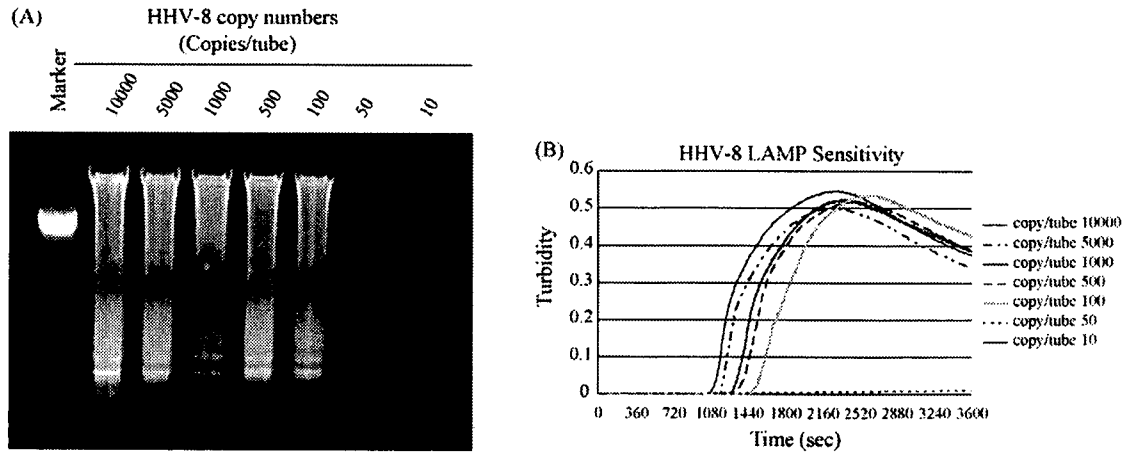


Fig. 3. Serial dilutions of the pGEMH8S12 plasmid were amplified to determine the sensitivity of the assay. Agarose gel electrophoresis (A) and a turbidity assay (B) were both used to determine the sensitivity of the assay. Marker, 123-base-pair DNA ladder.

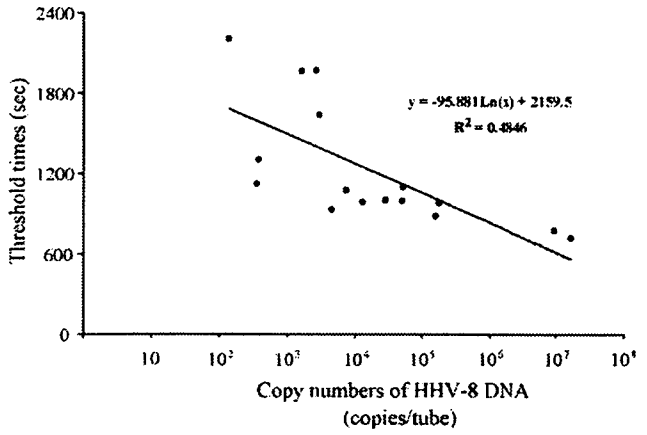


Fig. 5. Correlation between the threshold time (in s) with the viral copy number measured in clinical specimens by real-time PCR. Values on the *x*-axis are the threshold times, which are defined as the time it takes to reach the threshold level of turbidity (0.1).

significantly different in this study. Compared to agarose gel electrophoresis, turbidity assays allow a reduction in the operation time and reduce contamination risks, and are therefore thought to be an appropriate method for rapid diagnoses in hospital laboratories.

Quantitative analysis of the HHV-8 DNA load using real-time PCR analysis has been demonstrated to be a valuable tool for the management of patients with viral infections. It has been shown that HHV-8 DNA can no longer be detected after the resolution of Kaposi's sarcoma lesions in patients infected with HIV who are being treated with highly active antiretroviral therapies (Burdick et al., 1997; Martinelli et al., 1998). Moreover, the exacerbation of the symptoms in HIV-infected patients with multicentric Castleman's disease has been associated with an increase in the HHV-8 viral load in PBMCs (Grandadam et al., 1997). In order to determine whether or not the HHV-8 LAMP method could be used to quantify the viral DNA load, the threshold times for serial dilutions of a DNA plasmid were measured. Although the standard curve determined using the plasmid dilutions had high correlation efficiency (Fig. 4), the threshold time determined by the HHV-8 LAMP was weakly correlated with the viral copy number measured by HHV-8 real-time PCR analysis in clinical specimens (Fig. 5). One of the reasons for explanation of the low correlation efficiency in the analysis of clinical samples is the difference of the target genes between HHV-8 LAMP and real-time PCR. Additionally, as shown in Fig. 4, it is likely that the reliability of HHV-8 LAMP assay is low for quantitative analysis of samples containing low copies of viral DNA. These results suggest that this method can be used to quantify the high amount of HHV-8 DNA, as has been reported elsewhere (Mori et al., 2001, 2004; Hong et al., 2004; Parida et al., 2004; Suzuki et al., 2006). However, the accuracy of HHV-8 DNA load measured by HHV-8 LAMP method, in particular low amount of viral DNA, appears to be lower than real-time PCR assay. Therefore, if precise quantitation of viral DNA is necessary in the samples containing low copy of viral DNA, real-time PCR would be better than the LAMP method to use at present.

Table 1
Comparison between HHV-8 LAMP and the previously established real-time PCR

No. of samples	Materials	Real-time PCR (copies/tube)	Results of LAMP (s[a])
1	KS[b]	0	−2700<
2	KS[b]	0	−2700<
3	KS[b]	0	−2700<
4	KS	138	+2208
5	KS[b]	353	+1122
6	KS[b]	380	+1302
7	KS	1,640	+1962
8	KS[b]	2,730	+1968
9	KS	2,980	+1638
10	KS	4,570	+930
11	KS	7,560	+1074
12	KS[b]	13,402	+984
13	KS	28,483	+1002
14	KS	51,161	+996
15	KS	52,199	+1098
16	KS	157,878	+876
17	KS	177,798	+978
18	PEL cell line	9,416,765	+774
19	PEL cell line	16,594,820	+720

LAMP: loop-mediated isothermal amplification, KS: Kaposi's sarcoma, PEL: primary effusion lymphoma.
[a] Time required for the turbidity to exceed the cut-off value (0.1).
[b] Paraffin embedded samples.

The prevalence of HHV-8 and the number of patients with acquired immunodeficiency syndrome is low in Japan; accordingly, the frequency of HHV-8-associated diseases, including Kaposi's sarcoma and multicentric Castleman's disease, is also low. Thus, not only the number of clinical samples was limited in this study, but also all materials of clinical samples were restricted within biopsy specimens except for two primary effusion lymphoma cell lines. As it has been demonstrated that HHV-8 DNA loads in peripheral bloods collected from patients with Kaposi's sarcoma were lower than those in the affected tissues (tumoral skin) (Duprez et al., 2005), HHV-8 LAMP method might be less sensitive to detect viral DNA in blood samples. However, as demonstrated in the LAMP method for detection of other viral DNA (Poon et al., 2005), prior-heat denaturation of the template DNA could increase assay sensitivity to detect HHV-8 DNA. Further study is needed to determine whether the HHV-8 LAMP would be useful for detection of viral DNA in other clinical samples such as peripheral blood mononuclear cells and serum.

Although is was observed recently that serum might have slight inhibitory effect in LAMP reactions, a small amount of HHV-6 DNA was detected directly (without DNA extraction) in human serum using the LAMP method (Ihira et al., 2007). It has been demonstrated that the presence of HHV-8 DNA in plasma was associated significantly with the clinical status of Kaposi's sarcoma patients (Broccolo et al., 2002b). Therefore, direct detection of HHV-8 DNA in serum by the HHV-8 LAMP method might be valuable tool for the management of Kaposi's sarcoma patients. Omission of DNA extraction step would be highly advantageous for using the diagnostic method

in developing countries. Large number of sera collected in Kaposi's sarcoma endemic areas should be examined by direct HHV-8 LAMP in a future study.

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