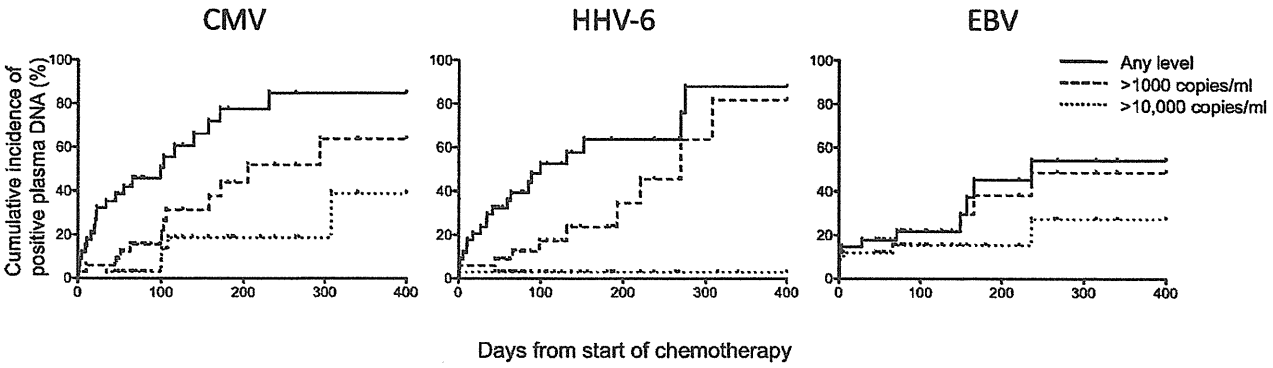

Fig. 1. Kaplan–Meier plot of the cumulative incidence of reactivation of CMV, HHV-6, or EBV, by number of copies of each viral DNA per ml in the day after start of chemotherapy.

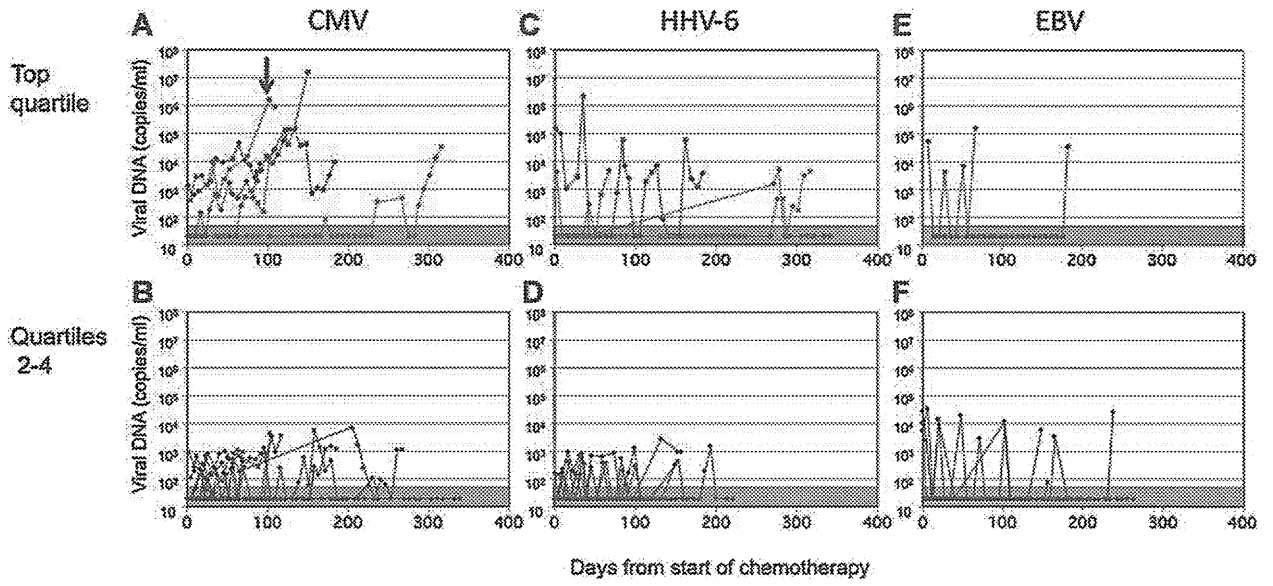

Fig. 2. Kinetics of the course of reactivation of CMV, HHV-6, and EBV. Each line represents an individual patient. The shaded area indicates values below the threshold for detection (<50 copies/ml of plasma). The top graphs (A,C, and E) show the kinetics of viral load among patients whose peak DNA load exceeded the upper one-fourth and the bottom graphs (B,D, and F) show those among patients whose peak DNA load were less than the upper one-fourth. The arrow in the (A) depicting the kinetics of CMV DNA reactivation indicates the day on which interstitial pneumonia developed in one patient.

TABLE II. Characteristics of Each Virus Reactivation Among Positive Cases

	CMV	HHV-6	EBV
Time to onset from start of chemotherapy			
Median, days (range)	28.5 (1–157)	38.5 (1–275)	28 (1–236)
Mean, days (SD)	61.4 (65.7)	70.8 (81.9)	73.7 (87.3)
Time to peak viral DNA from start of chemotherapy			
Median, days (range)	104.5 (2–315)	84 (1–315)	71 (1–238)
Mean, days (SD)	115.8 (74.9)	101.9 (91.8)	87.4 (81.8)
Duration of positive viral DNA in each patient			
Median, weeks (range)	5.5 (1–26)	2 (1–16)	1 (1–4)
Mean, weeks (SD)	7.1 (6.9)	3.8 (4.0)	1.9 (1.1)
Peak viral load			
Median, copies/ml (range)	1,216 (35–14,743,520)	1,117 (69–2,041,969)	10,810 (80–343,140)

SD, standard deviation.

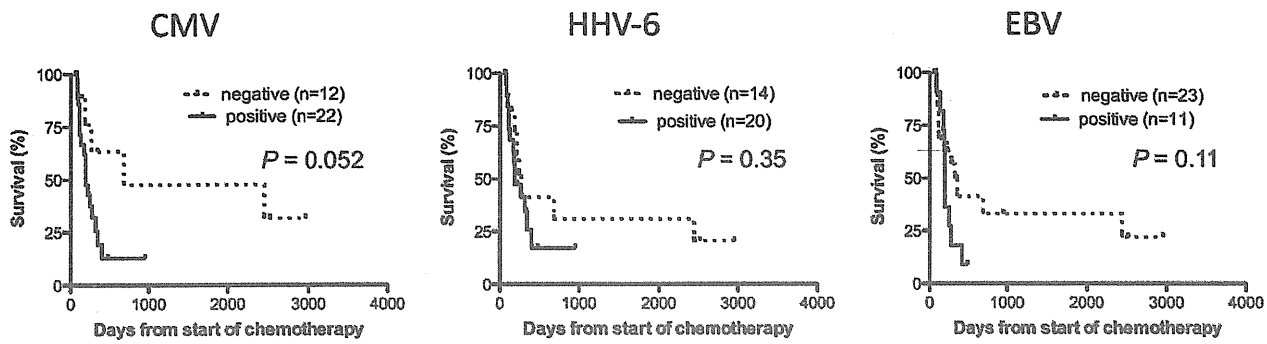

Fig. 3. Kaplan–Meier plot of the probability of survival according to viral reactivation. In each figure, "positive" indicates that tested positive for DNA of a viral genome in plasma at any time after start of chemotherapy, and "negative" indicates patients without positive DNA for a particular viral genome at any point after start of chemotherapy. Comparisons of survival were made using the log-rank test.

DISCUSSION

Immunosurveillance by virus-specific T cells prevents reactivation of herpesviruses. Compromised cellular immune responses are associated with reactivation of herpesviruses. In patients who have received solid-organ or allogeneic stem cell transplantation, reactivation of CMV, HHV-6, and EBV is closely related to serious complications—including CMV pneumonia [Boeckh and Ljungman, 2009], HHV-6 encephalitis [Zerr, 2006a; Ogata et al., 2010], and EBV-related post-transplant lymphoproliferative disorder [Shapiro et al., 1999; Omar et al., 2009]. Interestingly, reactivations of EBV and CMV are also common in patients with severe aplastic anemia who received immunosuppressive therapies but are rarely associated with clinical disease even in patients with high-level reactivation [Scheinberg et al., 2007]. These results indicate that clinical syndromes accompanied by herpesvirus reactivation depend on not only on viral reactivation but also on the patient's background or the cause of immunosuppression.

This study showed that CMV, HHV-6, or EBV reactivation was surprisingly frequent in ATL patients receiving chemotherapy. Some ATL patients developed profound reactivation. For example, plasma CMV viral load exceeded 10^6 copies/ml in two ATL patients. Such high levels of reactivation had not been observed in a previous study investigating CMV reactivation in patients who received stem cell transplantation at Oita University Hospital [Ikewaki et al., 2005]. A recent report demonstrated that plasma HHV-6 DNA levels of $\geq 10^4$ copies/ml are associated with the development of HHV-6 encephalitis in stem cell transplant recipients [Ogata et al., 2010]. In this study, one ATL patient repeatedly experienced such a high-level of HHV-6 reactivation throughout the clinical course.

Each virus had distinct reactivation kinetics. In patients with high levels of CMV DNA (i.e., $\geq 10^4$ coppies/ml plasma), plasma CMV DNA levels increased gradually and needed at least 4 weeks to reach a peak CMV viral load. Furthermore, plasma CMV DNA did not disappear if the CMV DNA load reached a level of $\geq 10^4$ copies/ml of plasma. Subsequent prognosis of patients whose CMV DNA load reached this level was very poor. These findings suggest that there is a threshold level of CMV reactivation after which spontaneous improvement of the reactivation cannot be expected. A CMV load $\geq 10^4$ copies/ml plasma is indicative of subsequent exacerbation of CMV reactivation and development of serious clinical course. In contrast, most HHV-6 reactivation peaked within 1 week, and high-level reactivations could disappear and reappear suddenly. The dynamic reactivation pattern of HHV-6 in ATL patients may be similar to that of recipients of stem cell transplant: in stem cell transplantation patients, plasma HHV-6 DNA can elevate to peak within 1 week but do not persist in most cases [Ogata et al., 2008]. Most EBV reactivation did not persist. Interestingly, the rate of EBV-DNA-positive samples near the initiation of chemotherapy was relatively high. The viral DNA observed in the early phase of chemotherapy disappeared rapidly. Polyclonal EBV-infected cells were frequently observed in the lymph nodes of patients with incipient ATL [Ohshima et al., 1997], and this observation may be associated with the EBV-DNA-positive samples observed in the early phase of chemotherapy. The characteristics of the reactivation kinetics of each herpesvirus are probably affected by the doubling-time of each virus, the ability of virus-specific T cells to control each virus in the host, and the responsiveness of each virus to the host immune response.

This study has several limitations—including the retrospective design, the small number of patients enrolled, research at only two institutes, and the irregularity of the sampling intervals. This study may underestimate the incidence of viral reactivation because some plasma-sampling intervals were long.

The results of this study raised the possibility that routine prospective monitoring for CMV DNA in plasma may be useful in preventing CMV-related disease in ATL patients. CMV pneumonia is major cause of death in ATL patients [Suzumiya et al., 1993]. In fact, one patient in this study developed fatal interstitial

pneumonia when CMV DNA reached peak levels. Pre-emptive anti-viral therapy using active agents, such as ganciclovir or foscarnet, when CMV DNA levels reach $\geq 10^4$ copies/ml may be justified. Because at least 3 weeks were needed to reach CMV DNA $\geq 10^4$ copies/ml, routine monitoring of CMV DNA once every 7–14 days is proposed. In contrast, the levels of HHV-6 and EBV DNA in plasma increased and decreased suddenly in patients experiencing high-level reactivations, and these dynamic kinetics make prediction of subsequent viral reactivation or disease development based on the plasma HHV-6 or EBV DNA load difficult. Although HHV-6 encephalitis or EBV-associated lymphoproliferative disorder in ATL patients has been reported, these complications are very rare. Therefore practical routine monitoring for HHV-6 and EBV to prevent disease from HHV-6 or EBV is not justified in patients with ATL.

In conclusion, this study showed that CMV, HHV-6, and EBV reactivation was common in ATL patients who were receiving cytotoxic chemotherapy. Routine monitoring of CMV reactivation may be useful for the early detection of CMV-related diseases. Clinical trials of plasma-CMV-DNA-guided, preemptive approaches against CMV-related diseases are warranted. In contrast, the practical usefulness of monitoring for HHV-6 or EBV was not evident based on the results of this study.

REFERENCES

Boeckh M, Ljungman P. 2009. How we treat cytomegalovirus in hematopoietic cell transplant recipients. Blood 113:5711–5719.

Boeckh M, Gooley TA, Myerson D, Cunningham T, Schoch G, Bowden RA. 1996. Cytomegalovirus pp65 antigenemia-guided early treatment with ganciclovir versus ganciclovir at engraftment after allogeneic marrow transplantation: A randomized double-blind study. Blood 88:4063–5719.

Chen S, Ishii N, Ine S, Ikeda S, Fujimura T, Ndhlovu LC, Soroosh P, Tada K, Harigae H, Kameoka J, Kasai N, Sakaki T, Sugamura K. 2006. Regulatory T cell-like activity of Foxp3+ adult T cell leukemia cells. Int Immunol 18:269–277.

Fujiwara H, Matsumoto T, Eizuru Y, Matsushita K, Ohtsubo H, Kukita T, Imaizumi R, Matumoto M, Hidaka S, Arima N, Tei C. 2000. Cytomegalovirus infection is not necessarily a poor prognostic factor in adult T-cell leukemia/lymphoma. J Med Virol 62:140–143.

Fujiwara H, Eizuru Y, Matsumoto T, Kukita T, Imaizumi R, Kawada H, Ohtsubo H, Matsushita K, Arima N, Tei C. 2001. The significance of cytomegalovirus infection over the clinical course of adult T-cell leukemia/lymphoma. Microbiol Immunol 45:97–100.

Han XY. 2007. Epidemiologic analysis of reactivated cytomegalovirus antigenemia in patients with cancer. J Clin Micrbiol 45:1126–1132.

Idutsu K, Abe Y, Otonari J, Tachikawa Y, Ohtsuka R, Choi I, Muta K, Takayanagi R. 2007. Human herpesvirus 6 encephalitis in a patient with adult T-cell leukemia/lymphoma. Rinsho Ketsueki 48:664–666 [article in Japanese].

Ikewaki J, Ohtsuka E, Satou T, Kawano R, Ogata M, Kikuchi H, Nasu M. 2005. Real-time PCR assays based on distinct genomic regions for cytomegalovirus reactivation following hematopoietic stem cell transplantation. Bone Marrow Transplant 35:403–410.

Kimura H, Morita M, Yabuta Y, Kuzushima K, Kato K, Kojima S, Matsuyama T, Morishima T. 1999. Quantitative analysis of Epstein–Barr virus by using a real-time PCR assay. J Clin Microbiol 37:132–136.

Ljungman P. 2010. Molecular monitoring of viral infections after hematopoietic stem cell transplantation. Int J Hematol 91:596–601.

Locatelli G, Santoro F, Veglia F, Gobbi A, Lusso P, Malnati MS. 2000. Real-time quantitative PCR for human herpesvirus 6 DNA. Clin Microbiol 38:4042–4048.

Meerbach A, Wutzler P, Hafer R, Zintl F, Gruhn B. 2008. Monitoring of Epstein–Barr virus load after hematopoietic stem cell transplantation for early intervention in post-transplant lymphoproliferative disease. J Med Virol 80:441–454.

Nguyen Q, Estey E, Raad I, Rolston K, Kantarjian H, Jacobson K, Konoplev S, Ghosh S, Luna M, Tarrand J, Whimbey E. 2001. Cytomegalovirus pneumonia in adults with leukemia: An emerging problem. Clin Infect Dis 21:539–545.

Ogata M, Kikuchi H, Satou T, Kawano R, Ikewaki J, Kohno K, Kashima K, Ohtsuka E, Kadota J. 2006. Human herpesvirus 6 DNA load in plasma after allogeneic stem cell transplantation: Incidence and clinical significance. J Infect Dis 193:68–79.

Ogata M, Satou T, Kawano R, Goto K, Ikewaki J, Kohno K, Ando T, Miyazaki Y, Ohtsuka E, Saburi Y, Saikawa T, Kadota J. 2008. Plasma HHV-6 viral load-guided preemptive therapy against HHV-6 encephalopathy after allogeneic stem cell transplantation: A prospective evaluation. Bone Marrow Transplant 41:279–285.

Ogata M, Satou T, Kawano R, Takakura S, Goto K, Ikewaki J, Kohno K, Ikebe T, Ando T, Miyazaki Y, Ohtsuka E, Saburi Y, Saikawa T, Kadota J. 2010. Correlations of HHV-6 viral load and plasma IL-6 concentration with HHV-6 encephalitis in allogeneic stem cell transplant recipients. Bone Marrow Transplant 45:129–136.

Ohshima K, Suzumiya J, Kato A, Tashiro K, Kikuchi M. 1997. Clonal HTLV-1-infected CD4+ T-lymphocytes and non-clonal non-HTLV-1-infected giant cells in incipient ATLL with Hodgkin-like histologic features. Int J Cancer 72:592–598.

Omar H, Hagglund H, Gustafsson-Jernberg A, LeBlanc K, Mattsson J, Remberger M, Ringden O, Sparelid E, Sudnin M, Winiarski J, Yun Z, Ljungman P. 2009. Target monitoring of patients at high risk of post-transplant lymphoproliferative disease by quantitative Epstein–Barr virus polymerase chain reaction. Transpl Infect Dis 11:393–399.

Scheinberg P, Fischer SH, Li L, Nunez O, Wu CO, Sloand EM, Cohen JI, Young NS, John Barrett A. 2007. Distinct EBV and CMV reactivation patterns following antibody-based immunosuppressive regimens in patients with severe aplastic anemia. Blood 109:3219–3224.

Shapiro R, Nalesnik M, McCauley J, Fedorek S, Jordan ML, Scantlebury VP, Jain A, Vivas C, Ellis D, Lombardozzi-Lane S, Randhawa P, Johnston J, Hakala TR, Simmons RL, Fung JJ, Starzl TE. 1999. Posttransplant lymphoproliferative disorders in adult and pediatric renal transplant patients receiving tacrolimus-based immunosuppression. Transplantation 68:1851–1854.

Shimoyama M. 1991. Diagnostic criteria and classification of clinical subtypes of adult T-cell leukaemia-lymphoma. A report from the Lymphoma Study Group (1984–87). Br J Haematol 79:428–437.

Suzumiya J, Marutsuka K, Nabeshima K, Nawa Y, Koono M, Tamura K, Kimura N, Hisano S, Tachibana N, Inoue S, Sumiyoshi A. 1993. Autopsy findings in 47 cases of adult T-cell leukemia/lymphoma in Miyazaki prefecture, Japan. Leuk Lymphoma 11:281–286.

Takeda N, Isonuma H, Sekiya S, Ebe T, Matsumoto T, Watanabe K. 2001. Studies of anti-cytomegalovirus IgG antibody positive rate and cytomegalovirus mononucleosis in adults. Kansenshogaku Zasshi 75:775–779 [article in Japanese].

Tanaka Y, Kondo T, Kishimoto W, Kanda J, Tashima M, Mizumoto C, Icinohe T, Ishikawa T, Uchiyama T, Haga H, Manabe T. 2008. Epstein–Barr virus-associated B cell lymphoproliferative disorder complicated in adult T cell leukemia. Rinsho Ketsueki 49:263–269 [article in Japanese].

Tobinai K, Ohtsu T, Hayashi M, Kinoshita T, Matsuno Y, Mukai K, Shimoyama M. 1991. Epstein–Barr virus (EBV) genome carrying monoclonal B-cell lymphoma in a patient with adult T cell leukemia-lymphoma. Leuk Res 15:837–846.

Tsukasaki K, Utsunomiya A, Fukuda H, Shibata T, Fukushima T, Takatsuka Y, Ikeda S, Masuda M, Nagoshi H, Ueda R, Tamura K, Sano M, Momita S, Yamaguchi K, Kawano F, Hanada S, Tobinai K, Shimoyama M, Hotta T, Tomonaga M. 2007. VCAP-AMP-VECP compared with biweekly CHOP for adult T-cell leukemia-lymphoma: Japan Clinical Oncology Group Study JCOG9801. J Clin Oncol 25:5458–5464.

Uchiyama T. 1997. Human T cell leukemia virus type 1 (HTLV-1) and human diseases. Annu Rev Immunol 15:15–37.

van Brussel M, Salemi M, Liu HF, Goubau P, Desmyter J, Vandamme AM. 1999. The discovery of two new divergent STLVs has

implications for the evolution and epidemiology of HTLVs. Rev Med Virol 9:155–170.

Yasunaga J, Sakai T, Nosaka K, Etoh Ki, Tamiya S, Koga S, Mita S, Uchino M, Mitsuya H, Matsuoka M. 2001. Impaired production of naive T lymphocytes in human T-cell leukemia virus type I-infected individuals: Its implications in the immunodeficient state. Blood 97:3177–3183.

Yoshikawa T, Asano Y, Ihira M, Suzuki K, Ohashi M, Suga S, Kudo K, Horibe K, Kojima S, Kato K, Matsuyama T, Nishiyama Y. 2002. Human herpesvirus 6 viremia in bone marrow transplant recipients: Clinical features and risk factors. J Infect Dis 185:847–853.

Zerr DM. 2006a. Human herpesvirus 6 and central nervous system disease in hematopoietic cell transplantation. J Clin Virol 37:52–56.

Zerr DM. 2006b. Human herpesvirus 6: A clinical update. Herpes 13:20–24.

Zerr DM, Meier AS, Selke SS, Frenkel LM, Huang ML, Wald A, Rhoads MP, Nguy L, Bornemann R, Morrow RA, Corey L. 2005a. A population-based study of primary human herpesvirus 6 infection. N Engl J Med 352:768–776.

Zerr DM, Corey L, Kim HW, Huang ML, Nguy L, Boeckh M. 2005b. Clinical outcomes of human herpesvirus 6 reactivation after hematopoietic stem cell transplantation. Clin Infect Dis 40:932–940.

Int J Hematol (2012) 95:399–408
DOI 10.1007/s12185-012-1020-2

ORIGINAL ARTICLE

Heterogeneity in clonal nature in the smoldering subtype of adult T-cell leukemia: continuity from carrier status to smoldering ATL

Shimeru Kamihira · Masako Iwanaga · Yuko Doi · Daisuke Sasaki · Sayaka Mori · Kazuto Tsurda · Kazuhiro Nagai · Naoki Uno · Hiroo Hasegawa · Katsunori Yanagihara · Yoshitomo Morinaga · Kunihiro Tsukasaki · Hiroaki Taniguchi

Received: 1 November 2011 / Revised: 24 January 2012 / Accepted: 24 January 2012 / Published online: 28 March 2012

Abstract To better understand indeterminate HTLV-1 carriers and smoldering (SM) subtype of adult T-cell leukemia (ATL), HTLV-1 proviral integrated status, proviral load (PVL) and ATL-related biomarkers were examined in 57 smoldering cases, including unusual carriers with a percentage of ATL-like cells. We found that according to Southern blot hybridization analytic features, 28 patients with SM ATL could be divided into 3 groups consisting of 16 (57.4%) patients with a monoclonal band, 6 (21.4%) with oligoclonal bands and the remaining 6 with smears. Although no clinical differences were observed among the 3 SM subtypes, HTLV-1-infected CD4 T-cell counts increased in order of poly-, oligo- and monoclonal subtypes. This trend began in the carrier stage and also was observed in PVL, CD25 and CCR4, indicating that a clone consisting of leukemic phenotypic cells was continuously growing. Moreover, the antigen modulation rates of CD26 and CD7 and the increasing rate of CD25 and CCR4 cells were closely correlated to growing clonal size, indicating that these markers had the possibility to predict a monoclonal band. In particular, CD26 or the ratio of CD26/CD25 had a validity differential for leukemic nature and predictive detection of clonal band. Conclusively, the present study shows that smoldering ATL is heterogeneous in the leukemogenic process, and the behavior of CD26 plays a central role in the evolution from early occult to overt smoldering ATL.

Keywords ATL · HTLV-1 · Provirus · Southern blot · Leukemogenesis

Abbreviations

HTLV-1	Human T-cell leukemia virus type-1
ATL	Adult T-cell leukemia
SBH	Southern blotting hybridization
PMNC	Peripheral blood mononuclear cell
LDH	Lactate dehydrogenase
sIL-2R	Soluble-interleukin-2 receptor

S. Kamihira (✉) · Y. Doi · D. Sasaki · S. Mori · K. Tsurda · K. Nagai · N. Uno · H. Hasegawa · K. Yanagihara · Y. Morinaga
Central Diagnostic Laboratory of Nagasaki University Hospital, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan
e-mail: kamihira@nagasaki-u.ac.jp

S. Kamihira · K. Nagai · N. Uno · H. Hasegawa · K. Yanagihara · Y. Morinaga
Department of Laboratory Medicine, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan

M. Iwanaga · K. Tsukasaki · H. Taniguchi
Department of Hematology, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan

Introduction

Human T-cell leukemia virus type 1 (HTLV-1) was found to be a causative retrovirus of a T-cell malignancy, designated as adult T-cell leukemia (ATL) [1, 2]. All ATL cells, without exception, carry the HTLV-1 provirus in the same genomic site in each case, indicating that provirus insertion is an excellent biomarker for the cellular clonality of ATL and the integrated provirus can be used as the proviral load (PVL) [3] to estimate viral and cellular burden. Proof of clonality is essential for a diagnosis of malignant neoplasm, but it should be noted that HTLV-1-infected cells also are clonally expanded to maintain

persistent infection [2, 4]. Thus, changes in the abundance of HTLV-1-infected cell clones play an important role in persistent infection and ATL leukemogenesis. The clonality of provirus-carrying cells is usually demonstrated by Southern blot hybridization (SBH). However, since the detection sensitivity of this assay is limited (about 5%) [5, 6], it is unavailable for samples including small clones with 5% or fewer monoclonal cell populations.

Recently, we have had many opportunities to see patients with smoldering ATL and unusual carriers with high HTLV-1 PVL or with a proportion of ATL-like cells. We sometimes struggle to distinguish such borderline cases between carriers and smoldering ATL. ATL cells phenotypically resemble Treg cells expressing CD4, CCR4 and CD25. On the other hand, ATL cells aberrantly express 100 or more cell surface receptors and ligands [7, 8]. Such aberrantly expressed receptors consist mainly of natural, adaptive and ectopic types, some of which are considered to be involved in leukemogenesis [9]. In particular, down-regulation of CD3, CD7 and CD26 are observed during the early phase of leukemogenesis [9]. However, little is known about the behavior of cells concurrently expressing CD4, CD25, CCR4 and CD26 in the carrier to SM stages using in vivo practical samples. Accordingly, to better understand indeterminate carriers and smoldering ATL, the present study was focused on the implication between the SBH features reflecting clone size and cellular changes in phenotype and number. In particular, CD26 is noted to be one of the prodromal cellular changes, because the down-regulation of CD26 begins in the carrier stage and persists continuously till the completion of ATL.

Materials and methods

White blood cell counts, morphological data, serum lactate dehydrogenase (LDH) activity and soluble interleukin-2 receptor (sIL-2R) were used from routine laboratory data. Peripheral blood samples were collected from our ATL and HTLV-1 carrier clinic, consisting of 28 patients with smoldering ATL carrying 5% or more ATL-like cells in blood, 12 unusual carriers with around 5% ATL-like cells, and 17 common (healthy) carriers. Thirty-four samples from patients with leukemic chronic or acute ATLs were used as a positive control. Morphological evaluation was microscopically conducted by hematological specialists.

High-molecular-weight DNA was extracted from peripheral blood mononuclear cells (PMNC) using a QIAmp DNA Blood Mini Kit (Qiagen GmbH, Hilden Germany). PVL was quantified by LightCycler Technology (Roche Diagnostic K.K., Tokyo, Japan) using hydro-probes and previously described primers [10–12]. Normalization was done using the β-globin gene and the PVL was expressed as copy number per 10^4 cells or percent for PMNC. This study was done under the approval of our institutional board.

Clone assay of SBH

SBH analysis was performed by a method described previously with modification, using 7 mixtures of probes covering the total region of the digoxigenined provirus and the restriction enzymes of EcoR-1 and Pst-1 [13, 14]. Pst-1 cuts 4 sites of the provirus, but Eco-R1 cannot cut within the provirus. Therefore, to determine clonally related sharp band or polyclonally related smear bands, Eco-R1 digestive genomic fragments were used. To assay clonality accurately, we monitored 1.5, 3 and 5% clonal cell controls every time. Band patterns were estimated using a densitometer (Fujifilm Life Science, Science Lab 2005, and Tokyo, Japan).

Flow cytometry analysis for cell surface antigens

The positive rate for CD3, CD4, CD7, CD25, CD26 and CCR4 was measured by a routine method using whole blood according to the manufacturer's instructions (BD FACSCato-II, Nixon BD. Inc, Tokyo, Japan). The association between CD7 and CD26 antigen modulation and the positivity of CD4, CD25 and CCR4 cells was assessed by using the four-color staining method according to the manufacturer's instructions. Co-expression with CD4, CD25, CCR4 and CD26 was assessed by a four-color flow cytometric method using CD26-FITC, CD4-PerCP, CD25-APC and CCR4-PE. The rate (%) of $CD4^{(+)}CD25^{(+)}CCR4^{(+)}$ cells relative to all CD4 cells and the co-expression rate of CD26 with $CD4^{(+)}CD25^{(+)}CCR4^{(+)}$ cells was measured according to the BD FACSCato-II protocol (BD, Inc., Tokyo, Japan).

Statistical analysis

Data are expressed mainly as the median and analyzed using the Mann–Whitney test. P value of below 0.05 was considered to be statistically significant. Analyses were performed with Stat Flex version 6.5 software packages (Artech Inc., Osaka, Japan).

Results

Classification features of band patterns using a densitometer

The band patterns in SBH analysis using Eco-R1-digestive genomic fragments were mainly estimated by a densitometer graph. The densitometer graphs equivalent to band

status are classified into five patterns (Fig. 1a): patterns-1 and -2 are light and dense smear bands with no difference in staining density; pattern-3 is a hill ridge, irregular shape with vertical stripes or a low and broad projection with smear bands; pattern-4 is a low/sharp peak type; and pattern-5 is a sharp high peak. In vivo clonal status of the five patterns based on SBH features, as shown in Fig. 1b, is considered to correspond to few small clones in pattern (P)-1, many small clones with few growing clones in P-2, oligoclones of a detection limit in P-3, and clearly detectable monoclone(s) with various background clonal status in P-4 and 5.

SBH analysis allocated 57 cases to 17 of P-1, 11 of P-2, 12 of P-3, 6 of P-4 and 11 of P-5. The relations between intra- or inter-disease status and the band patterns are summarized in Table 1. Consequently, smoldering ATL was the most heterogeneous for SBH patterns; 16 (57.1%) of 28 smoldering ATLs were P-4 and -5 (large clone consisting of at least 5% monoclonal cells), while the remaining 12 (42.9%) were P-1, -2 and -3 (equivalent to polyclonal or oligoclonal band). Actually, Fig. 2 is an interesting example of an SM subtype showing dense smear bands and abnormal cells with an aberrant phenotype of 73% CD4, 77% CD25 and 21% CD26.

Cyto-oncological characteristics of the three SM subtypes

To characterize ATL-related biomarkers in the three subtypes of SM, the findings were comprehensively compared with those of healthy carriers, unusual carriers and patients with chronic ATL (Table 2). First, clonal expansion-associated biomarkers, such as PVL, HTLV-1-infected CD4 T-cell counts in 1 μL peripheral blood and the serum level of sIL-2R increased regularly in the order of poly-, oligo- and monoclonal SM subtypes. Figure 3 shows the line graphs on increasing fold (rate) of PVL and total lymphocyte, all CD4 T-cell and HTLV-1-infected CD4 T-cell counts converted from Table 2. The graph shows two distinctive patterns (solid lines of PVL and infected CD4 T-cell vs. broken lines of total lymphocyte and all CD4 T-cell). In contrast to the horizontal part of the broken lines, the solid lines are gradually elevated, meaning that the provirus-carrying CD4 T-cells gradually increase

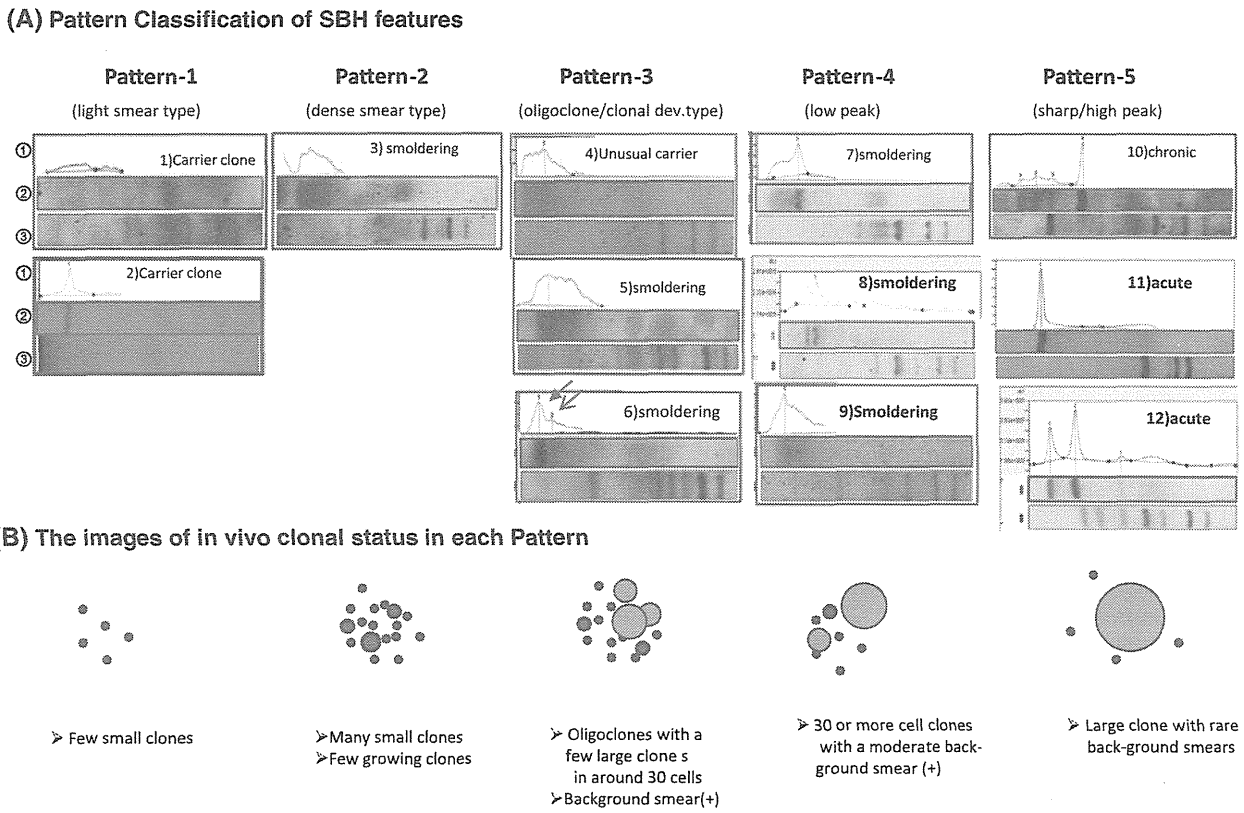

Fig. 1 a The densitometry patterns for HTLV-1 proviral integration status according to SBH band features using a restrictive enzyme of Eco-R1 and representative cases. Subjects were mainly classified into five patterns according to densitometer images. ① Densitometry graph. ② SBH analysis for Eco-R1-digestive genomic fragments. ③ SBH analysis for Pst-1-digestive genomic fragments. b The image of in vivo clonal status in each pattern

Table 1 The pattern distribution of SBH features in intra- and inter-diseases

	Smear band		Oligoclonal bands	Monoclonal bands		Total
	P-1	P-2	P-3	P-4	P-5	
Common carriers	11	5	0	0	(1)[a]	17
Unusual carriers	5	1	6	0	0	12
Smoldering ATL	1	5	6	6	10	28

The criteria of the classification is explained in the text. P-1, P-2, P-3, and P-4 and P-5 generally correspond to light smears, dense smears, oligoclonal bands and monoclonal bands, respectively. Of leukemic type ATL, including the chronic and acute type, SBH features in smoldering ATL were the most heterogeneous

Common carriers HTLV-1-seropositive individuals without any HTLV-1-associated disorders

Unusual carriers those who have clinico-cytological findings similar to that of the smoldering subtype of ATL

[a] Carrier clone

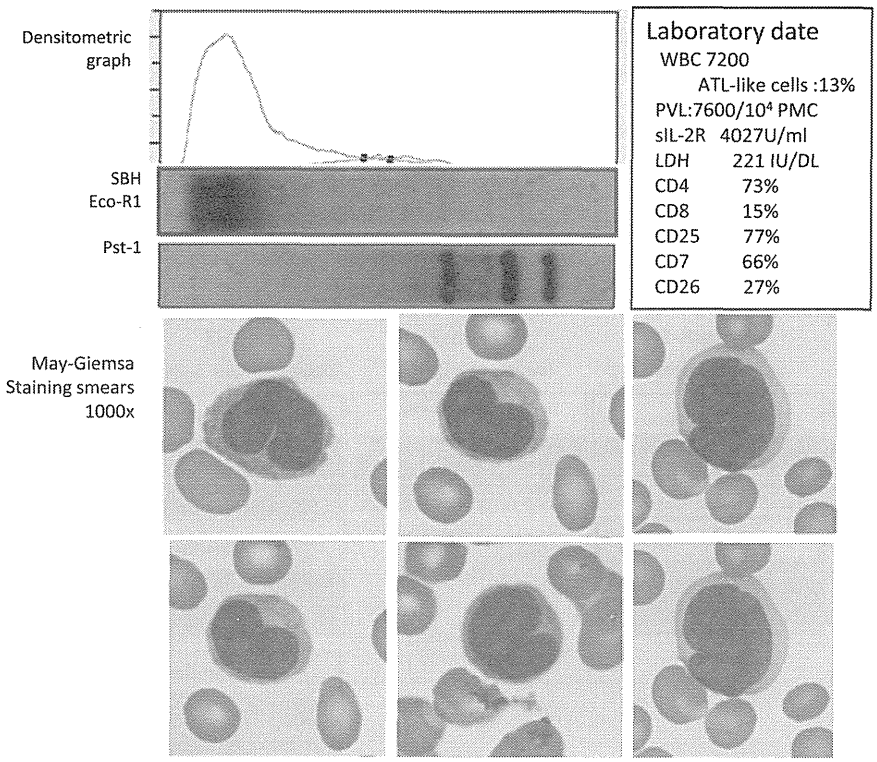

Fig. 2 A representative case presentation of the polyclonal smoldering subtype (SM) showing a polyclonal dense smear in SBH analysis and the smoldering subtype with full hematological criteria

regardless of the almost stable counts of non-infected CD4 T-cells during the entire period of smoldering ATL.

The positive values of CD4, CD25, CCR4, CD7 and CD26 subsets (%) were observed to change continuously and concurrently in the order of common carriers, unusual carriers, polyclonal SM, oligoclonal SM and monoclonal SM. In order to interpret these data in detail, a line graph was used (Fig. 4). CCR4 and CD25 cells increased concurrently and sharply from the common carrier stage to the oligoclonal stage. The down-regulation of CD26 was initiated in the unusual carrier stage and kept falling continuously by chronic stage. The fluctuations of CD4 and CD26 showed an opposing trend, and the interval between CD4 and CD26 (solid triangle and gray circle) gradually enlarged with the increasing cell number of 32, 54, 115 and 163 cells. Such behavior of CD26 was expected to play a central role in budding of ATL in the early stage of multistep leukemogenesis.

Clinico-oncological usefulness of CD26

From the results described above, CD26 appears to be closely associated with the evolution of SM. In contrast to characteristic phenotypes in overt ATL cells, those of

Table 2 Comparison of the measurement value (mean) of ATL-related biomarkers among the polyclonal, oligoclonal and monoclonal SM subtypes

	Carrier stage		Smoldering stage			Chronic
	Common	Unusual	Polyclonal	Oligoclonal	Monoclonal	
PVL (%)	5.9	17.9	22.6*	28.0	39.3*	78.3
Total Ly counts	1750	1950	2308	1732	2306	7659
All CD4 T-cell	786	882	1570	1513	1377	7220
Infected CD4	102	330	480*	628	744*	8346
LDH (IU/mL)	199	200	222	186	179	257
sIL-2R (U/mL)	868	765	1425	1877	1887	6106
CD4 (%)	43	48	52	55	56	79
CD25 (%)	14	22	32	45	44	75
CCR4 (%)	14	25	38*	50	58**	76
CD7 (%)	63	65	70	47	50	11
CD26 (%)	43	41	41*	28	22**	7

Statistically significant ($P < 0.05$) between * and ** in PVL, infected CD4 cell number, CCR4 (%) and CD26 (%)

HTLV-1-infected CD4 T-cell number/1 μL p-blood = total Ly counts × CD4%/100 × PVL (%/100)

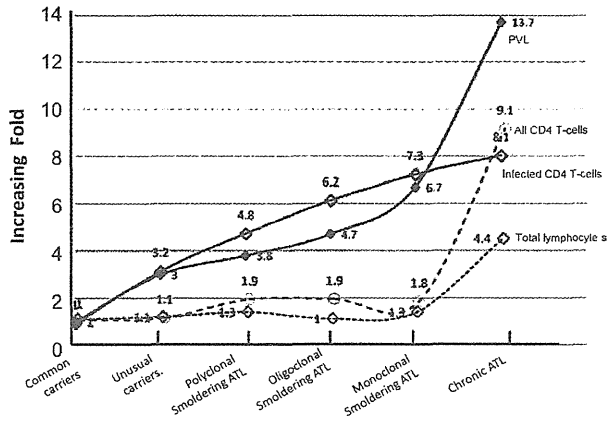

Fig. 3 The difference in the line-graph patterns between HTLV-1-infected and uninfected cells. PVL and infected CD4 T-cell counts gradually increased from the early carrier stage to the last stage of smoldering, while the HTLV-1-uninfected cell population was stable, indicating that the discrepancy was explained by the infected leukemic clonal expansion alone

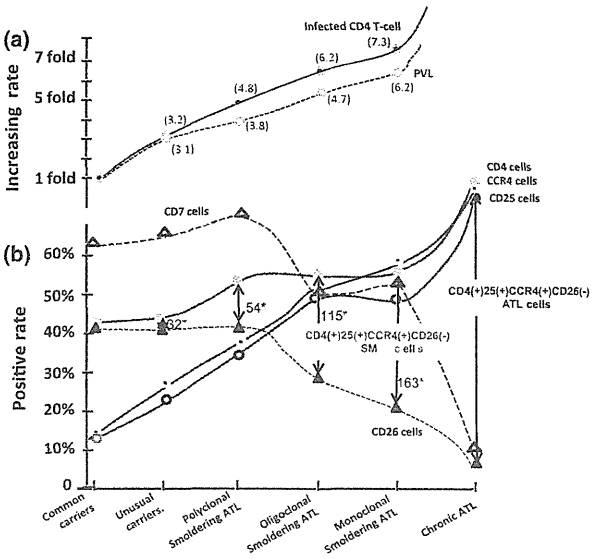

Fig. 4 The rate of change in each ATL-related biomarker. There were two major types of curves; a concurrent increasing type with tumor burden and a decreasing type with aberrant down-regulation. **a** The increasing rate (fold) relative to 102 HTLV-1-infected CD4 T-cells in 1 μL of blood. **b** Comparison of the positive rates in each disease state equivalent to P-1 (common carriers), P-2 (dense smears), P-3 (oligoclonal), and P-4 and P-5 corresponding to the monoclonal phase. *Asterisks* represent predicted $CD4^{(+)}CD26^{(-)}$ cell number equivalent to a major clonal expansion representing the absolute increased tumor burden

occult ATL (SM) cells are now controversial. One of the reasons for this is thought to be the difficulty in identifying SM cells. Therefore, to overcome these problematic issues, a dot-plot graph for CD26 versus CD25 and a four-color staining method were applied. The dots of CD26 and CD25 were mainly clustered into two areas (Fig. 5): 11 of monoclonal SM were clustered into a solid line circle, while oligoclonal and polyclonal SM were widely distributed. On the other hand, carriers were compactly clustered within the broken line circle. This indicates that also the 3 SM subtypes are not always homogeneous in biological character. Since the CD25 versus CD26 dot graph only hinted at the heterogeneity of SM, we examined the clinico-oncological role of CD26 using 3 parameters of HTLV-1-infected cell counts, the CD26/CD25 ratio and SBH features. As shown in Fig. 6, the number of HTLV-1-infected CD4 T-cells was closely correlated to the ratio of

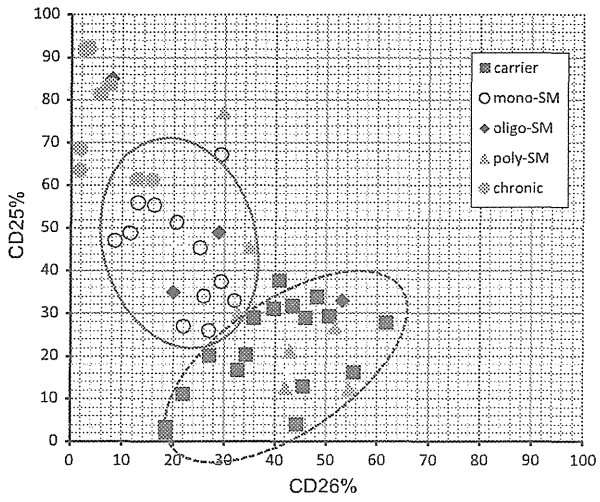

Fig. 5 CD26 versus CD25 twin dot graph, showing that there were two hot areas. The cells clustering in the same area have uniform biocharacteristics. Monoclonal SM was concentrated in the same area, but polyclonal SM and carriers were distributed sparsely and widely. *Red squares within dotted line circle* 3SM subtypes were scattered into both circles

CD26/CD25 ($R^2 = 0.6586$), and the clustering patterns were characteristic.

Samples with monoclonal band were mainly clustered in a high area within 0 to <1 of the *X*-axis. Most other samples were widely distributed in a area of around 1.00–11.00 of the *Y*-axis. Thus, the CD26/CD25 ratio represents the degree of advance in the leukemic process, comparable to the growing level of an ATL cell clone. Actually, this was demonstrated to be an indicator of a monoclonal band using a distribution graph and a receiver operating curve (ROC). That is, as shown in Fig. 7a, the ratios of CD26/CD25 were clearly separated by the presence or absence of a monoclonal band. The ROC analysis gave a high area under the curve (AUC) of 0.90, and sensitivity, specificity, and positive and negative predictive values were 87.0, 83.0, 80.0 and 89.0%, respectively (Fig. 7b), when the ratio of cutoff value (COV) was 1.04. The detective test performances of CD26 and CD7 alone for a monoclonal band were 0.82 and 0.81 AUC, respectively. This simple predictive method as an alternative to the SBH test, which is time- and labor-consuming, may be practically useful.

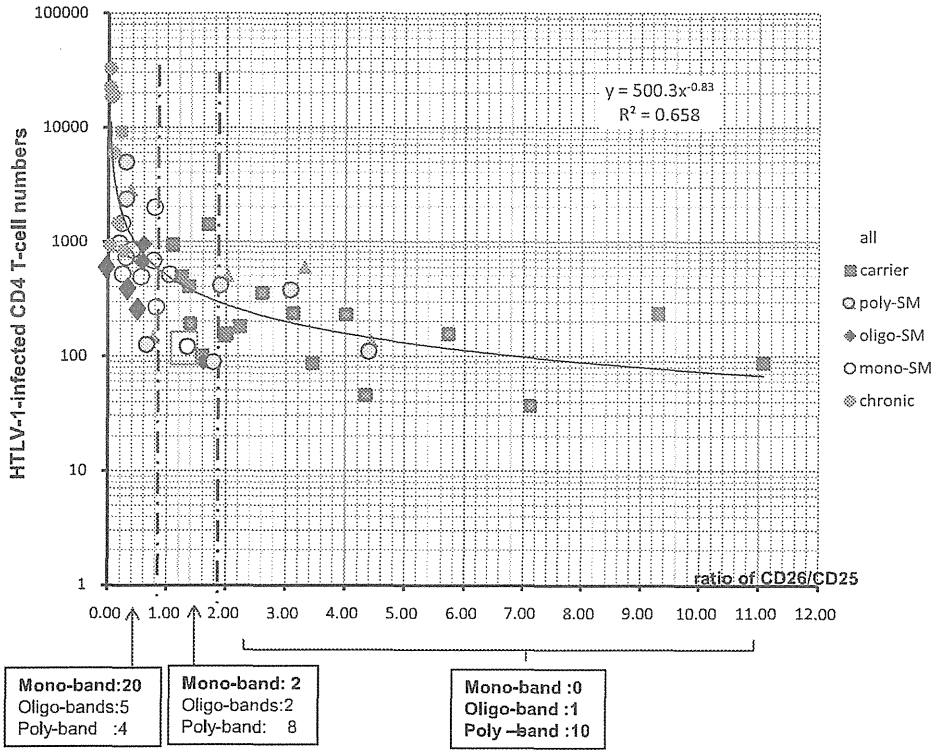

Fig. 6 Three parameter "twin dot-plot graph" between HTLV-1-infected CD4 T-cell numbers and the ratio of CD25/CD26, and the carrier or disease subtypes. Samples with the same band pattern showed a tendency to gather in the same areas bordered by the CD25/26 ratio lines, such as most samples with monoclonal band (momo-band) in an area within 0.00–1.00 of the *X*-axis, and most samples with smears (poly-band) in an area within 1.00–11.00 of the *X*-axis

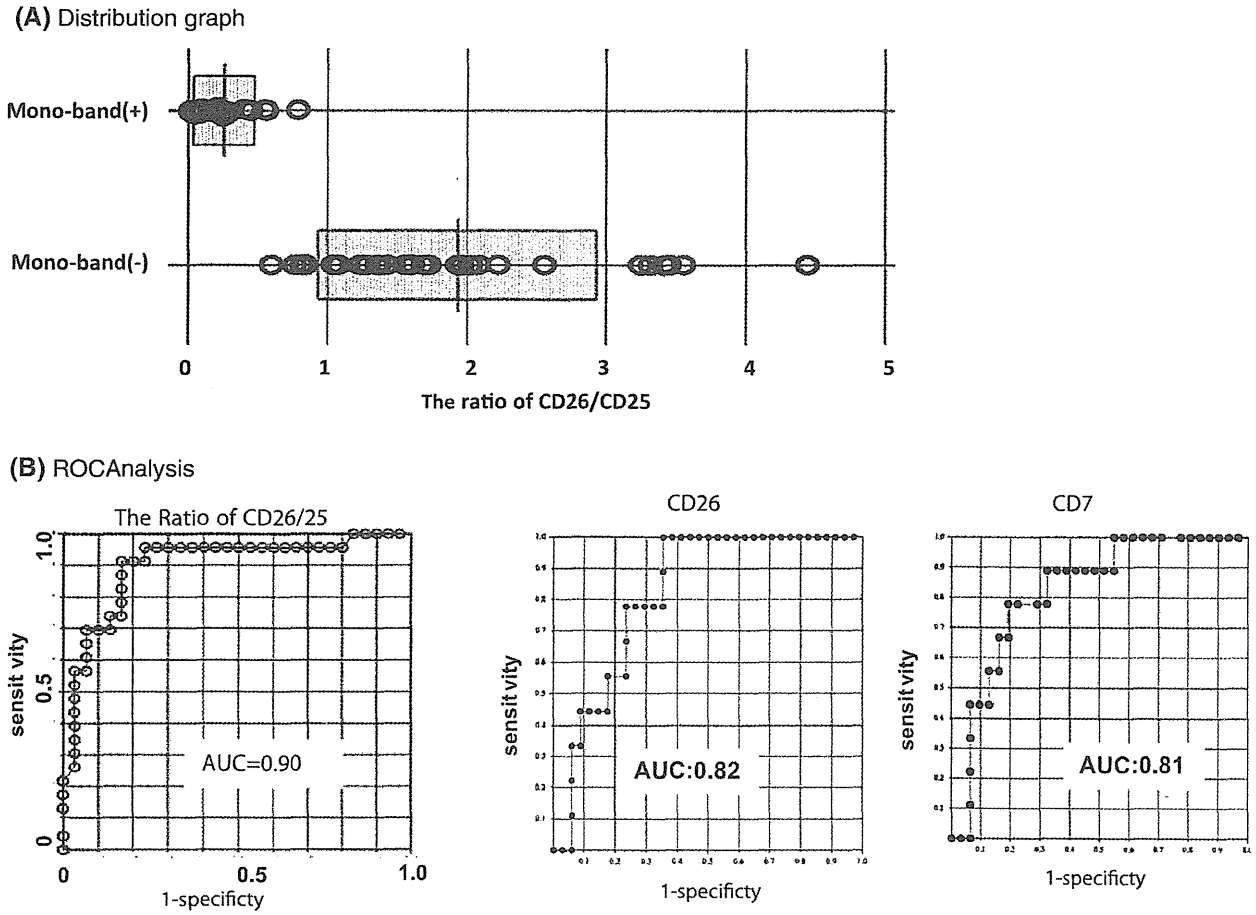

Fig. 7 **a** The distribution graph of each sample with or without a monoclonal band. The median values of mono-band (+) or (−) were 0.32 and 1.98. **b** The receiver operating curve (ROC) analysis

Correlation between down-regulation of CD26 antigens and SBH features

It is known that CD7 and CD26 antigens are lost in chronic and acute ATL cells. The present study revealed that the loss of CD26 antigens was initiated early in the pattern-2 or -3 stages. To confirm whether $CD4^{(+)}$ $CD25^{(+)}$ $CCR4^{(+)}$ cells were concurrently expressed, a four-color staining flow cytometric method for CD4, CD25, CCR4 and CD26 was used (Fig. 8). $CD4^{(+)}$ $CD25^{(+)}$ $CCR4^{(+)}$ cells (P1 square) were 1% or less, of which 75% (0.3% of total CD4 cells) were CD26 negative and 25% were CD26 positive in a healthy individual seronegative for HTLV-1 (case 1). That is, the $CD25^{(-)}/CD26^{(+)}$ ratio was 3.0. On the other hand, the ratio in common carriers and SM subtypes was about 3.0–10.0 (cases 2–4) and 10 or more (cases 5–9), respectively. This phenomenon regarding the loss of CD26 antigens was observed in other ATL cells [20].

Discussion

More than 35 years have passed since ATL was found and HTLV-1 was identified as its causative virus several years later. After that, a better molecular understanding of ATL pathology has been advancing. However, at the forefront of clinical practice, many problematic issues, such as a correct diagnosis of smoldering ATL, discrimination from unusual carriers with a percentage of ATL-like cells and promising therapeutic strategies, remain unclear. Recently, understanding of ATL pathology has deepened, but there is no point of contact between clinical and molecular aspects.

The results of the present study revealed that SM was heterogeneous in clonally related SBH features (mainly clone size) and lymphocyte subset profiles. We here designated such cases as monoclonal, oligoclonal and polyclonal smoldering (SM) subtypes. Although there was no difference in clinical manifestations, increase of only

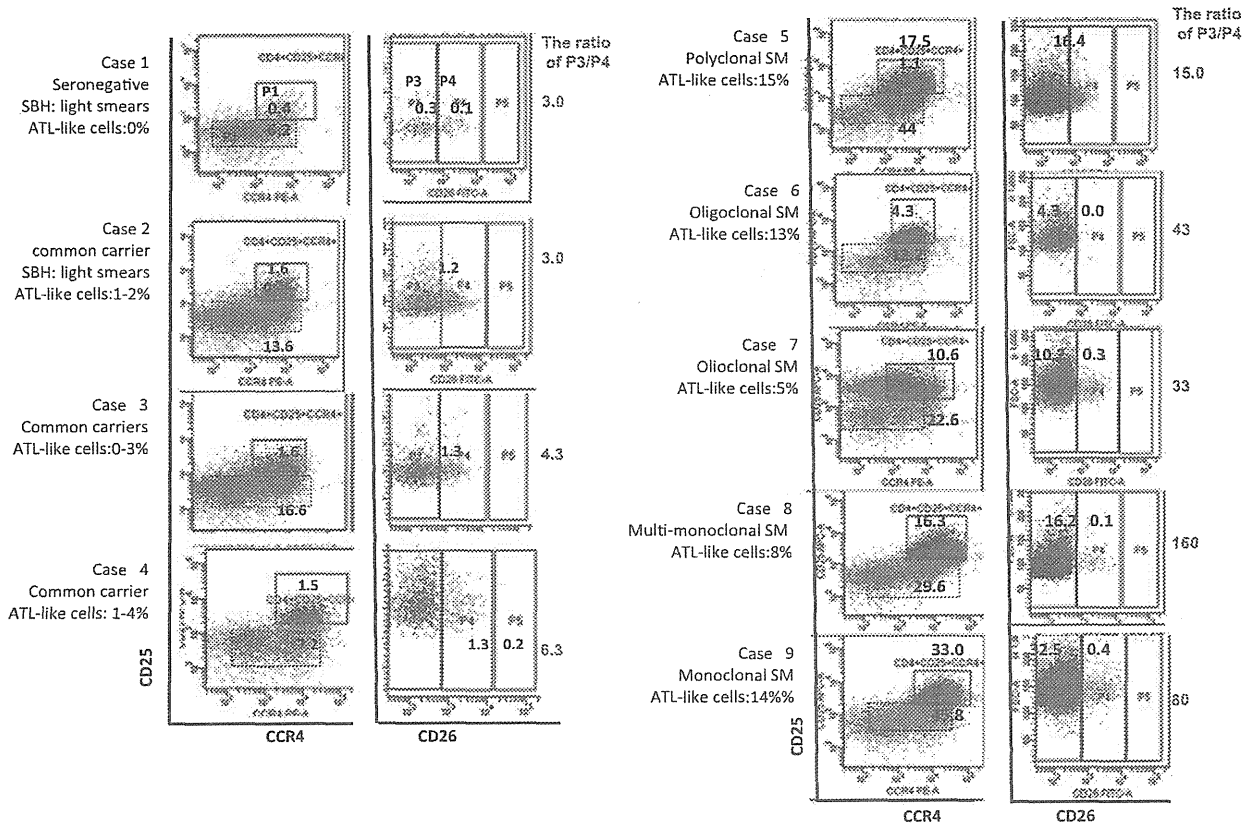

Fig. 8 Four-color flow cytometry for CD4, CD25, CCR4 and CD26. After CD4 gating, gating-CD4 T-cells were developed into a cytograph (a CCR4 = *X*-axis, and CD25 = *Y*-axis), and then P1 gating cells were developed into a cytograph (b CD26 down-regulation positive (*blue* P3 area meaning loss of CD26 antigen) or negative (*red* P4 area)

HTLV-1 provirus-carrying cells with a phenotype of $CD4^+CD25^+CCR4^+CD26^-$ was characteristic, regardless of the stable lymphocyte counts. Moreover, the ratio of CD26/CD25 was defined to be useful as an indicator of the grade of advance into ATL. Such findings were observed partially in unusual carriers with oligoclonal bands. These suggest that the expansion of leukemic clone begins in the unusual carrier stage and reaches large clone detected by SBH analysis in the SM stage. Thus, continuous changes of all ATL-related biomarkers would be explained by growing leukemic clonal cell population [15]. This is easily understood by a diagram shown in Figs. 3 and 4, which was derived from the increase of absolute CD4 T-cells infected by HTLV-1. The SM period seems to oncologically mean one of the turning points for multi-step leukemogenesis of ATL.

Now, it is interesting to develop such a subtype manifestations. Although clinical over-diagnosis cannot be completely neglected, there are in fact such cases with a highly dense smear for Eco-R1 genomic fragments and internal bands for Pst-1 genomic fragments, like the case in Fig. 2. As a possibility, a cluster of small clones may work co-operatively to develop SM manifestations. Subsequently, this appears to give rise to frequently multiclonal ATL and genomic diversity of leukemic clones [16].

Another interest is the behavior of CCR4, CD7 and CD26. So far, little is known about CD26 associated with ATL pathology. CD26/dipeptide peptidase IV (DPPIV), which is an antigenic enzyme expressed on the surface of most cell types, suppresses the development of cancer and tumors. CD26 plays an important role in tumor biology, and is useful as a marker for various cancers [17–19]. Now, why would down-regulation of CD26 first occur? The down-regulation preceding the increase in HTLV-1-infected CD4 T-cells may be indispensable to an environment for growing immature ATL cells. On the other hand, down-regulation of CD7, a glycoprotein member of the immunoglobulin (Ig) superfamily, is also one of the most commonly seen antigenic aberrations in T-lymphoproliferative disorders, but there is no specificity for malignant cell types, including a variety of reactive conditions [20, 21]. The changes in expression of CCR4 and CD26 may be the results of transformation.

Furthermore, to reveal other roles of CD26, statistical methodology and a dot plot were used. Consequently, two twin dot-plot graphs of CD25 versus CD26, and HTLV-1-CD4 T-cell number versus the ratio of CD26/CD25 revealed that the ratio of CD26/CD25 is useful as a surrogate marker for the prediction of the provirus clonal status. When the COV of the ratio is 1.04, the diagnostic validity is 87.0% in sensitivity and 83% in specificity. However, the ratio of CD26/CD25 in polyclonal SM was widely distributed, indicating that the polyclonal SM was distinctive from the other two subtypes of oligo- and monoclonal SM. This simple predictive method, alternative to the SBH test which is time- and labor-consuming, may be practically useful for screening in rapid turn-around test or epidemiological mass test.

Finally, using four-color flow cytometry, the usefulness of the CD26 antigen monitor was verified in actual cases. The antigen status was evaluated as the ratio of $CD26^{(-)}$ versus $CD26^{(+)}$ within a fraction of $CD4^{(+)}CD25^{(+)}CCR4^{(+)}$ cells. The ratio went up with increases in the $CD4^{(+)}CD25^{(+)}CCR4^{(+)}$ cell populations, reflecting occult ATL cells or transforming cells. In the present study, the border line of the ratio between carriers and patients with SM was about 10.0. That is, if the $CD26^{(-)}$:$CD26^{(+)}$ ratio is 10 or more, the case is predicted to be smoldering ATL.

Taken together, the present study showed that smoldering ATL was heterogeneous in a clone size and the quality of its constituent cells. This suggests that it is relevant to classify the current smoldering ATL into two subtypes of SM with or without a monoclonal band. Indeterminate HTLV-1 carriers and smoldering ATL can be discriminated according to the patterns of SBH densitometer images and CD26 antigen status. Moreover, CD26 is expected to be used as a novel biomarker for prediction of clonal bands and discrimination of carriers or SM subtypes. CD26 may become one of the central molecules in understanding the early leukemogenic process.

Acknowledgments This work was supported by the Japanese Government, Kakenn B No. 21390182. This study was performed as a cooperative study, mainly organized by SK and with the other authors YD, SD, MO, KT, KN, NU, HH, KY, KT and HT.

Conflict of interest The authors have no conflict of interest.

References

1. Poiesz BJ, Ruscetti FW, Gazdar AF, Robbert Gallo C. Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of patients with cutaneous T-cell lymphoma. Proc Natl Acad Sci USA. 1980;77(12):7415–9.
2. Matsuoka M, Jeang KT. Human T-cell leukaemia virus type 1 (HTLV-1) infectivity and cellular transformation. Nat Rev Cancer. 2007;7(4):270–80.
3. Yoshida M. Discovery of HTLV-1, the first human retrovirus, its unique regulatory mechanisms, and insights into pathogenesis. Oncogene. 2005;24(39):5931–7.
4. Tanaka G, Okayama A, Watanabe T, Aizawa S, Stuver S, Mueller N, Hsieh CC, Tsubouchi H. The clonal expansion of human T lymphotropic virus type 1-infected T cells: a comparison between seroconverters and long-term carriers. J Infect Dis. 2005;91(7):1140–7.
5. Kamihira S, Sugahara K, Tsuruda K, Minami S, Uemura A, Akamatsu N, Nagai H, Murata K, Hasegawa H, Hirakata Y, Takasaki Y, Tsukasaki K, Yamada Y. Proviral status of HTLV-1 integrated into the host genomic DNA of adult T-cell leukemia cells. Clin Lab Haematol. 2005;27(4):235–41.
6. Sugahara K, Yamada Y. Southern blot hybridization analysis for lymphoid neoplasms. Rinsho Byori. 2000;48(8):702–7.
7. Kamihira S, Sohda H, Atogami S, Fukushima T, Toriya K, Miyazaki Y, Ikeda S, Yamada Y, Tomonaga M. Unusual morphological features of adult T-cell leukemia cells with aberrant immunophenotype. Leuk Lymphoma. 1993;12(1–2):123–30.
8. Maeda T, Yamada Y, Moriuchi R, Sugahara K, Tsuruda K, Joh T, Atogami S, Tsukasaki K, Tomonaga M, Kamihira S. Fas gene mutation in the progression of adult T cell leukemia. J Exp Med. 1999;189(7):1063–71.
9. Tsuji T, Sugahara K, Tsuruda K, Uemura A, Harasawa H, Hasegawa H, Hamaguchi Y, Tomonaga M, Yamada Y, Kamihira S. Clinical and oncologic implications in epigenetic down-regulation of CD26/dipeptidyl peptidase IV in adult T-cell leukemia cells. Int J Hematol. 2004;80(3):254–60.
10. Sasaki D, Doi Y, Hasegawa H, Yanagihara K, Tsukasaki K, Iwanaga M, Yamada Y, Watanabe T, Kamihira S. High human T cell leukemia virus type-1 (HTLV-1) provirus load in patients with HTLV-1 carriers complicated with HTLV-1-unrelated disorders. Virol J. 2010;7:81.
11. Kamihira S, Dateki N, Sugahara K, Yamada Y, Tomonaga M, Maeda T, Tahara M. Real-time polymerase chain reaction for quantification of HTLV-1 proviral load: application for analyzing aberrant integration of the proviral DNA in adult T-cell leukemia. Int J Hematol. 2000;72(1):79–84.
12. Kamihira S, Dateki N, Sugahara K, Hayashi T, Harasawa H, Minami S, Hirakata Y, Yamada Y. Significance of HTLV-1 proviral load quantification by real-time PCR as a surrogate marker for HTLV-1-infected cell count. Clin Lab Haematol. 2003;25(2):111–7.
13. Uemura A, Sugahara K, Nagai H, Murata K, Hasegawa H, Hirakata Y, Tsukasaki K, Yamada Y, Kamihira S. An ATL cell line with an IgH pseudo-rearranged band pattern by Southern blotting: a pitfall of genetic diagnosis. Lab Hematol. 2005;11(1):8–13.
14. Shimoyama M. Diagnostic criteria and classification of clinical subtypes of adult T-cell leukaemia-lymphoma. A report from the Lymphoma Study Group (1984–87). Br J Haematol. 1991;79(3):428–37.
15. Gillet NA, Malani N, Melamed A, Gormley N, Carter R, Bentley D, Berry C, Bushman FD, Taylor GP, Bangham CR. The host genomic environment of the provirus determines the abundance of HTLV-1-infected T-cell clones. Blood. 2011;117(11):3113–22.
16. Ariyama Y, Mori T, Shinomiya T, Sakabe T, Fukuda Y, Kanamaru A, Yamada Y, Isobe M, Seto M, Nakamura Y, Inazawa J. Chromosomal imbalances in adult T-cell leukemia revealed by comparative genomic hybridization: gains at 14q32 and 2p16-22 in cell lines. J Hum Genet. 1999;44(6):357–63.
17. Tian Y, Kobayashi S, Ohno N, Isobe M, Tsuda M, Zaike Y, Watanabe N, Tani K, Tojo A, Uchimaru K. Leukemic T cells are specifically enriched in a unique CD3 (dim) CD7 (low) subpopulation of CD4(+) T cells in acute-type adult T-cell leukemia. Cancer Sci. 2011;102(3):569–77.

18. Kelemen K, Guitart J, Kuzel TM, Goolsby CL, Peterson LC. The usefulness of CD26 in flow cytometric analysis of peripheral blood in Sézary syndrome. Am J Clin Pathol. 2008;129(1): 146–56.
19. Sato T, Yamochi T, Yamochi T, Aytac U, Ohnuma K, McKee KS, Morimoto C, Dang NH. CD26 regulates p38 mitogen-activated protein kinase-dependent phosphorylation of integrin beta1, adhesion to extracellular matrix, and tumorigenicity of T-anaplastic large cell lymphoma Karpas 299. Cancer Res. 2005; 65(15):6950–6.
20. Narducci MG, Scala E, Bresin A, Caprini E, Picchio MC, Remotti D, Ragone G, Nasorri F, Frontani M, Arcelli D, Volinia S, Lombardo GA, Baliva G, Napolitano M, Russo G. Skin homing of Sézary cells involves SDF-1-CXCR4 signaling and down-regulation of CD26/dipeptidylpeptidase IV. Blood. 2006;107(3):1108–15.
21. Abe M, Uchihshi K, Tsuruda K, Kamihira S. Foxp3 expression on normal and leukemic CD4+CD25+ T-cells implicated in HTLC-1 is inconsistent with Treg cells. Europ J Haematol. 2008;81:209–17.

Cancer Epidemiology 36 (2012) 560–565

Contents lists available at SciVerse ScienceDirect

Cancer Epidemiology

The International Journal of Cancer Epidemiology, Detection, and Prevention

journal homepage: www.cancerepidemiology.net

Impact of miR-155 and miR-126 as novel biomarkers on the assessment of disease progression and prognosis in adult T-cell leukemia

Kaori Ishihara [a,b,*], Daisuke Sasaki [a,b], Kazuto Tsuruda [a], Naoko Inokuchi [a], Kazuhiro Nagai [a,b], Hiroo Hasegawa [a,b], Katsunori Yanagihara [a,b], Shimeru Kamihira [a,b]

[a] Central Diagnostic Laboratory of Nagasaki University Hospital, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan
[b] Department of Laboratory Medicine, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan

ARTICLE INFO

Article history:
Received 12 March 2012
Received in revised form 19 June 2012
Accepted 5 July 2012
Available online 10 August 2012

Keywords:
miRNA
Biomarker
ATL
Microarray
Plasma RNA

ABSTRACT

Objective: Micro RNAs (miRNAs) provide new insight in the development of cancer, but little is known about their clinical relevance as biomarkers in the assessment of diagnosis, classification, progression and prognosis of various cancers. To explore a potential novel biomarker, we examined the cellular and plasma miRNA profiles in adult T-cell leukemia (ATL) characterized by diverse clinical features. *Methods and results*: Using CD4-positive cells isolated from 2 non-infected healthy individuals, 3 chronic ATL patients and 3 acute ATL patients, cellular miRNAs were profiled by microarray. The microarray screened 5 miRNAs namely miR-155, let-7g, miR-126, miR-130a and let-7b because of the large difference in their expression in diseased vs. that of healthy controls. The expression levels of before 5 miRNAs re-quantified by reverse transcription quantifiable polymerase chain reaction (RT-qPCR) were not always accordant in cells and plasma. The high and low plasma levels of miR-155 and miR-126 changed with ATL stage. *Conclusion*: The present study revealed that there is a quantitative discrepancy between cellular and plasma miRNAs. The elevation of plasma miR-155 and the reduction in miR-126 correlated with poor prognosis, indicating their usefulness as a novel biomarker for the assessment of disease stage.

1. Introduction

Adult T-cell leukemia (ATL) is a T-cell neoplasm originating from human T-cell leukemia virus type-1 (HTLV-1)-infected cells [1]. Although HTLV-1 is thought to be a causative agent for ATL [2], the oncogenic mechanism of the virus remains to be fully elucidated. ATL is sub-classified into four categories; smoldering, chronic, acute and lymphoma. ATL is characterized by diverse and complex clinical and pathological features, but there are few relevant biomarkers to evaluate disease progression. In particular, since the survival time of indolent (smoldering and chronic) ATL varies [3], novel biomarkers are likely identified or discovered. Recently, in an effort to understand the complex mechanism of ATL pathology, various types of RNAs that do not encode proteins have emerged, including the minus strand RNA of HTLV-1 (HBZ or HBZ-SI*) [4] and micro RNAs (miRNAs) [5,6].

miRNAs are small noncoding RNAs of 19–24 nucleotides in length that are important in the regulation of basic biological processes such as cell growth, apoptosis and differentiation. With regard to the relationship between cancer and miRNAs, it was found that miR-15a and miR-16-1 are down-regulated or deleted in patients with B-cell type chronic lymphocytic leukemia (CLL) [7]. These 2 miRNAs are not expressed by CLL cells, leading to increased levels of an oncogenic target, Bcl-2. On the other hand, miRNA-17–92 was first shown to be functional via the study of the miRNA-induced c-Myc activation [8]. This may indicate that there is a biological significance of high miRNA expression in many cancers, suggesting that it plays an oncogenic role. At present, the primarily studies on miRNAs are mainly focused on their functions as tumor suppressors and oncogenes (onco-miR).

miRNAs are known to be present in both cells and circulating blood [9]. Although, the physiological role of plasma miRNAs remains to be elucidated, they may be useful biomarkers in diagnostic, monitoring and therapeutic applications. In fact, there have been several reports on miRNAs in patients with solid tumors [10], but little is known about their comprehensive expression in ATL. Our aim was to see if cell/plasma miRNAs differentiate between chronic and acute ATL to investigate the general profiles of miRNAs in chronic and acute ATL, and to look for novel type plasma bio-markers.

* Corresponding author at: Central Diagnostic Laboratory of Nagasaki University Hospital, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan. Tel.: +81 95 819 7414; fax: +81 95 819 7414.
E-mail address: ishiha@nagasaki-u.ac.jp (K. Ishihara).

1877-7821/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.canep.2012.07.002

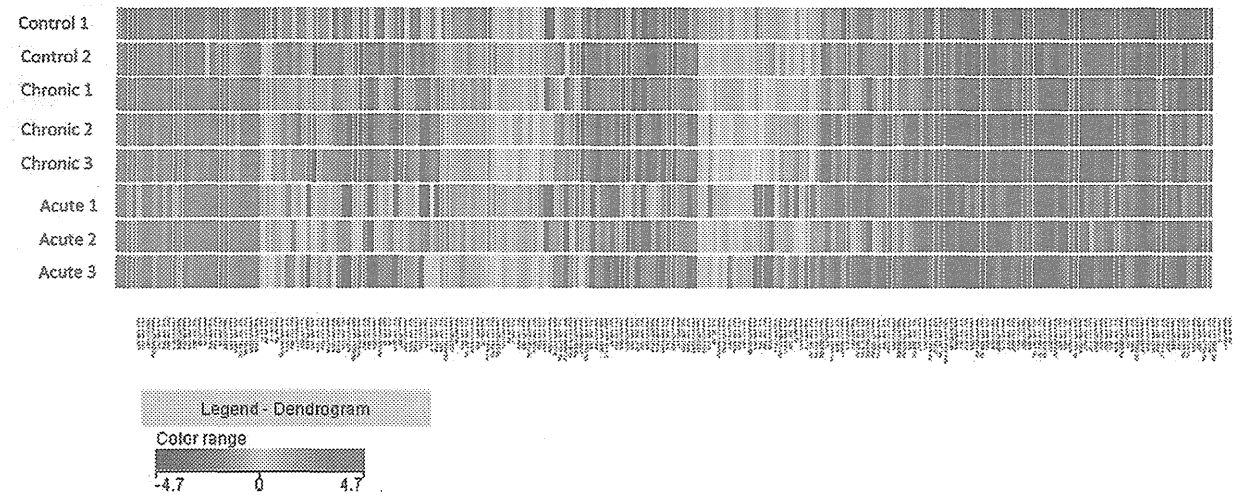

Fig. 1. Heatmap of 2 normal controls, 3 chronic ATL patients and 3 acute ATL patients. Methods and analyses were as described in Section 2. Comprehensive profiles among the eight samples were similar.

2. Materials and methods

2.1. Materials

Peripheral blood mononuclear cells samples from 6 ATL patients (acute: 3 patients; chronic: 3 patients) and plasma samples from 35 ATL patients (acute: 17 patients; chronic: 18 patients), 17 HTLV-1 carriers and 11 HTLV-1 uninfected volunteers were used to evaluate cellular and plasma miRNAs under the approval of the ethics committees of Nagasaki University. Plasma and blood samples were processed immediately and stored at −80 °C after collection.

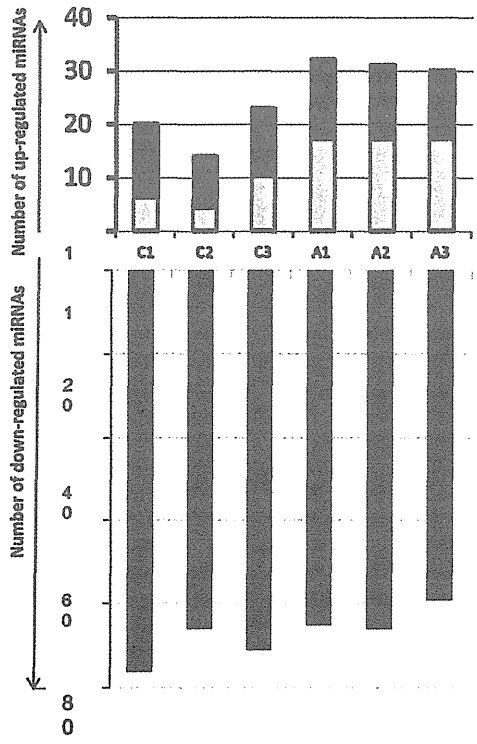

Fig. 2. Comparison of the number of up- and down-regulated miRNAs. Red column: up-regulation of 2-fold or more relative to expression in healthy samples. Yellow columns: number of up-regulation miRNAs at levels from 1.0 to 2.0. Blue columns: number of down-regulation miRNAs at levels from 1.0 to 2.0. Black column: down-regulation of 2-fold or more relative to expression in healthy samples. Only 17–23 (1.7–2.4%) of 955 miRNAs examined were up-regulated. On the other hand, the number of down-regulated miRNAs was 3–5 times higher. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3. Methods

3.1. RNA isolation

To purify CD4-positive cells, magnetic beads (CD4 MicroBeads, Miltenyi Biotec, Auburn, CA) were used according to the manufacturer's instructions. Total RNAs were extracted from CD4-positive cells and from plasma using a commercially available mirVana PARIS kit (Applied Biosystems, Calsbad, CA, US).

3.2. miRNA microarray analysis

We used Human miRNA microarray Rel.12.0 (Agilent, Santa Clara, CA, US) containing 866 human miRNAs and 89 human virus miRNAs.

3.3. Real-time reverse transcription quantifiable polymerase chain reaction (RT-qPCR)

In order to evaluate differences in the expression levels between cellular and plasma miRNA, we quantified several types of miRNAs using a TaqMan MicroRNA RT kit and TaqMan MicroRNA assays (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. RNU6B and has-miR-16 were used as internal controls for cells and plasma, respectively [11,12]. Expression levels were adjusted relative to those of control miRNAs.

4. Results

4.1. Profiles of cellular miRNAs in fresh chronic and acute ATL cells

In order to precisely evaluate the cellular miRNA profiles of ATL cells, we used selected CD4-positive samples from patients with a leukocyte fraction of 80% or more ATL cells. Blood samples tested consisted of 2 cells from HTLV-1 uninfected normal volunteers

Table 1
Five miRNAs extracted based on relative and absolute expression density.

miRNA	Chronic						Acute					
	Chronic 1		Chronic 2		Chronic 3		Acute 1		Acute 2		Acute 3	
	Log2 ratio	Chronic 1 Normal	Log2 ratio	Chronic 2 Normal	Log2 ratio	Chronic 3 Normal	Log2 ratio	Acute 1 Normal	Log2 ratio	Acute 2 Normal	Log2 ratio	Acute 3 Normal
miR-155	0.35	1366.1 1071.6	1.64	597.5 191.4	1.77	653.1 191.4	2.57	6384.4 1071.6	4.28	20,884 1071.6	3.94	2936.1 191.4
let-7g	−0.82	18,448 32,649	−0.59	4358.4 6540.4	−0.91	3486 6540.4	−2.16	7282.5 32,649	−1.84	9091.7 32,649	−1.24	2773.4 6540.4
let-7b	−1.2	1606.5 3685.9	−2.19	269.1 1223.8	−1.22	524.9 1223.8	−1.99	929.6 3685.9	−1.87	1008.4 3685.9	−3.37	118.7 1223.8
miR-126	−1.42	192.6 516.8	−2.16	260.8 1166.3	−2.46	211.8 1166.3	−9.39	0.8 516.8	−5.6	10.6 516.8	−13.51	0.1 1166.3
miR-130a	−1.05	196.7 408.5	−2.11	144.5 625.6	−2.57	105.3 625.6	−12	0.1 408.5	−3.38	39.3 408.5	−12.61	0.1 625.6

The left 3 cases represent Chronic ATL, and the right 3 cases represent Acute ATL. In each case, numbers of ATL patient expression density (upper) and healthy volunteer expression density (lower) are shown on the right side. The left line represents the Log 2 ratio comparing ATL patients and healthy volunteers. Log 2 ratio indicates log 2 (ATL patient/healthy volunteer).

(N1 and 2), 3 chronic ATL cell (C1, 2, and 3) and 3 acute ATL cell (A1, 2, and 3) specimens. Using microarray assay technology, miRNA status was screened and displayed as characteristic heatmap, as shown in Fig. 1. The heatmap showed similar patterns among controls (N1 and N2), chronic (C1–3) and acute (A1–3) samples.

Of the 955 miRNAs tested, the number of up-regulated miRNAs was significantly higher in acute ATL (30–32, mean: 31) than in chronic ATL (14–23, mean: 17.6) ($p = 0.005$).

On the other hand, down-regulated miRNAs showed an inverse correlation (91acute vs. 83 chronic; $p = 0.05$), as shown in Fig. 2. We profiled a total of 5 up- or down-regulated miRNAs based on large differences in relative and/or absolute expression intensity, as summarized in Table 1. These 5 miRNAs (miR-155, let-7g, let-7g, miR-126 and miR-130a) were further characterized based on their relative and absolute expression, as shown in Fig. 3(a and b); that is, clear elevation of miR-155 in the acute phase, a plateau in let-7g, a reduction in miR-130a and let-7b in the acute stage and slight variation in miR-126 in all stages.

4.2. Real-time RT-qPCR assay for plasma miRNAs

As a next step, to evaluate of plasma miRNA status, we quantified the plasma density of before 5 miRNAs (let-7b, let-7g, miR-130a and miR-126) in HTLV-1 seropositive or seronegative individuals and patients with ATL. As shown in Fig. 4, plasma samples contained detectable levels of miRNA, increasing in the order of the carrier, chronic ATL and acute ATL. The plasma level of let-7g was higher in HTLV-1 uninfected volunteers than HTLV-1 carriers.

4.3. Survival rate and the level of miR-155 or miR-126

First of all, a total of 35 cases were divided into two groups according to the expression level of miR-155 and miR-126 by the median. As shown in Fig. 5, ATL patients with high miR-155 showed poor prognosis ($p = 0.04$), whereas patients with high miR-126 showed a better prognosis as compared to low miR-126 ($p = 0.004$). Since ATL has characteristics of diverse prognosis

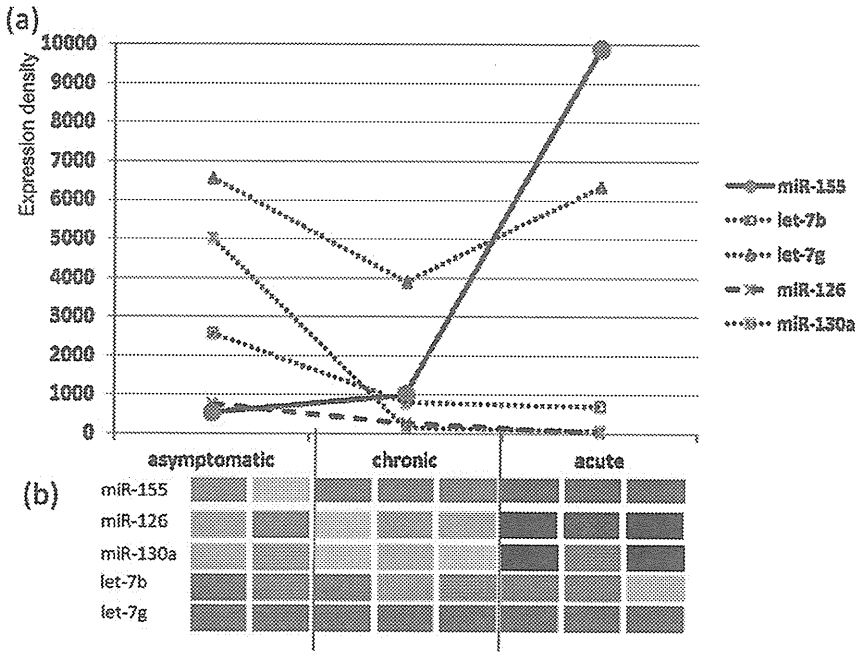

Fig. 3. Subcellular 5 miRNAs leads at the time of each ATL stage, selected based on relative (b) and absolute expression (a) fluctuating with disease progression from chronic to acute ATL.

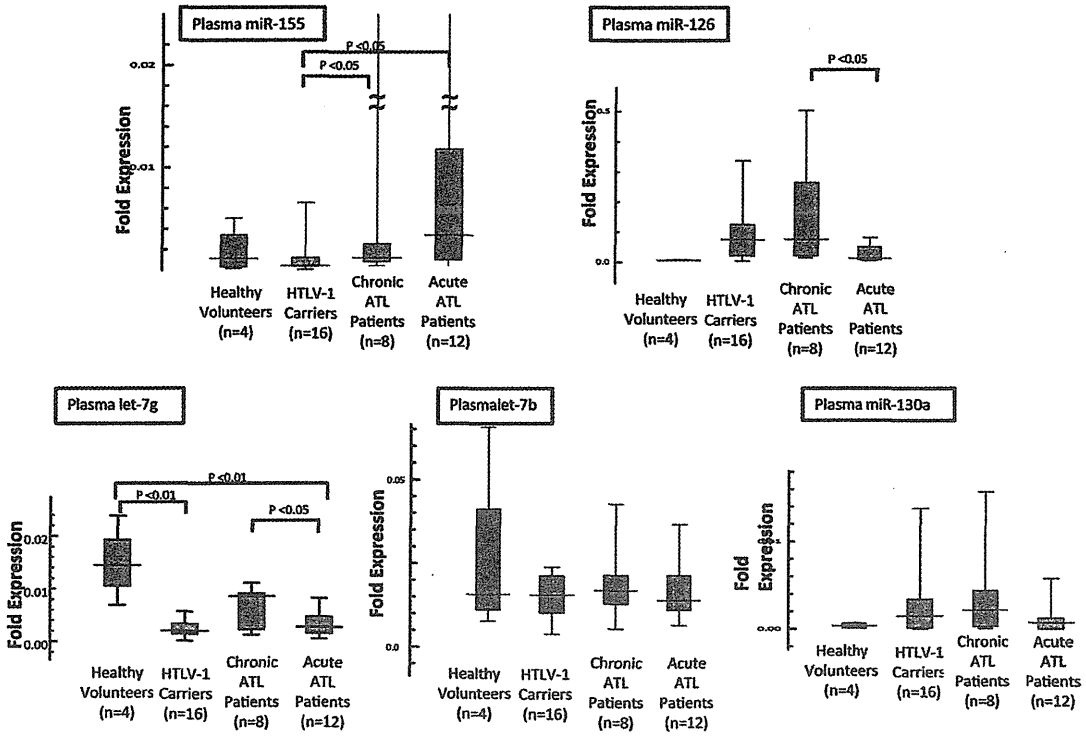

Fig. 4. Comparison of plasma miRNAs levels that were down-regulated in ATL cells of HTLV-1 uninfected volunteers, healthy HTLV-1 carriers and ATL patients.

among acute and chronic subtypes, we compared the prognosis within subtypes. Consequently, as shown in Fig. 6, miR-126 may be a factor to decide prognosis of acute or chronic subtypes. We also compared the usefulness of miRNAs with the known prognostic factors LDH and sIL-2R (Fig. 7). Similar to miRNAs, the overall survival rate was compared between the two groups stratified by the median of LDH and sIL-2R. The median survival rate for each biomarker is summarized in Table 2. miR-155 and miR-126 are equivalent prognostic factor to LDH and better than sIL-2R.

5. Discussion

We evaluated cellular miRNA status in chronic and acute ATL cells freshly isolated from patients with ATL. The expression status in ATL cells was almost similar to that in normal HTLV-1 un-infected CD4 T-cells. Cellular miRNA expression levels examined using microarray are generally prone to down-regulation or partial disappearance with disease progression from chronic to acute subtype. Here, we focused on miRNAs to clarify if they could be potent biomarkers for evaluation of disease progression and degree of malignancy.

Initially, we profiled 12 mature miRNAs as candidates for evaluation of malignant ATL behavior based on the microarray findings. Considering the density levels of circulating miRNAs, 5 miRNAs (miR-155, let-7g, let-7g, miR-126 and miR-130a) were finally extracted. However, since the circulating density levels of each miRNAs were not always accordant with the expression levels of cellular miRNAs, we focused on the kinetic pattern in the plasma and cells of the two miRNAs, miR155 and miR-126, because there was an inverse association between the change of miR155 and

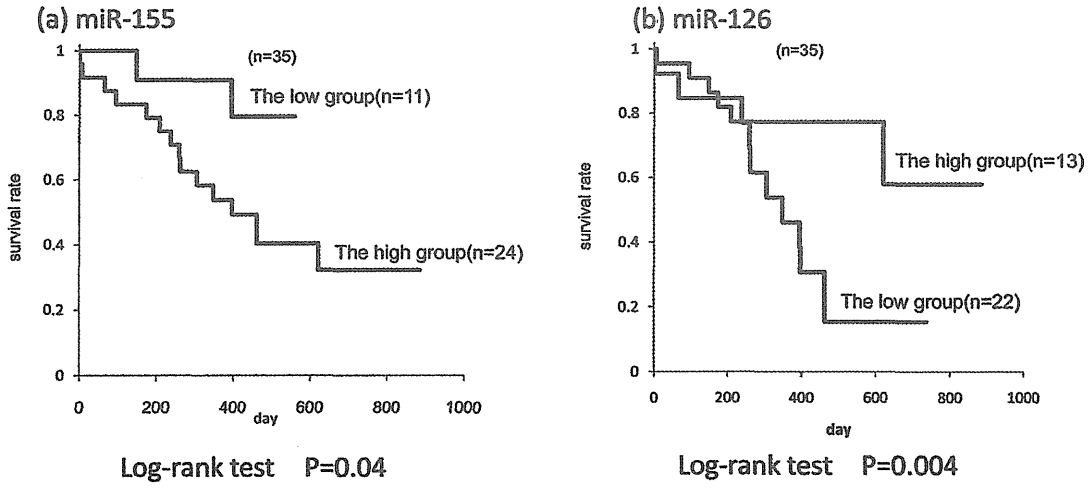

Fig. 5. Kaplan–Meier estimates of the probability of overall survival in 35 ATL patients according to expression levels of miR-126 and miR-155 in all subtypes of ATL patients.

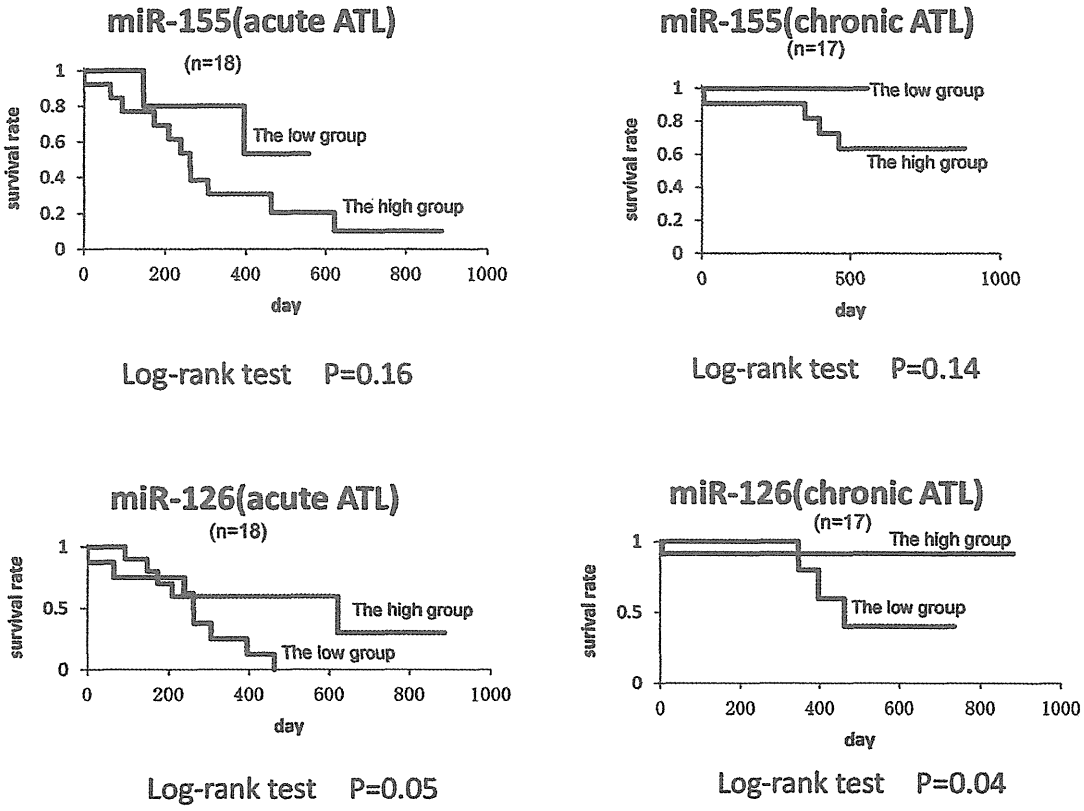

Fig. 6. Kaplan–Meier estimates of the probability of overall survival in 35 ATL patients according to expression levels of miR-126 and miR-155 in acute and chronic ATL.

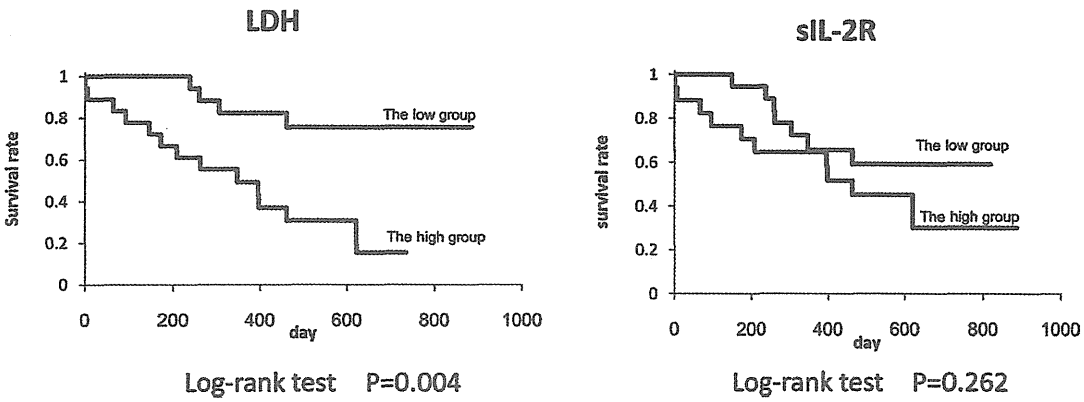

Fig. 7. Kaplan–Meier estimates of the probability of overall survival in 35 ATL patients according to levels of the known factors LDH and sIL-2R in all subtypes of ATL patients.

miR-126 levels and the disease state. In fact, The Kaplan–Meier survival curve indicates that the two markers (miR-155 and miR-126) could be stratified more clearly into two groups of long and short survival rather than that of the known predictor markers of LDH and sIL-2R. We think that cause of the distinct levels of miRNAs is reduced production or consumption of the miRNAs.

Currently, the profiling status of ATL cells is not matched and confusing as shown in Table 3 [13–15]. Up-and down-regulation status appears to rely on differences in the cell types and maturation grade. However, 4 studies examined [5,6,14,15] gave similar findings on only miR-155 and miR-126. In particular, marked up-regulation of miR-155 at the acute stage is noted from the view-point of specificity for HTLV-q-infected cell lines and freshly isolated ATL cells. The present study also found that all except one of 7 ATL cell lines showed high expression of miR-155, while in 8 non-ATL cell lines, only the SKW cell line derived from EBV-infected B-cells showed marked expression of miR-155 (data not shown). Additionally, a recent study reportedly clarified that diffuse large B-cell lymphoma (DLBCL) cells in which the germinal

Table 2
Comparison of prognostic prediction between known prognostic factors and miRNA (miR-155 and -126).

	Median survival rate		p value
	The high group	The low group	
LDH	547	[a]	0
sIL-2R	462	[a]	0.26
miR-155	397	[a]	0.04
miR-126	[a]	347	0

[a] Did not reach 50% survival.

Table 3
Differences in up- or down-regulation of miRNAs in ATL-related cellsal., Pichler et al. and Tomita et al. in the present study and in previous reports by Yeung et al. and Bellon et al.

	Present study		Yeung et al. [6]		Bellon et al. [5]		Pichler et al. [14]	Tomita et al. [15]
	Chronic fresh ATL cells	Acute fresh ATL cells	Patients PBMC	A Venn diagram	HTLV-1 cell lines	ATL cells	HTLV-1 transformed cells	HTLV-1 infected T-cell lines
miR-155	Up	Up	Up	?	Up	Up	Up	?
miR-130a	Down	Down	Down	Down	?	?	?	?
miR-126	Down	Down	?	Down	Down	Down	?	?

Up: up-regulated; down: down-regulated; stable: no significant change; ?: data not shown or not tested.

cells were activated highly expressed miR-155 [16]. This indicates that up-regulation of miR-155 with disease progression does not appear to be present only in HTLV-1-associated tumors.

With regard to miR-126 as a partner biomarker along with miR-155, there have been numerous studies on solid tumors, but few on hematopoietic tumors. miR-126 is aberrantly down-regulated in many solid cancers, and a ubiquitous loss of miR-126 is seen in colon cancer lines [17]. Donnem et al. reported that miR-126 is a strong negative prognostic factor in cancer [18]. This indicates a clinical and biological significance similar to ATL, as our data suggest an inverse correlation between these miRNA expression levels and disease progression. Since miR-155 is predicted to inhibit any TGFβR2 function search for complementary genes, we consider that up-regulation of miR-155 leads to cancer growth. Inversely, with miR-126 targeting EGFL7, Crk or SLC7A5, which yield growth advantages [19–22], it is considered that down-regulation plays a role in cancer growth. Our findings on ATL also supported the opinions described above. Furthermore, miR-155 and miR-126 may be superior at predicting survival of ATL compared to the known factors (LDH and sIL-2R).

In conclusion, the subcellular miRNA profile of ATL cells was similar to that of resting peripheral CD4+ T cells, while the expressions of some miRNAs such as miR-155 vs. miR-126 showed different densities. We found plasma that two miRNAs namely miR-155 and -126 could have an impact as novel biomarkers to evaluate disease progression and prognosis.

Funding

Supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Health, Labour, and Welfare of Japan (Nos. 21659149, 21390182).

Conflict of interest statement

None of the authors have any conflicts of interest in connection with this work.

References

[1] Poiesz BJ, Ruscetti FW, Gazdar AF, Bunn PA, Minna JD, Gallo RC. Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. Proc Natl Acad Sci USA 1980;77(December (12)):7415–9.
[2] Yoshida M, Seiki M, Yamaguchi K, Takatsuki K. Monoclonal integration of human T-cell leukemia provirus in all primary tumors of adult T-cell leukemia suggests causative role of human T-cell leukemia virus in the disease. Proc Natl Acad Sci USA 1984;81(April (8)):2534–7.
[3] Takasaki Y, Iwanaga M, Imaizumi Y, Tawara M, Joh T, Kohno T, et al. Long-term study of indolent adult T-cell leukemia–lymphoma. Blood 2010;115(June (22)):4337–43 [Epub 2010 Mar 26].
[4] Usui T, Yanagihara K, Tsukasaki K, Murata K, Hasegawa H, Yamada Y, et al. Characteristic expression of HTLV-1 basic zipper factor (HBZ) transcripts in HTLV-1 provirus-positive cells. Retrovirology 2008;5(April):34.
[5] Bellon M, Lepelletier Y, Hermine O, Nicot C. Deregulation of microRNA involved in hematopoiesis and the immune response in HTLV-I adult T-cell leukemia. Blood 2009;113(May (20)):4914–7 [Epub 2009 Feb 26].
[6] Yeung ML, Yasunaga J, Bennasser Y, Dusetti N, Harris D, Ahmad N, et al. Roles for microRNAs, miR-93 and miR-130b, and tumor protein 53-induced nuclear protein 1 tumor suppressor in cell growth dysregulation by human T-cell lymphotrophic virus 1. Cancer Res 2008;68(November (21)): 8976–85.
[7] Calin GA, Dumitru CD, Shimizu M, Bichi R, Zupo S, Noch E, et al. Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. Proc Natl Acad Sci USA 2002;99(November (24)):15524–29 [Epub 2002 Nov 14].
[8] He L, Thomson JM, Hemann MT, Hernando-Monge E, Mu D, Goodson S, et al. A microRNA polycistron as a potential human oncogene. Nature 2005;435(June (7043)):828–33.
[9] Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, et al. Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci USA 2008;105(July (30)):10513–18 [Epub 2008 Jul 28].
[10] Tavazoie SF, Alarcón C, Oskarsson T, Padua D, Wang Q, Bos PD, et al. Endogenous human microRNAs that suppress breast cancer metastasis. Nature 2008;451(January (7175)):147–52.
[11] Sasaki D, Imaizumi Y, Hasegawa H, Osaka A, Tsukasaki K, Choi YL, et al. Overexpression of enhancer of zeste homolog 2 with trimethylation of lysine 27 on histone H3 in adult T-cell leukemia/lymphoma as a target for epigenetic therapy. Haematologica 2011;96(May (5)):712–9 [Epub 2011 Jan 12].
[12] Davoren PA, McNeill RE, Lowery AJ, Kerin MJ, Miller N. Identification of suitable endogenous control genes for microRNA gene expression analysis in human breast cancer. BMC Mol Biol 2008;9(August):76.
[13] Jeang KT. Human T cell leukemia virus type 1 (HTLV-1) and oncogene or oncomiR addiction? Oncotarget 2010;1(October (6)):453–6.
[14] Pichler K, Schneider G, Grassmann R. MicroRNA miR-146a and further oncogenesis-related cellular microRNAs are dysregulated in HTLV-1-transformed T lymphocytes. Retrovirology 2008;5(November):100.
[15] Tomita M, Tanaka Y, Mori N. MicroRNA miR-146a is induced by HTLV-1 tax and increases the growth of HTLV-1-infected T-cells. Int J Cancer)2009;(December) [Epub ahead of print].
[16] Eis PS, Tam W, Sun L, Chadburn A, Li Z, Gomez MF, et al. Accumulation of miR-155 and BIC RNA in human B cell lymphomas. Proc Natl Acad Sci USA 2005;102(March (10)):3627–32.
[17] Guo C, Sah JF, Beard L, Willson JK, Markowitz SD, Guda K. The noncoding RNA, miR-126, suppresses the growth of neoplastic cells by targeting phosphatidylinositol 3-kinase signaling and is frequently lost in colon cancers. Genes Chromosomes Cancer 2008;47(November (11)):939–46.
[18] Donnem T, Lonvik K, Eklo K, Berg T, Sorbye SW, Al-Shibli K, et al. Independent and tissue-specific prognostic impact of miR-126 in nonsmall cell lung cancer: coexpression with vascular endothelial growth factor-A predicts poor survival. Cancer)2011;(January) [Epub ahead of print].
[19] Sun Y, Bai Y, Zhang F, Wang Y, Guo Y, Guo L. miR-126 inhibits non-small cell lung cancer cells proliferation by targeting EGFL7. Biochem Biophys Res Commun 2010;391:1483–9.
[20] Feng R, Chen X, Yu Y, Su L, Yu B, Li J, et al. miR-126 functions as a tumour suppressor in human gastric cancer. Cancer Lett 2010;298(December (1)):50–63 [Epub 2010 Jul 8].
[21] Fathers KE, Rodrigues S, Zuo D, Murthy IV, Hallett M, Cardiff R, et al. CrkII transgene induces atypical mammary gland development and tumorigenesis. Am J Pathol 2010;176:446–60.
[22] Kaira K, Oriuchi N, Imai H, Shimizu K, Yanagitani N, Sunaga N, et al. l-type amino acid transporter 1 and CD98 expression in primary and metastatic sites of human neoplasms. Cancer Sci 2008;99:2380–6.