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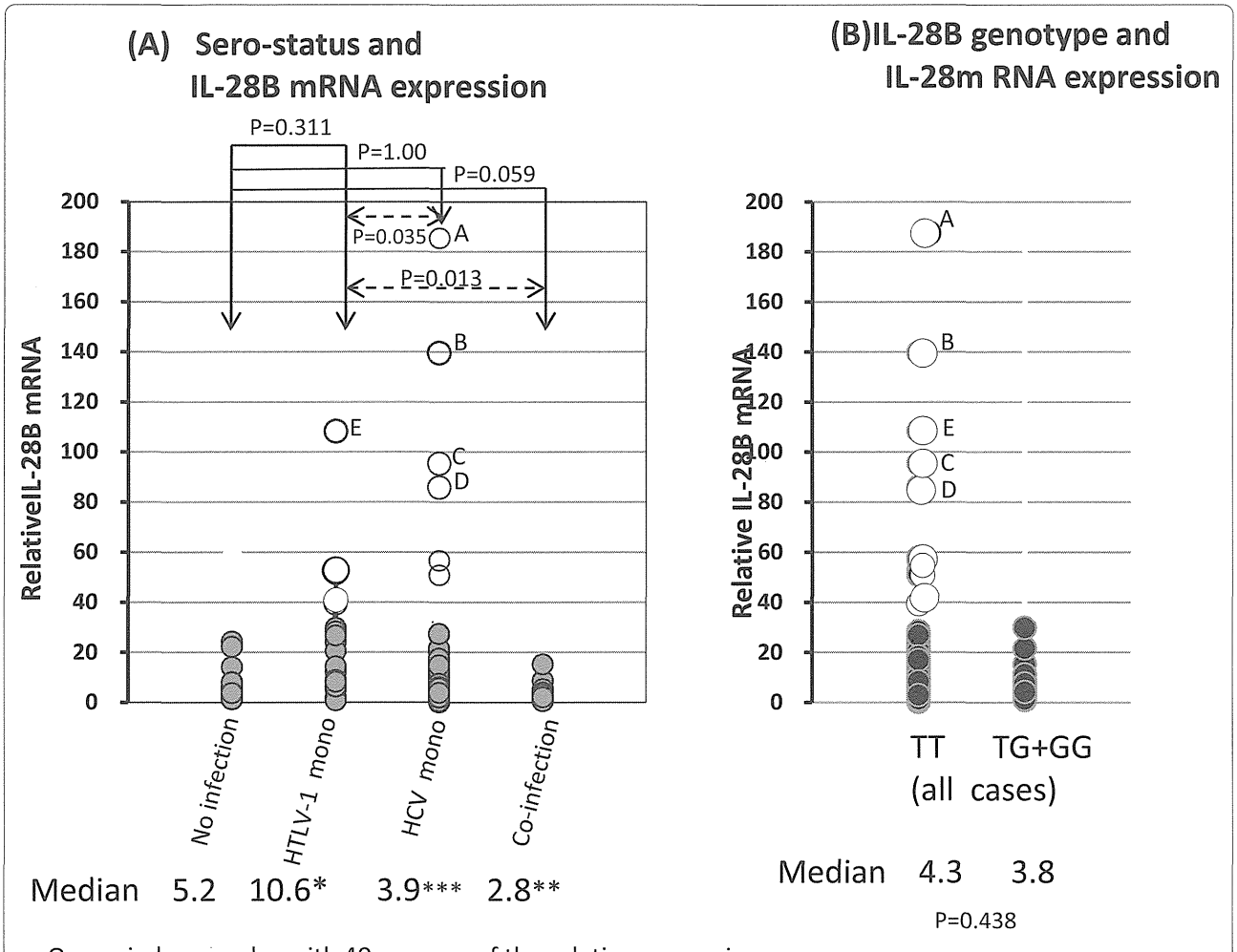

Figure 1 Relative individual IL-28B mRNA levels in peripheral blood mononuclear cells among viral infectious groups (a) and among the TT and TG/GG genotypes (b). The median values were significantly different between * vs ** and *** vs **. Open circles; those with high IL28BmRNA with TT homozygotes.

co-infection with HCV and ATL was associated with TT genotype (OR = 6.25). On the other hand, down-regulation of the mRNA density was defined as HTLV-1/HCV co-infection (OR = 9.5 p = 0.004), but low expression was not associated with ATL development (OR = 0.8, p = 0.81).

Discussion

Although co-infection with HTLV-1 and HCV has been shown to result in higher rates of cirrhosis and increased death from liver diseases [1,2], the caustic mechanism by which the co-infection affects HCV pathogenesis remains to be elucidated. Some clue to the mechanism may be found by studying the relation between IL-28B genotypes and co-infection, because IL-28B encoding IFN-λs are categorized as type 3 IFNs and are potent endogenous anti-viral cytokines. They signal via JAK/STAT intracellular pathways and up-regulated transcription of IFN-stimulated genes (ISGs) that are required to control viral infection [13]. Here, we investigated whether IL-28B polymorphism rs8099917 is associated with co-infection status.

The present study is the first to reveal that the IL-28B genotype is not associated with stratification based on the combination of HTLV-1 and HCV infection; no infection for both (double negative; DN), HTLV-1 mono-infection, HCV mono-infection and HTLV-1/HCV co-infection. Similarly, the frequency of the major TT homozygotes was not associated among ATL patients and HTLV-1 carriers (Table 2). These two

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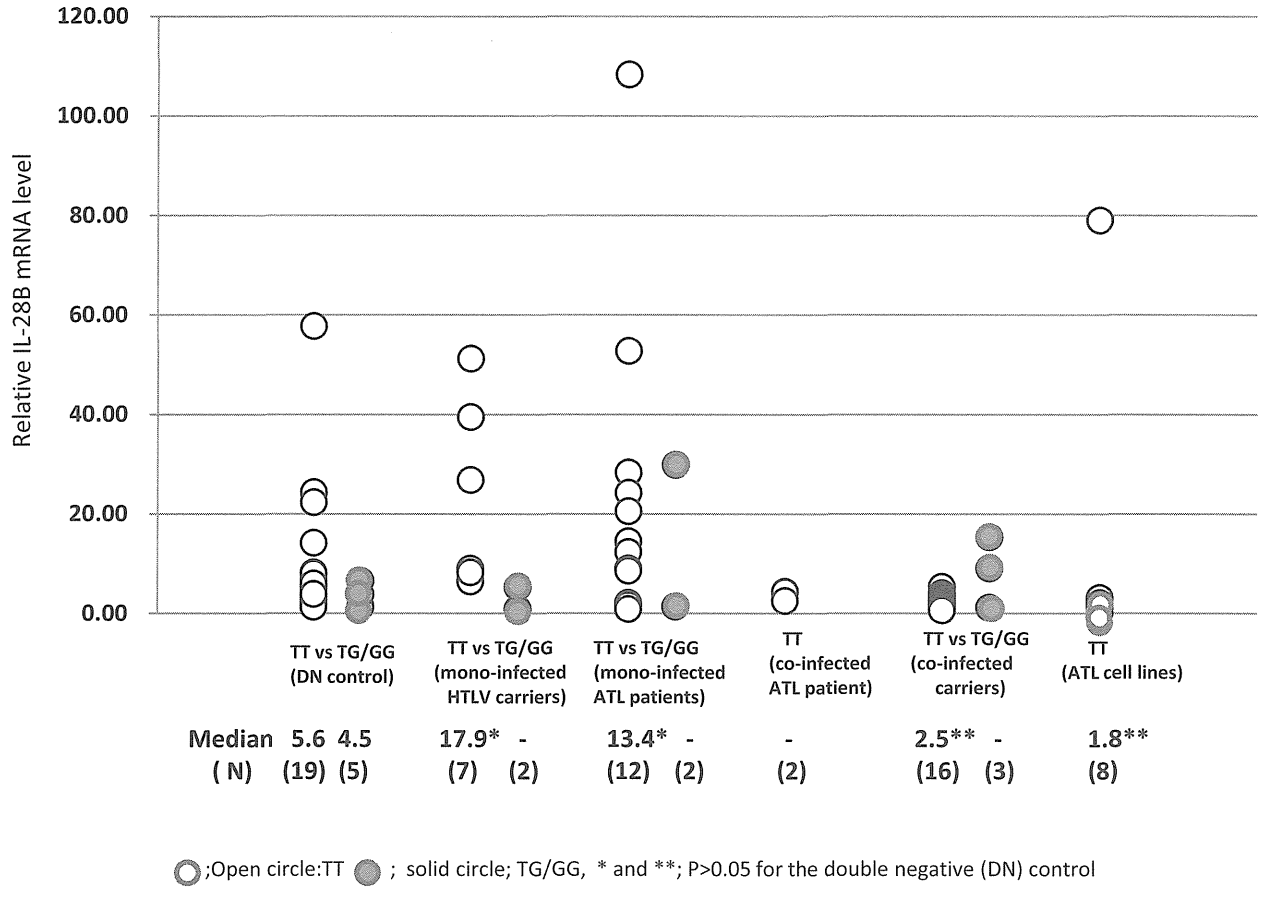

Figure 2 Individual relative expression density of IL-28B mRNA in TT vs TG/GG derived from 24 double-negative (DN) controls consisting of 19TT and 5TG/GG, 9 mono- infected HTLV-1 carriers, 14 mono-infected and 2 co-infected ATL patients, 19 co-infected carriers and 8 ATL cell lines samples.

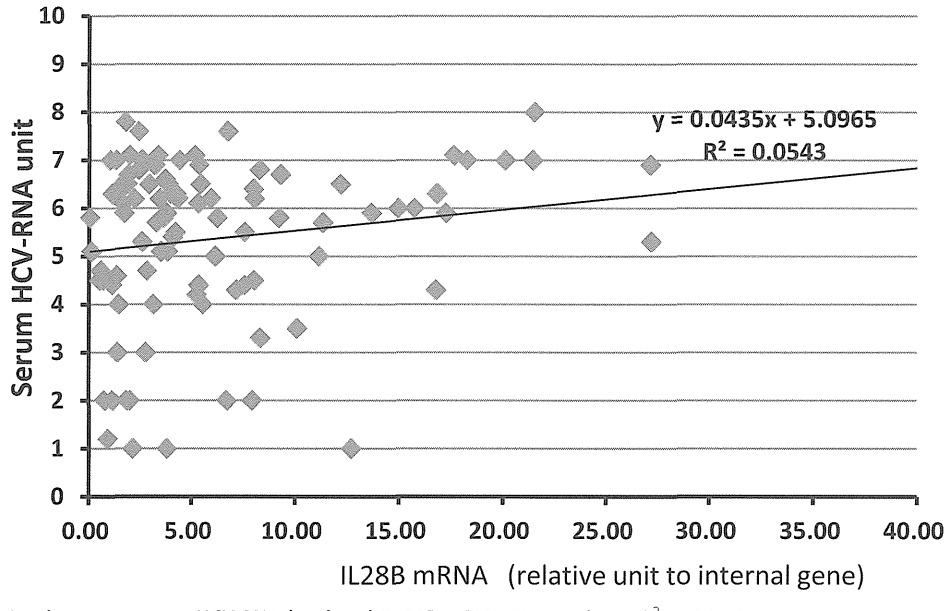

Figure 3 Correlation between serum HCV-RNA level and IL-28B mRNA. No-correlation (r^2) = 0.0543.

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Table 2 Assessment by odds ratio analysis for an outcome if a risk factor is present

	factor			
(A) Outcome	dependent independent	Odds ratio	95%CI	P
1) HTLV-1 mono-infection	TT vs TG/GG	1.11	0.62-1.99	0.72
2) Co-infection	TT vs TG/GG	0.54	0.04-6.88	1.00
3) High mRNA Expression*	TT vs TG/GG	6.25	1.16-33.75	0.04
4) ATL	TT vs TG/GG	1.50	0.60-3.75	0.39
(B)				
5) Low mRNA Expression	HTLV-1 mono vs DN	0.34	0.06-2.04	0.24
6) Low mRNA Expression	HCV mono vs DN	0.29	0.07-2.23	0.15
7)) Low mRNA Expression	Co-Inf** vs HTLV-1-mono**	9.5	2.06-43.76	0.004
8) ATL	low expression or not	0.8	0.14-4.74	0.81

(A) Upper 4 lines; assessing the risk of 1) HTLV-1 persistent infection, 2) super-imposed HTLV-1 infection with HCV (co-infection), 3) high IL-28B mRNA expression, and 4) ATL development when the genotype is a risk factor (B) Lower 4 lines; assessing the risk factors described in the outcome, the IL-28B mRNA expression level in peripheral blood (5, 6, and 7), and ATL development (8). Consequently, similarly to HCV, HTLV-1 is associated with up-regulation of IL-28B mRNA along with the TT homozygote, and co-infection with HTLV-1 and HCV paradoxically down-regulates the mRNA level

*; IL-28B Expression level, Co-inf = co-infection with HTLV-1 and HCV, mono = mono-infection

findings suggest that the SNP rs8099917 is not associated with susceptibility to HTLV-1 infection or the development of ATL. On the other hand, all of ATL cell- or HTLV-1-infected T-cell- lines examined were exclusively TT homozygous, implying that HTLV-1-infected cells carrying TT homozygotes may immortalize easily in vitro.

Next, we found a strange phenomenon that the IL-28B mRNA expression levels in peripheral blood were lower in samples with HTLV-1/HCV co-infection than in samples with either HTLV-1 or HCV alone, especially significantly for HTLV-1 mono-infection. In particular, samples carrying TT homozygotes were strongly down-regulated, more than the minor TG hetero- and GG-homozygotes. Why are the mRNA expression levels different in mono- and dual-infection? Although it is not known how rs8099917 affects the action of IL-28B, presumably it alters the immune function to viruses. In addition to a common anti-viral IFN-stimulating signal pathway, HTLV-1 may use an alternative anti-viral pathway like HBV [14], because the HTLV-1 provirus is integrated into host genomic DNA and replicates in distinctive life cycle kinetics. Moreover, ATL originates from Treg cells, which play a central role in suppressing immunity [15]. However, this cannot fully explain the impairment in the HTLV-1 carrier's immunity because no ATL cells are present during the carrier period. Thus, we noted IFN-λ(IL-29, IL-28), which was recently discovered as a type III IFNs with anti-virus ability, anti-tumor and immune responses [16-18].

From our results, the IL-28B expression level was higher in HTLV-1 mono-infected individuals including ATL patients. IFN-λis usually up-regulated through activation of the NF-kappaB pathway after viral infection. Actually, the Sendai virus, an influenza A virus, and others have been demonstrated to activate the NF-kappa B pathway, resulting in up-regulated IL-28B expression [19,20]. Accordingly, the highest up-regulation of IFN-λ3 in HTLV-1 mono-infection may be explained by virtue of a viral protein of HTLV-1 having strong NF-kB activating ability. Moreover, it is instructive that IFN-λhas a potent function to expand Treg cells [21], which are mainly infected with HTLV-1, predisposing development of ATL. However, there has not yet been evidence that co-infection with HCV damages Tax action.

Of IL-28B producing cells in the literature, most cells in the blood are described as having a weak or absent expression under the steady state conditions. Li et al. [9] reported that IL-28B mRNA is not always expressed in virally infected cells. Actually, our findings in HTLV-1-infected cases also showed that at least the main producing cells are likely to be cells other than ATL cells because most cell lines from ATL and some blood samples containing ATL cells were expressed faintly. At present, plasmacytoid dendritic cells are indicated to be the most potent producers of IFN-λs [19]. On the other hand, IFN-λ3 reportedly has the functions of proliferating Treg cells which are the origin of ATL cells, suggesting that HTLV-1 is associated with up-regulation via Treg cells infected with HTLV-1.

In conclusion, we found an unusual phenomenon in that the expression of IL-28B mRNA was affected by not only the IL-28B rs8099917 genotype, but also co-infected HTLV-1 with HCV. This will contribute to a better understanding the enigmatic impairment of immunity in the HTLV-1 carrier state, including co-infection with HTLV-1 and HCV.

Abbreviations

HTLV-1: Human T-cell leukemia virus type -1; HCV: Hepatitis C virus; SNP: Single nucleotide polymorphism; IFN: Interferon; PBMC: Peripheral blood

mononuclear cell; PCR: Polymerase chain reaction; MAF: Minor-allele frequency.

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Author details
[1]Department of Laboratory Medicine, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, 852-8501, Japan. [2]Central Diagnostic Laboratory of Nagasaki University Hospital, Nagasaki, 852-8501, Japan. [3]Department of Gastroenterology and Hepatology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, 852-8501, Japan. [4]Faculty of Wellness Studies, Kwassui Women's University, Nagasaki, 850-8515, Japan. [5]Division of Surgical Oncology, Department of Translational Medical Science, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, 852-8501, Japan.

Authors' contributions
SK designed the study and wrote the manuscript, and SM, TU, KN, DS HH, KY, NU, YM analyzed the genotype, TK collected samples, and TK, KN, MI and SK organized and assessed the data. All authors interpreted the data and were financially supported. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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ORIGINAL ARTICLE

Human T-cell leukemia virus type 1 infection worsens prognosis of hepatitis C virus-related living donor liver transplantation

Tatsuki Ichikawa,[1] Naota Taura,[1] Hisamitsu Miyaaki,[1] Toshihisa Matsuzaki,[1] Masashi Ohtani,[1] Susumu Eguchi,[2] Mitsuhisa Takatsuki,[2] Akihisa Soyama,[2] Masaaki Hidaka,[2] Sadayuki Okudaira,[2] Tetsuya Usui,[4] Sayaka Mori,[4] Shimeru Kamihira,[3,4] Takashi Kanematsu,[2] and Kazuhiko Nakao[1]

1 Department of Gastroenterology and Hepatology, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan
2 Department of Transplantation and Digestive Surgery, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan
3 Department of Laboratory Medicine, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan
4 Central Diagnostic laboratory of Nagasaki University Hospital, Nagasaki, Japan

Keywords
donor age, HCV, HTLV-1, liver transplantation.

Correspondence
Tatsuki Ichikawa, Department of Gastroenterology and Hepatology, Graduate School of Biomedical Sciences, Nagasaki University, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan. Tel.: +81-95-819-7481; fax: +81-95-849-7482; e-mail: ichikawa@net.nagasaki-u.ac.jp

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Summary

Severe and life-threatening donor-transmitted human T-cell leukemia virus type 1 (HTLV-1) infections after solid organ transplantation have been reported. However, in HTLV-1-infected recipients, graft and patient survival were not fully evaluated. A total of 140 patients underwent living donor liver transplantation (LDLT). Of these, 47 of 126 adult recipients showed indications of hepatitis C virus (HCV)-related liver disease. The HTLV-1 prevalence rate was 10 of 140 recipients (7.14%) and three of 140 donors (0.02%). In HCV-related LDLT, graft and patient survival was worsened by HTLV-1 infection in recipients (seven cases). The 1-, 3-, and 5-year survival rates in the HCV/HTLV-1-co-infected group were 67%, 32%, and 15%, respectively, and the corresponding rates in the HCV-mono-infected group were 80%, 67%, and 67%, respectively. Only the 5-year survival rates were statistically significant ($P = 0.04$, log-rank method). HTLV-1 infection in recipients is also an important factor in predicting survival in HTLV-1 endemic areas.

Introduction

Human T-cell leukemia virus type 1 (HTLV-1) is highly endemic in the southwestern area of Japan, including Nagasaki as well as in Saharan Africa, South America, the Caribbean islands, and aboriginal Australia [1,2]. However, HTLV-1 infects approximately 15–25 million people worldwide [3] and is associated with adult T-cell leukemia/lymphoma (ATL), HTLV-1-associated myelopathy (HAM), uvetitis, sialadenitis-like Sjögren syndrome (SjS), and a wide variety lymphocyte-mediated disorders [2,4,5]. Severe and life-threatening donor-transmitted HTLV-1 infections after solid organ transplantation have been reported [6–8]. However, in HTLV-1-infected recipients, graft and patient survival has not been fully evaluated. The development of three ATL cases in eight HTLV-1 infected recipients after living donor liver transplantation (LDLT) was reported in Japan [9]. We also reported the development of HAM [10] and sialadenitis-like SjS [11] resulting from HTLV-1 in LDLT recipients. Previous reports state that HTLV-1 infection is associated with a nonresponse to interferon (IFN) monotherapy for chronic hepatitis C (CHC) [12] and flare-up of alanine aminotransferase in hepatitis C virus (HCV)-RNA carriers [13]. HTLV-1/HCV co-infection may affect the course of HCV-associated liver disease and liver cancer [14].

Additionally, HTLV-1 interferes with intracellular signaling by type 1 IFN and upregulates HCV replication [14–16]. However, the influence of HTLV-1 in recipients on the grafted liver has not been explored. The effect of HTLV-1/HCV co-infections in recipient compared with HCV mono-infections has similarly not been explored.

Between 1988 and 2000, 0.027% of donors reporting to the United Network for Organ Sharing (UNOS) were diagnosed with HTLV-1 infections [6]. However, the prevalence of anti-HTLV-1 antibodies in patients visiting Nagasaki University Hospital between 2000 and 2007 was 13.57% [2], indicating that HTLV-1 carriers are clustered in Nagasaki. To prevent vertical transmission of HTLV-1, the ATL Prevention Program, which is a prefecture-wide breastfeeding intervention study for HTLV-1 carrier mothers, was initiated in Nagasaki in 1987 [17]. As a result, age-specific rates of HTLV-1 among residents in Nagasaki have annually declined (Seropositive rate, 14.5% in 2000; 12.7% in 2007) [2]. The prevalence of anti-HCV antibody increased with age and was higher in populations in the southwestern area of Japan (including Nagasaki) [18]. In endemic areas of HTLV-1 infection, HTLV-1/HCV co-infected patients are frequently observed and increase the probability a person will have a liver transplantation.

The HTLV-1 infection rate is lower in Western countries; however, the influence of HTLV-1 on HCV infection after transplantation has not been examined. It is necessary to evaluate HTLV-1 infection rates in HTLV-1 endemic areas. We examined whether HTLV-1 infection influences patient and graft survival in cases of liver transplantation in endemic areas of HTLV-1 infection in Nagasaki.

Patients and methods

Patients

In total, 126 consecutive adult LDLT patients, 47 of who were HCV-infected, were enrolled in this study. This retrospective cohort study of LDLT recipients included a comparative analysis of HTLV-1-positive and HTLV-1-negative recipients to determine graft and patient survival. In particular, we evaluated whether HTLV-1 infection influenced HCV-related LDLT. Anti-HTLV-1 antibody was detected using an enzyme immunoassay (EIA). In addition, in HTLV-1-positive patients, we used polymerase chain reaction (PCR) analysis to evaluate HTLV-1 proviral DNA in the peripheral blood mononuclear cells. We diagnosed patients with the anti-HTLV-1 antibody and proviral DNA as being HTLV-1 positive. In our hospital, HTLV-1-positive grafts are not used for negative recipients, but are used for positive recipients. Recipient characteristics such as age, gender, body mass index, Child-Pugh score and medical model for end-stage liver disease (MELD) score at the time of transplantation, presence or absence diabetes mellitus (DM), and presence of hepatocellular carcinoma (HCC) were also analyzed. Surgical factors examined included blood type matching, bleeding volume, (ml), and surgery time (min). Donor age was categorized into those less than 50 years old and 50 years old and older. Additional donor characters, such as donor gender, donor BMI, and donor HTLV-1 status were analyzed. HCV factors included genotype (1b or non-1b), titer in 1b, core amino acid mutation in 1b, and IL28B SNP. The HCV-RNA high group (100 000 IU/ml or more in the serum) of patients was analyzed using real-time PCR.

Primary outcomes evaluated included recipient and graft survival. The cause of death was determined using various factors together with biopsy and necropsy. Liver biopsy was performed each year and at exacerbation of liver function.

Methods

The study design, which also included the collection of data from medical records from the associated hospitals mentioned above, was approved by the Ethics Review Board of our hospital.

In this study, 3 *IL28B* SNPs, i.e., rs8099917, rs12979860, and rs12980275, were examined (Nagasaki University Institutional Review Board approval number: 100511184). SNPs were detected using pyrosequence analysis. The sense, antisense, and pyrosequence primers were **B**-5′-TCCTCCTTTTGTTTTCCTTTCTG-3′, 5′-AAAAAG CCAGCTACCAAACTGT-3′, and 5′-TGGTTCCAATTT GGG-3′ for rs8099917, 5′-GTCGTGCCTGTCGTGTAC TGA-3′, 5′-**B**-GGAGCGCGGAGTGCAATT-3′, and 5′-GG AGCTCCCCGAAGG-3′ for rs12979860, and 5′-GCTG TATGATTCCCCCTACATG-3′, 5′-**B**-TACATTGTTCGG CAAGCAATCT-3′, and 5′-AGAAGTCAAATTCCTAG AAA-3′ for rs12980275, respectively. "**B**" in the primer sequences indicates that the primer is biotin-labeled.

Statistical analysis

Data were processed on a personal computer and analyzed using StatView 5.0 (SAS Institute, Inc., Cary, NC, USA).Graft and patient survival was determined using the Kaplan–Meier method and survival curves were compared using a log-rank test. A cox proportional hazard model was used to determine risk factors for graft and patient survival. Differences between each laboratory data were analyzed using the Mann–Whitney *U*-test and χ^2 test. *P*-values < 0.05 were considered statistically significant.

Results

We evaluated the impact of HTLV-1 on general graft and patient survival in HCV-infected patients. Of the 140 patients who had undergone LDLT at the Nagasaki University Hospital between 1997 and January 2011, 47 of 126 adult recipients showed indications of HCV-related liver disease. The HTLV-1 prevalence rate was 7.8% (11/140) in the recipients and 2% (3/140) in the donors. Fourteen of the 140 recipients were pediatric recipients. HCV-related LDLT was observed only in adults. All HTLV-1 infected recipients were adult cases. First, we evaluated impact of HTLV-1 for LDLT in adult cases. In HCV-related LDLTs (Fig. 1a), graft and patients survival was worsened by the presence of HTLV-1 infection of recipients. The 1-, 3-, and 5-year survival rates in the HCV/HTLV-1-co-infected group were 67%, 32%, and 15%, respectively, and the corresponding rates in the HCV-mono-infected group were 80%, 67%, and 67%, respectively. Only the 5-year survival rate was found to be statistically significant ($P = 0.04$, log-rank method). However, adult recipients without HCV infection did not develop graft loss and patient death (Fig. 1b). In HCV-related LDLTs, clinical and demographic characteristics in HTLV-1-positive and HTLV-1-negative recipients did not differ between groups, except for donor age (Table 1). We attempted to clarify the factors of graft and patient survival in HCV-infected recipients by univariate analysis. MELD score and donor age at transplantation in the HTLV-1-infected recipients were shown to be significant factors. However, according to multivariate analysis, only donor age was a factor in worsening prognosis ($P < 0.05$; Relative risk 1.048). Three types of IL28B SNPs were not associated with graft and patient survival in HCV infected recipients according to a log rank test and univariate analysis of a Cox proportion hazard test.

Second, we analyzed stratification by donor age. Clinical characteristics, shown in Table 1, in the recipients who tested positive and negative for HTLV-1 did not differ between groups. In HCV-related LDLT recipients from old age donor group (age, 50 years and more; co-infected, 3 cases; HCV mono-infected, 13 cases), graft and patient survival was not worsened by recipient HTLV-1 infection (log-rank test, not significant). However, in the young age donor group (age less than 50 years; co-infected, 4 cases; HCV mono-infected, 34 cases), graft and patient survival was significantly worsened by recipient HTLV-1 infection (log-rank test, $P < 0.05$). However, graft and patient survival in HCV/HTLV-1-co-infected patients did not differ between the old and young donors, and the outcomes of HCV-mono-infected patients differed between the old and young donors according to the log-rank test. On the basis of multivariate analysis using a Cox proportional hazard test, HTLV-1 infection in HCV-infected recipients who received the transplant from younger donors was the only factor contributing to a worsened prognosis ($P = 0.03$; relative risk, 0.207).

Finally, we present the profile of seven cases of HCV/HTLV-1 co-infected recipients (Table 2). In the HCV/HTLV-1 co-infected group, chronic rejection (CR) developed in 3 patients, cases 60, 80, and 117, during peg-interferon/ribavirin treatment. CR did not develop in HCV-mono-infected patients. However, the CR rate was not statistically significant between the HCV/HTLV-1-co-infected group and the HCV-mono-infected group. Patients with CR did not have a prior history of acute rejection and used cyclosporine as an immunosuppressant. HCV-RNA disappearance during peg-interferon combination treatment with ribavirin was not observed in 3 CR cases. The period of peg-interferon combination treatment with ribavirin is 47, 23, and 2 months for HTLV-1/HCV co-infected CR patients. The treatment regimen of the

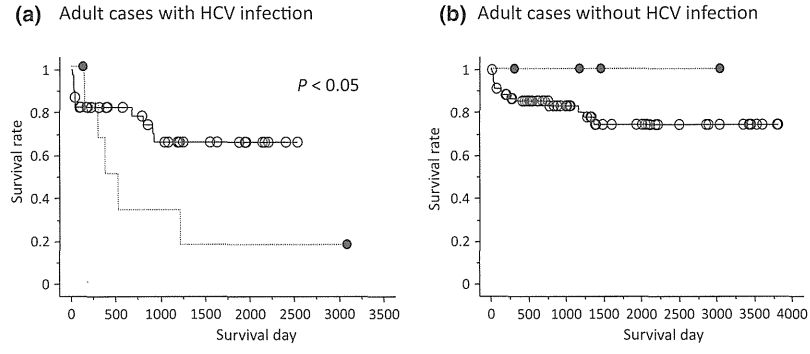

Figure 1 Kaplan–Meier curves for graft and recipient survival in adult transplant cases. A Kaplan–Meier curve revealed adult recipients with HCV infection (a) and without HCV infection (b). Black circle and dot line indicate HTLV-1 positive recipients and white circle and solid line indicate HTLV-1 negative recipients. In adult case with HCV-related LDLT, graft and patient survival of HTLV-1-infected recipients significantly decreased (a, $P < 0.05$).

Table 1. Clinical characteristics in patients with HCV infection.

	HTLV-1 positive	HTLV-1 negative	*P*-value
Number	7	40	
Age (years)	57.6 (7.16)	58.5 (6.48)	NS
Gender M/F	6/1	20/20	NS
BMI	25.6 (5.17)	25.0 (3.08)	NS
CP-score	10.6 (3.25)	9.85 (2.27)	NS
MELD	11.8 (8.38)	13.6 (8.16)	NS
DM +/-	2/5	20/20	NS
HCC +/-	4/3	27/13	NS
Donor age	48 (14.5)	37.6 (11.8)	0.04
Donor gender M/F	6/1	18/22	NS
Donor BMI	21.9 (2.42)	22.0 (2.62)	NS
Matching +/-	7/0	32/8	NS
Bleeding volume (ml)	5222 (3840)	14 182 (21 230)	NS
Surgery time (min)	902 (171)	934 (215)	NS
HCV GT1/non GT1	6/1	30/10	NS
HCV high titer in GT1	5	28	NS
IL28B SNP Major/Minor	1/2	24/6	NS
SVR rate	1/7	9/40	NS

Data are shown as means (standard deviation) and numbers, with statistical analysis assessed using a Mann–Whitney test for means and χ^2 test for numbers. Statistically significant difference between HTLV-1 positive and negative groups is $P < 0.05$. CP-score, Child–Pugh score; HCV GT, HCV genotype; Matching, Blood type matching; IL28B SNP Major, TT of rs8099917 in recipient and donor; Minor, TG or GG of rs8099917 in recipient and/or donor. BMI, body weight (kg)/height (m)/height (m). SVR, sustained viral response.

Table 2. Clinical characteristics in recipients with HCV and HTLV-1 co-infection.

Case number	20	59	60	80	112	117	132
Age (years)	58	50	68	65	48	54	58
Gender	M	M	M	F	M	M	M
Survival	+	+	–	–	–	–	–
Survival time (day)	3086	1210	528	378	139	301	132
Cause of death	–	HCC	CR	CR	Infect.	CR	–
IFN	+	–	+	+	–	+	–
Viral response	–	–	–	–		–	
IFN period (month)	48		47	23		2	
HCV GT1b	1b	1b	1b	1b	1b	N	1b
BMI	19.3	31.1	23.2	30.5	19.2	30.2	25.9
HCC	–	outside	Milan	Milan	–	Milan	–
MELD	8.1	8.5	4.4	7.3	29.3	15.2	9.9
DM	–	+	+	–	+	+	+
Donor	Sister	Brother	Child	Brother	Brother	Uncle	Child
Donor age	56	45	41	61	46	65	22
Donor HTLV-1	+	–	–	–	–	–	–

Viral response is the disappearance of HCV-RNA in patients under peg-IFN/ribavirin treatment. IFN period is treatment length (month) of peg-IFN/ribavirin treatment. Infect., infection; AIH, autoimmune hepatitis; BA, biliary atresia; LCN, cryptogenic cirrhosis; LCB, hepatitis B virus infected liver cirrhosis. Milan, HCC within Milan criteria, Outside, HCC without Milan criteria.

peg-interferon combination with ribavirin was performed under the rules of our hospital and was the same as was conducted for other HCV-related transplanted patients [19]. Hence, as an immunosuppressive therapy, tacrolimus was used for all HCV-infected patients as an induction therapy combined with steroid tapering; subsequently, tacrolimus treatment was intentionally replaced with cyclosporine treatment to facilitate interferon therapy [20,21] except in case 20. Case 20, which involved an HTLV-1 infected donor, suffered an onset of HAM and sialadenitis under the tacrolimus immunosuppressant regime [11]. Five cases of death occurred in the co-infected group. Causes of death in patients with HTLV-1/HCV co-infection included hepatoma recurrence, infection, and CR. ATL was not observed in this study. Progression of HCV and/or HTLV-1 infection was not always related to death. In particular, all CR cases developed during interferon treatment. Poor survival of HTLV-1/HCV-co-infected patients may have been caused by CR. HCV-RNA levels decreased in the CR cases when the length of peg-IFN/ribavirin treatment was less than 1 year.

Discussion

In this study, we clarified that HTLV-1 infection in HCV-infected recipients is an exacerbation factor involved in survival of both the graft and the patient. Particularly, young donors suffer detrimental effects caused by HTLV-1 infection. Survival of HCV-infected recipients is affected by donor age, MELD score, and HTLV-1 infection. Donor age is the most significant factor in graft and patient survival, and HTLV-1 infection in recipients is the second most important factor in survival in HTLV-1 endemic areas. Donors of advanced age and high MELD scores have been reported as complicating factors [22,23]. We report the impact of HTLV-1 infection on graft and patient survival for the first time.

The presence of HTLV-1 infection as a complicating factor in recipients was revealed after adjusting for age. As HCV/HTLV-1 co-infection occurred in three cases in older donors and four cases in younger donors, it was necessary to determine the role of donor age in HCV/HTLV-1-co-infected recipients. In the HTLV-1 infection-negative group, graft and patient survival was shorter in older donors than in younger donors, but in the HCV/HTLV-1-co-infected group, graft and patient survival did not differ between the old and young donors. Donor age is a complicating factor for graft and patient survival regardless of HTLV-1 infection [23]. The survival rate of the young donor group may initially be high, but survival rate decreases in the presence of HTLV-1 infection. HTLV-I possesses a unique and innate (or acquired) capacity to preserve cellular immunity, such as IL-2 and

IL-2-receptor induction [24]. HTLV-1 infection may lead to a stronger immune response in recipients when the donor is young than when the donor is old.

The relationship among IFN, HCV, and CR at post-liver transplantation has been previously studied. It is reported that peg-IFN/ribavirin treatment for HCV may trigger rapid CR in patients with therapeutic immunosuppressant trough levels, with or without first inducing acute cellular rejection [25]. Other reports state that the use of cyclosporine, ribavirin discontinuation, a peg-IFN treatment duration of over 1 year, and HCV infection elimination for IFN treatment appear to be associated with CR [26,27]. We suspect that HTLV-1-infected recipients under peg-IFN/ribavirin treatment may be associated with CR for young graft donors and have different immunological mechanisms than HTLV-1 negative recipients.

Recently, the relationship of IL28B SNP and HCV infection has been studied [28]. It has been reported that IL28B SNP is not only related to the effect of IFN treatment, but also to the natural course of HCV infection [29]. We conducted an analysis of IL28B SNP in only 33 pairs of donors and recipients who had obtained agreement in 47 cases of HCV-related liver transplantation. In this study, Il28B SNP was not related to graft and patient survival. However, upon analysis of three types of IL28B SNPs, the survival rate was the same for all three SNPs. Previous reports state that there are no statistical differences in overall graft survival according to recipient and donor IL28B SNPs [30,31].Since it is reported that IL28B SNPs in both recipients and donors is associated with IFN response [30–32], differences in long-term survival between IL28B SNP groups has been examined.

Due to the low prevalence HTLV-1 infection in western countries, the association of liver disease and HTLV-1 infection has not been evaluated. In this study, performed in an HTLV-1 endemic area, we determined that HTLV-1 increases mortality after HCV-related LDLT. Presently, to improve mortality rates, the presence of CR should be determined when HCV/HTLV-1 co-infected transplanted patients are treated using IFN/ribavirin. However, as CR treatment has not been fully evaluated, the mechanism of HTLV-1 infected T cells in HCV-infected graft liver patients under peg-IFN/ribavirin treatment should be determined. The follow-up period of the seven HCV/HTLV-1-co-infected patients was 132–3086 days. As our study population was small and follow-up periods were short, we will extend the follow-up period to validate our results.

Authorship

TI, NT, HM, TM, MO, SE, MT, AS, MH, SO, TU, SM: performed study. SK, TK and KN: designed the study. TI: wrote the manuscript.

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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

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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

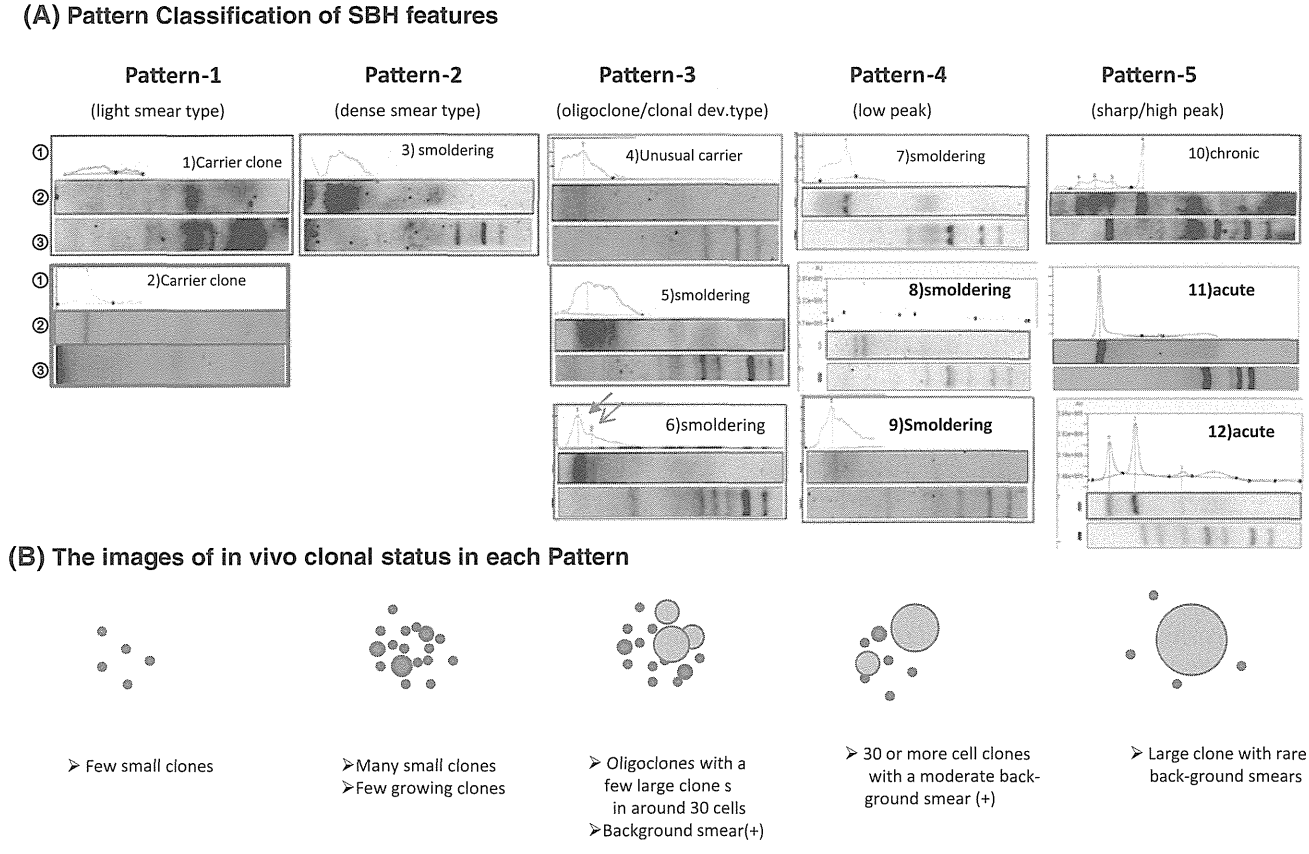

Fig. 1 **a** The densitometry patterns for HTLV-1 proviral integration status according to SBH band features using a restrictive enzyme of Eco-R1 and representative cases. Subjects were mainly classified into five patterns according to densitometer images. ① Densitometry graph. ② SBH analysis for Eco-R1-digestive 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

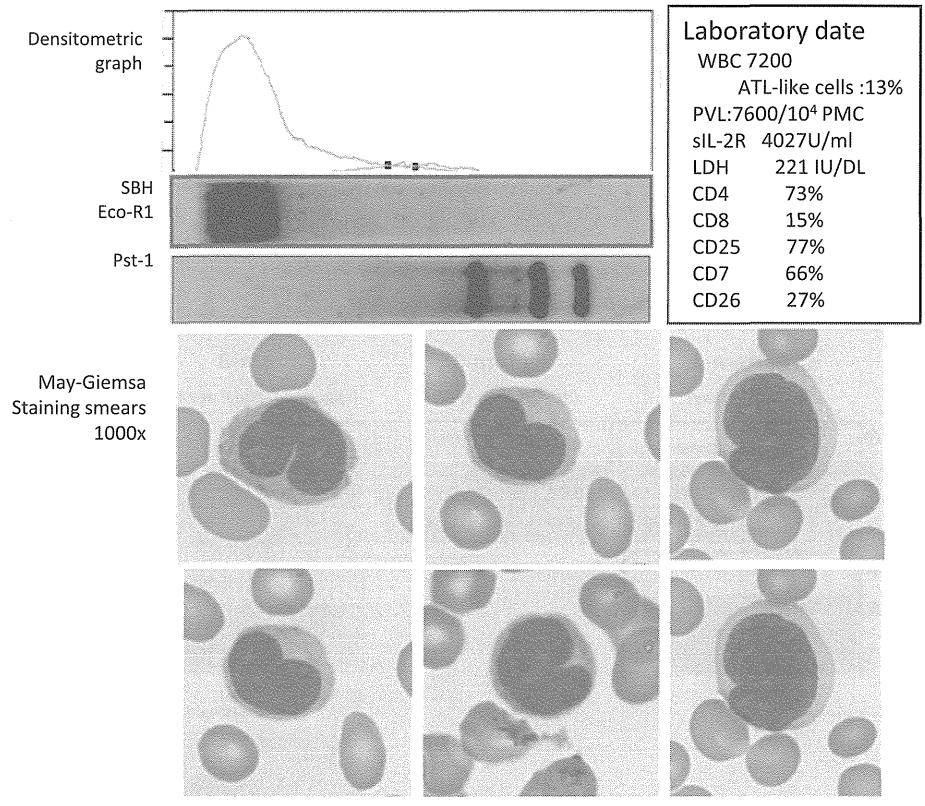

Fig. 2 A representative case presentation of the polyclonal smoldering subtype (SM) showing a polyclonal dense smear in SBH analysis and the smoldering subtype with full hematological criteria

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 multi-step 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)

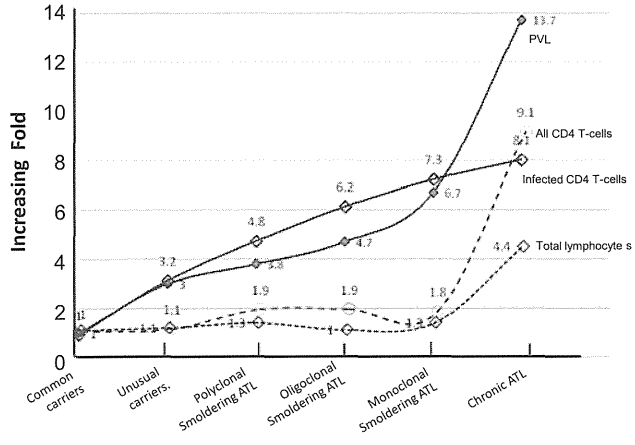

Fig. 3 The difference in the line-graph patterns between HTLV-1-infected and uninfected cells. PVL and infected CD4 T-cell counts gradually increased from the early carrier stage to the last stage of smoldering, while the HTLV-1-uninfected cell population was stable, indicating that the discrepancy was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

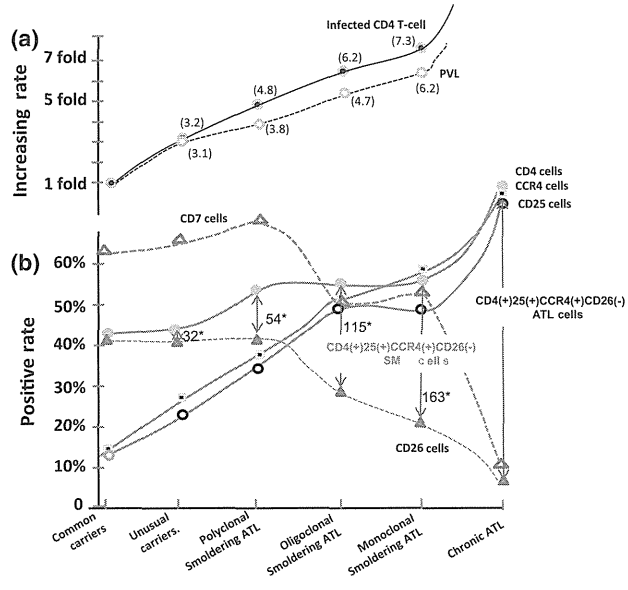

Fig. 4 The rate of change in each ATL-related biomarker. There were two major types of curves; a concurrent increasing type with tumor burden and a decreasing type with aberrant down-regulation. **a** The increasing rate (fold) relative to 102 HTLV-1-infected CD4 T-cells in 1 μL of blood. **b** Comparison 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

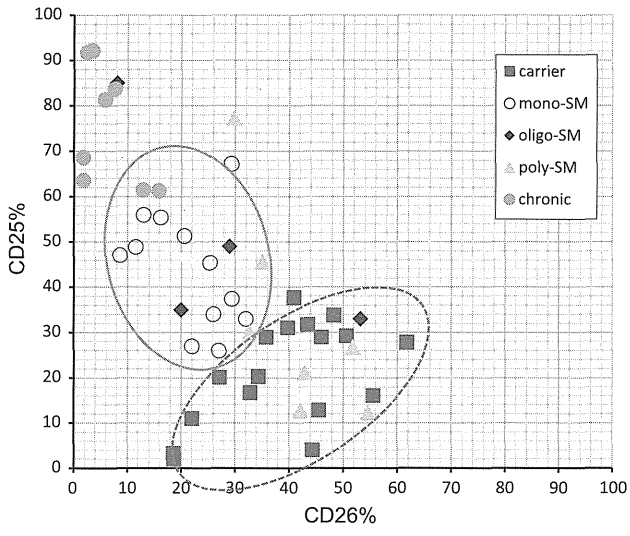

Fig. 5 CD26 versus CD25 twin dot graph, showing that there were two hot areas. The cells clustering in the same area have uniform biocharacteristics. Monoclonal SM was concentrated in the same area, but polyclonal SM and carriers were distributed sparsely and widely. *Red squares within dotted line circle*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

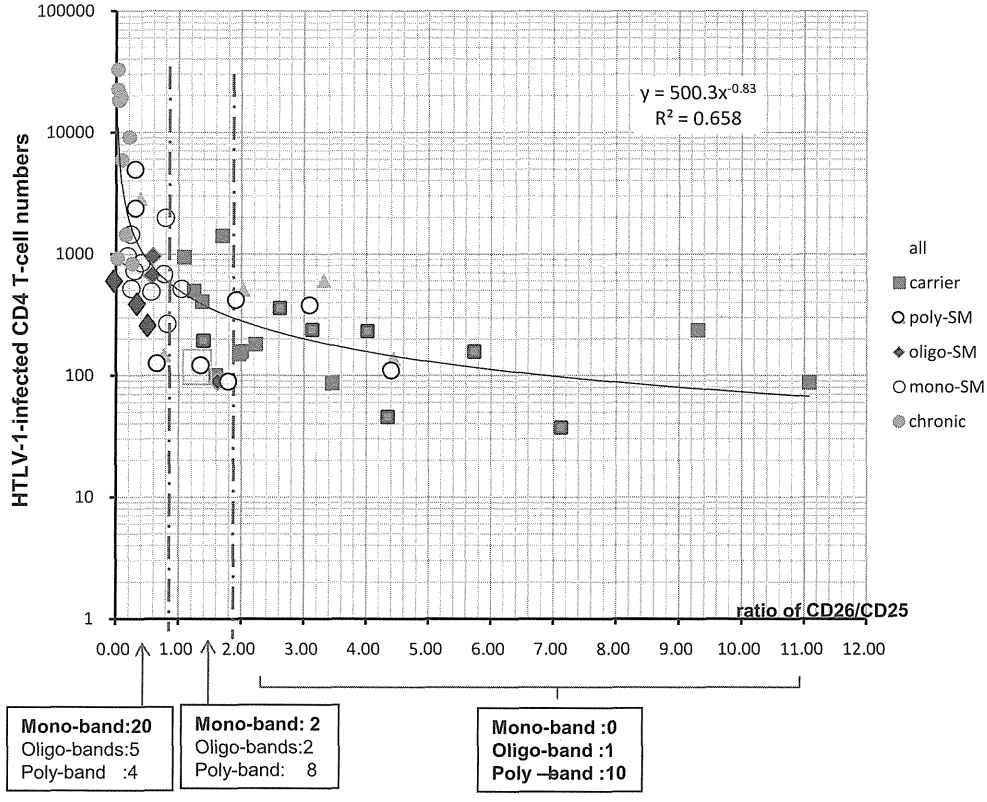

Fig. 6 Three parameter "twin dot-plot graph" between HTLV-1-infected CD4 T-cell numbers and the ratio of CD25/CD26, and the carrier or disease subtypes. Samples with the same band pattern showed a tendency to gather in the same areas bordered by the CD25/26 ratio lines, such as most samples with monoclonal 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

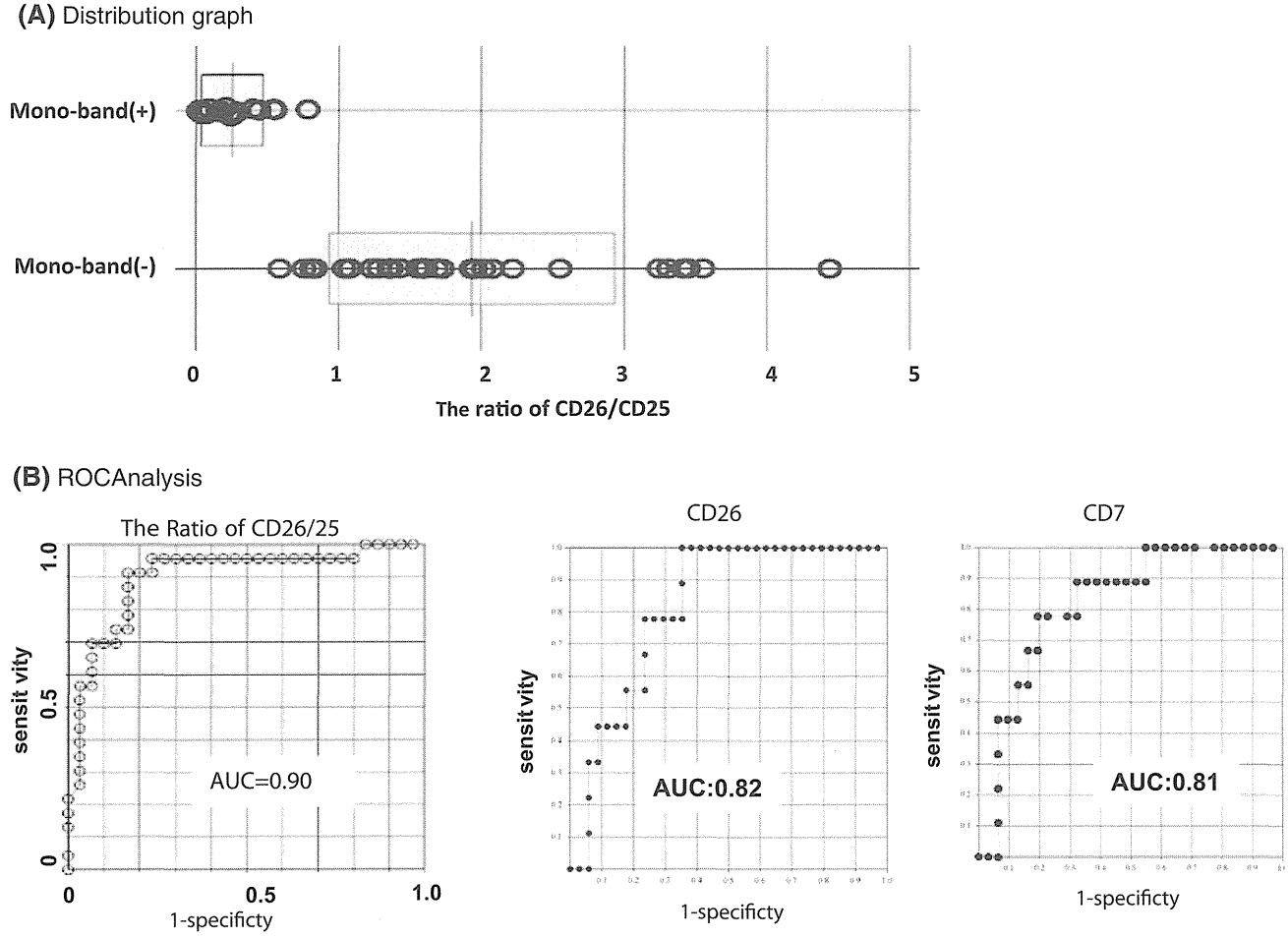

Fig. 7 **a** The distribution graph of each sample with or without a monoclonal band. The median values of mono-band (+) or (−) were 0.32 and 1.98. **b** The receiver operating curve (ROC) analysis

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

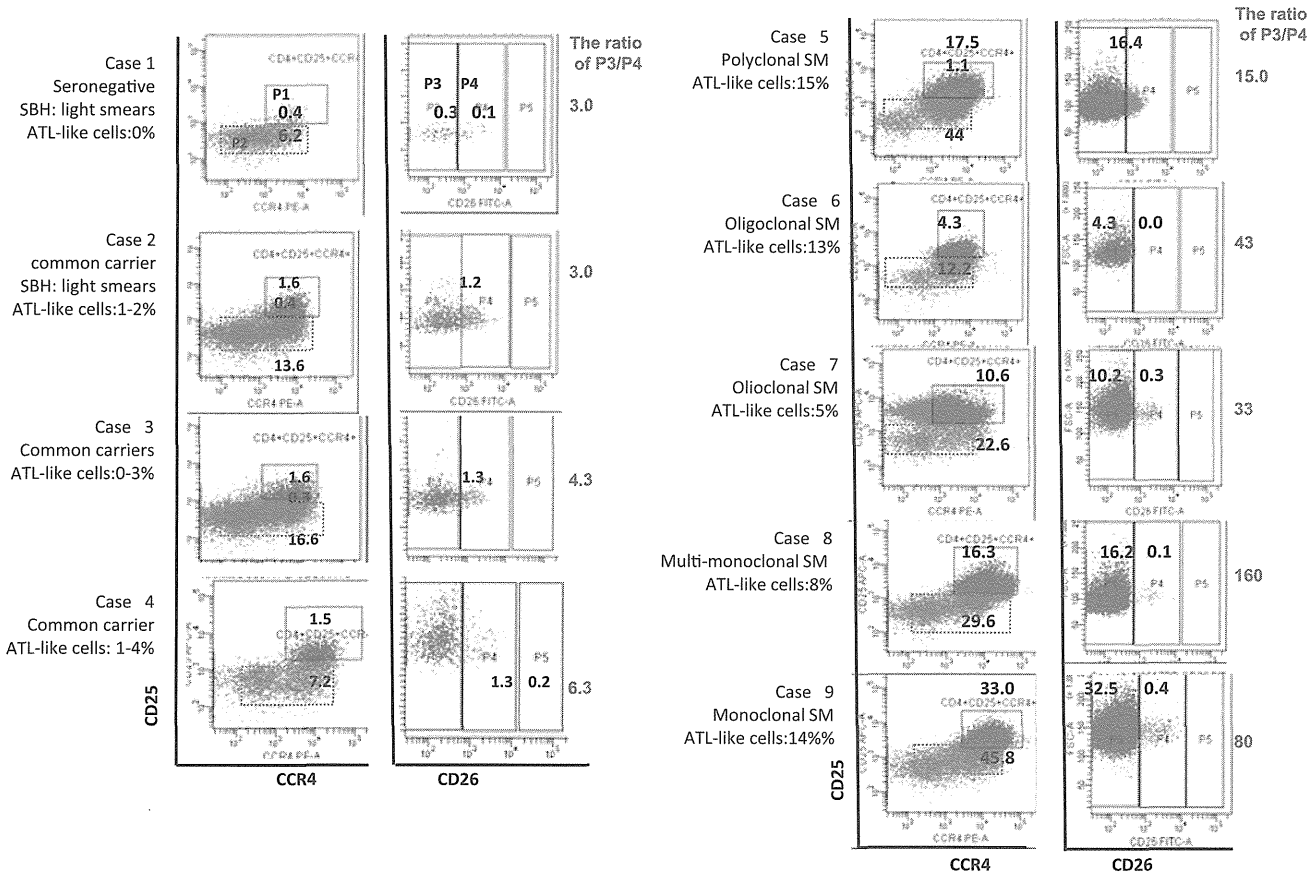

Fig. 8 Four-color flow cytometry for CD4, CD25, CCR4 and CD26. After CD4 gating, gating-CD4 T-cells were developed into a cytograph (a CCR4 = *X*-axis, and CD25 = *Y*-axis), and then P1 gating cells were developed into a cytograph (b CD26 down-regulation positive (*blue* P3 area meaning loss of CD26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

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Conflict of interest The authors have no conflict of interest.

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