subpopulation was a minor but distinct subpopulation. In contrast, the highest cell density was observed in the $CD3^{dim}CD7^{low}$ subpopulation in all acute-type ATL samples except for patient no. 4, from whom the sample was obtained under conditions of well-controlled ATL during chemotherapy. These subpopulations were distinct but the expression pattern of the CD3 *vs* CD7 plot, such as the degree of downregulation of CD3 and CD7, was variable among patients. The proportion of the $CD3^{dim}CD7^{low}$ subpopulation was significantly higher in acute-type ATL $CD4^+$ lymphocytes than in normal controls (Fig. 1C).

**Analysis of the HTLV-I proviral load in $CD3^{high}CD7^{high}$, $CD3^{dim}CD7^{dim}$ and $CD3^{dim}CD7^{low}$ subpopulations.** We next estimated the HTLV-I proviral load by quantitative real-time PCR in each FACS-sorted subpopulation. Representative results from three patients with acute-type ATL (patients no. 6, 7 and 8) are shown in Figure 2. In all patient samples, HTLV-I proviral integration, analyzed by real-time PCR, was detected in all subpopulations. However, the proviral load (%) was significantly higher in $CD3^{dim}CD7^{dim}$ and $CD3^{dim}CD7^{low}$ subpopulations compared with the $CD3^{high}CD7^{high}$ subpopulation. The proviral load of the $CD3^{dim}CD7^{low}$ subpopulation in patients no. 7 and 8 was nearly 200%, indicating integration of two copies of the HTLV-I viral genome and that almost all of the cells were infected with HTLV-I. Similarly, in patient no. 6, the majority of the $CD3^{dim}CD7^{low}$ subpopulation was infected with HTLV-I. A substantial proportion of the $CD3^{dim}CD7^{dim}$ subpopulation was infected with HTLV-I in patients no. 7 and 8, and nearly all the cells in the same subpopulation in patient no. 6 were infected with HTLV-I.

**Differences in the immunophenotype of $CD3^{high}CD7^{high}$, $CD3^{dim}CD7^{dim}$ and $CD3^{dim}CD7^{low}$ subpopulations in patients with acute-type ATL.** To further characterize these three subpopulations, we next examined CCR4 and CD25 expression in each subpopulation. Representative results of a normal control and a patient with acute-type ATL are shown in Figure 3A. The mean fluorescence intensities (MFI) of CD25 and CCR4 of each sub-

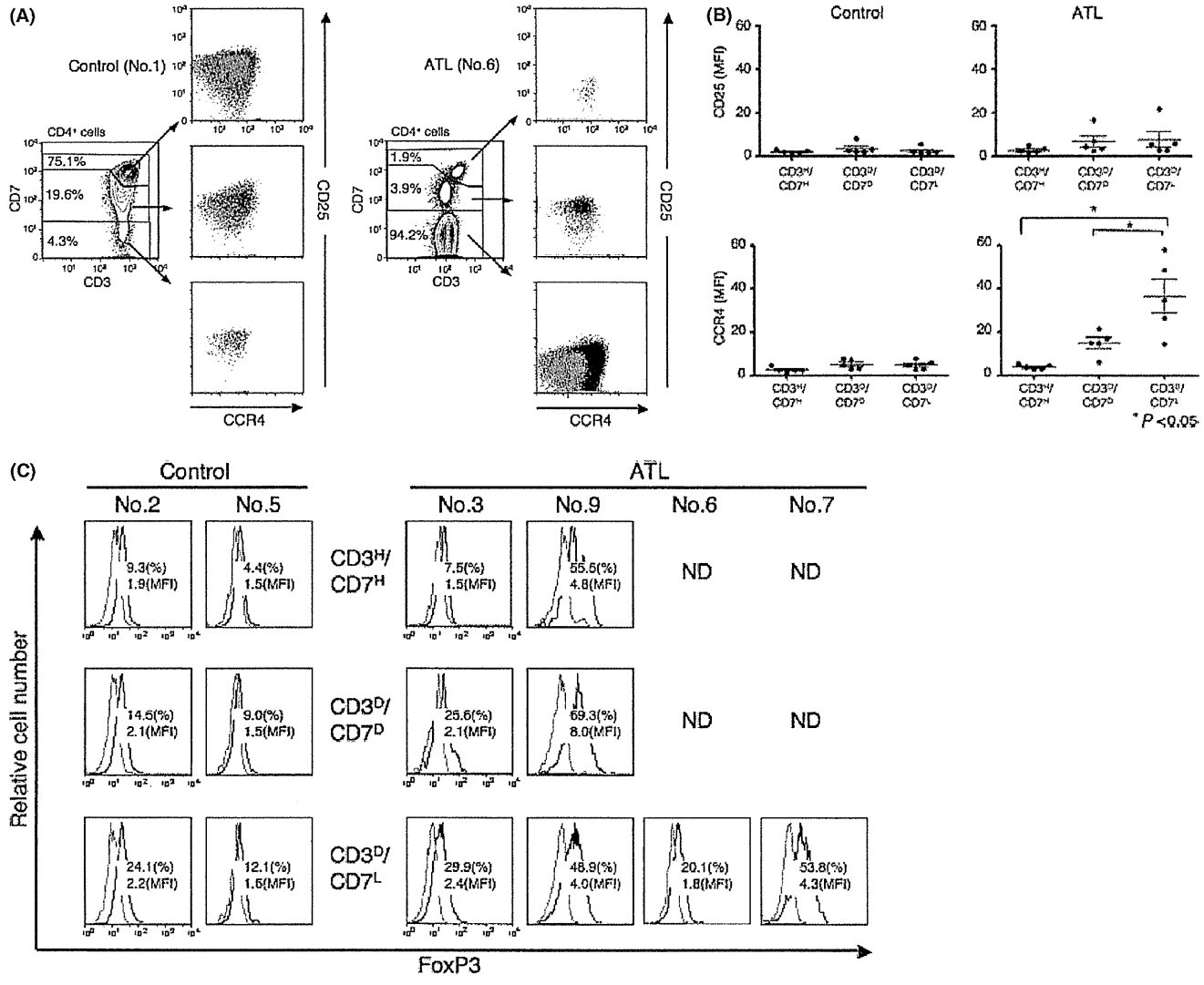


**Fig. 3.** Immunophenotypic analysis in $CD3^{high}CD7^{high}$, $CD3^{dim}CD7^{dim}$ and $CD3^{dim}CD7^{low}$ subpopulations. (A) Expression of CCR4 and CD25 in each subpopulation. Representative FACS data of a normal control (no. 1) and a patient with adult T-cell leukemia (ATL) (no. 6) are shown Gray dots, isotype antibody-stained cells; black dots, specific antibody-stained cells. (B) Mean fluorescence intensity (MFI) of CD25 and CCR4 in each subpopulation from all normal controls and patients with ATL. The MFI is shown in arbitrary units defined as follows: MFI of specific antibody/MFI of isotype antibody. Each dot represents a sample $*P < 005$ by ANOVA. (C) Expression of FoxP3 in each subpopulation. ND, analysis could not be performed in the $CD3^{high}CD7^{high}$ and $CD3^{dim}CD7^{dim}$ subpopulations in patients no. 6 and 7 due to an insufficient number of cells.



doi: 10.1111/j.1349-7006.2010.01833.x

population in all patients with ATL and normal controls are shown in Figure 3B. Both CCR4 and CD25 expression levels were very low and maintained at similar levels throughout all subpopulations in normal control cells and in the $CD3^{high}CD7^{high}$ subpopulation of patients with ATL. In contrast, CCR4 expression was significantly upregulated in the $CD3^{dim}CD7^{dim}$ and $CD3^{dim}CD7^{low}$ subpopulations of patients with ATL compared with the $CD3^{high}CD7^{high}$ subpopulation. The expression of CD25 was also upregulated in these subpopulations but this difference was not significant ($P = 0.36$). The expression of Forkhead box P3 (FoxP3), a master regulator in the development and function of regulatory T (Treg) cells,[14] was also analyzed in some patients. As shown in Figure 3C, FoxP3 expression in the $CD3^{dim}CD7^{low}$ subpopulations was variably upregulated among patients. In addition, in patient no. 9, FoxP3 was upregulated in the $CD3^{high}CD7^{high}$ and $CD3^{dim}CD7^{dim}$ subpopulations.

**Analysis of clonality in the $CD3^{high}CD7^{high}$, $CD3^{dim}CD7^{dim}$ and $CD3^{dim}CD7^{low}$ subpopulations by inverse long PCR.** To further analyze the enrichment of ATL cells in the $CD3^{dim}CD7^{low}$ subpopulation, we estimated clonality in each FACS-sorted subpopulation by inverse long PCR in four patients with acute-type ATL (Fig. 4). An intense band, suggesting a major clone, was detected in the $CD3^{dim}CD7^{low}$ subpopulations in all patients. In the same subpopulation, multiple bands with weak intensity were also observed. As the levels of DNA extracted from the $CD3^{dim}CD7^{low}$ subpopulation were sufficient, we performed duplicate PCR in three patient samples (Fig. 4B–D). Detection of the major bands was consistent, but the presence of the minor bands was variable. In the $CD3^{dim}CD7^{dim}$ subpopulations, bands of the same size as those of the $CD3^{dim}CD7^{low}$ subpopulations were observed, indicating that a distinct population in the $CD3^{dim}CD7^{dim}$ subpopulations belonged to identical clones.

**Clonality in the $CD3^{high}CD7^{high}$, $CD3^{dim}CD7^{dim}$ and $CD3^{dim}CD7^{low}$ subpopulations by flow cytometry-based TCR-Vβ repertoire analysis.** To further confirm clonality and to evaluate the degree of enrichment in each subpopulation, we performed TCR-Vβ repertoire analysis by flow cytometry[15] in three ATL cases. The representative results are shown in Figure 5. In patient no. 3, over 95% of the $CD3^{dim}CD7^{low}$ subpopulation used specific TCR-Vβ (Vβ9) and their proportion was quite low in the $CD3^{high}CD7^{high}$ and $CD3^{dim}CD7^{dim}$ subpopulations. In addition, in the two other cases, over 90% of cells in the $CD3^{dim}CD7^{low}$ subpopulation used the same TCR-Vβ (data not shown). These results indicate that ATL cells are highly purified in the $CD3^{dim}CD7^{low}$ subpopulation.

**Differences in morphology of the $CD3^{high}CD7^{high}$, $CD3^{dim}CD7^{dim}$, and $CD3^{dim}CD7^{low}$ subpopulations in patients with acute-type ATL.** We reviewed the glass-slide specimens of FACS-sorted samples to evaluate the morphology of each subpopulation on the CD3 *vs* CD7 plots. Representative results for two patients (no. 6 and 7) are shown in Figure 6A. In both patients, atypical lymphocytes with notched nuclei and/or basophilic cytoplasm were observed in all three subpopulations. In contrast, abnormal lymphocytes, including cells with multilobulated nuclei (flower cells) were mainly observed in the $CD3^{dim}CD7^{low}$ subpopulation in patient no. 6 (Fig. 6, left) and in the $CD3^{dim}CD7^{dim}$ and $CD3^{dim}CD7^{low}$ subpopulations in patient no. 7 (Fig. 6, right panel).

## Discussion

To investigate the characteristics of ATL cells, the purification of tumor cells is essential. In the present study, we successfully discriminated the $CD3^{dim}CD7^{low}$ subpopulation in $CD4^+$ T cells in the peripheral blood of patients with acute-type ATL by constructing a CD3 *vs* CD7 plot of $CD4^+$ T cells from multi-color FACS (Fig. 1). Previously, Yokote *et al.*[10] reported that $CD3^{low}$ gating facilitated the discrimination of ATL cells by flow cytometry. If we constructed a CD4 *vs* either CD3 or CD7 plot, in which the downregulated cell subpopulation was not clearly separated, then we could not define distinct cell subpopu-

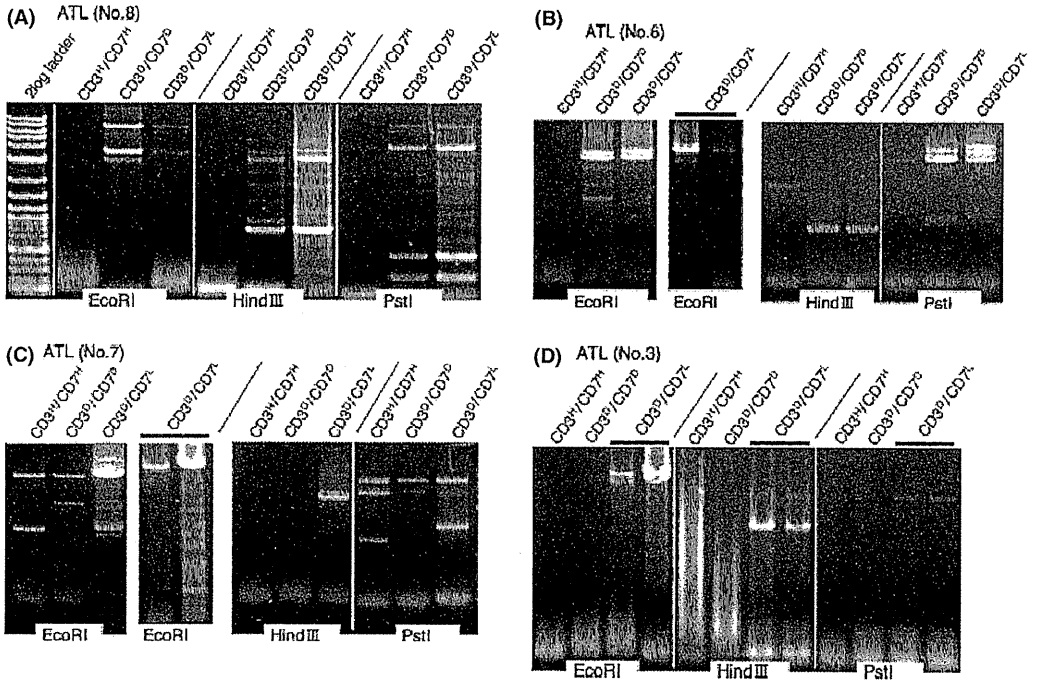


Fig. 4. Analysis of clonality in the $CD3^{high}CD7^{high}$, $CD3^{dim}CD7^{dim}$ and $CD3^{dim}CD7^{low}$ subpopulations using inverse long PCR. (A–D) Genomic DNA was extracted from FACS-sorted cells of each subpopulation and subjected to inverse long PCR. Representative data of four cases (patients no. 3, 6, 7 and 8) are shown For the $CD3^{dim}CD7^{low}$ subpopulations of patients no. 3, 6 and 7, PCR was performed in duplicate (black bars). ATL, adult T-cell leukemia.

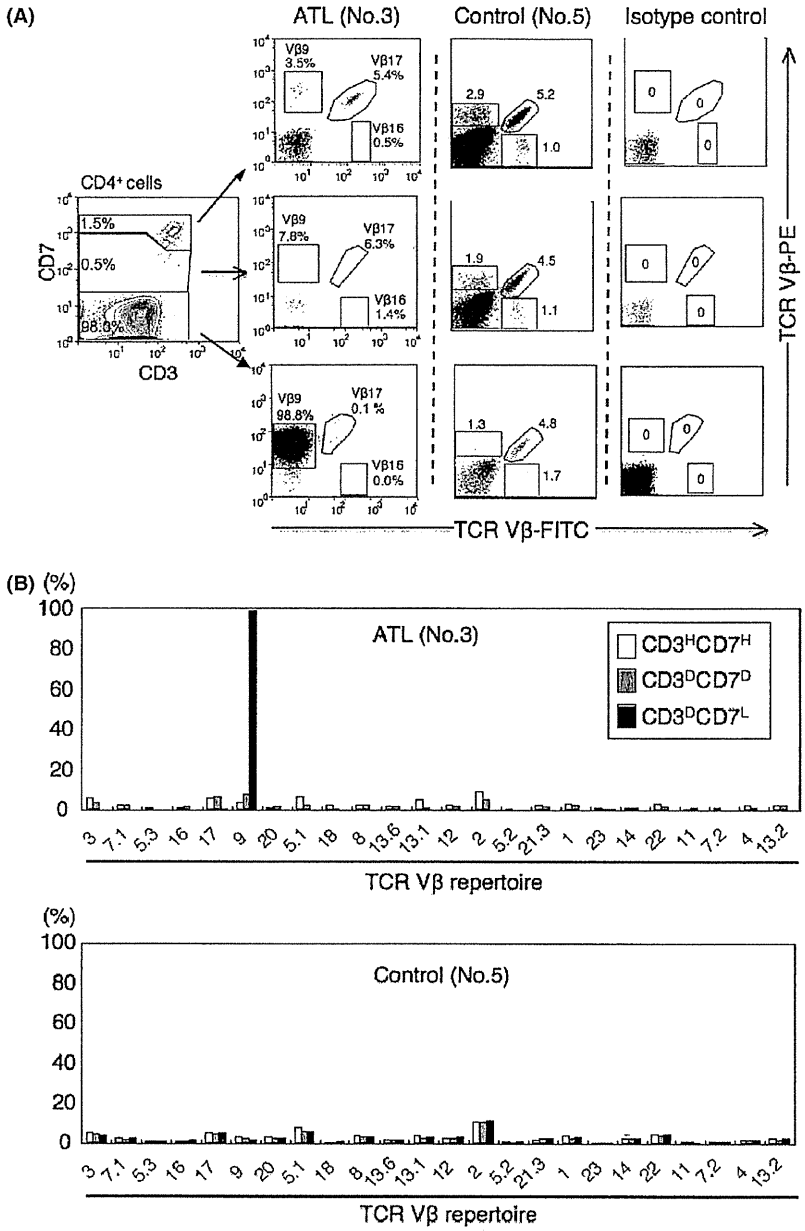


**Fig. 5.** Clonality in the $CD3^{high}CD7^{high}$, $CD3^{dim}CD7^{dim}$ and $CD3^{dim}CD7^{low}$ subpopulations using flow cytometry-based T-cell receptor (TCR)-Vβ repertoire analysis. (A) Representative data are shown A monoclonal pattern of TCR-Vβ9 expression was evident in the $CD3^{dim}CD7^{low}$ subpopulation of the adult T-cell leukemia (ATL) sample. Representative dot plots of 3 of the 24 TCR-Vβ repertoire (Vβ9, 16 and 17) are shown. (B) Bar graph representation of the data from Figure 5A. The percentages of cells positive for each TCR-Vβ repertoire in the $CD3^{high}CD7^{high}$, $CD3^{dim}CD7^{dim}$ and $CD3^{dim}CD7^{low}$ subpopulations. White bar, $CD3^{high}CD7^{high}$; gray bar, $CD3^{dim}CD7^{dim}$; black bar, $CD3^{dim}CD7^{low}$.

lations using the CD3 or CD7 marker alone, because the degrees of downregulation of CD3 and CD7 are variable. It should be noted that the combination of CD3 downregulation and diminished expression or absence of CD7 clearly indicates this subpopulation. In addition, gating-out monocytes in the CD4 *vs* CD14 plot is important for the CD3 *vs* CD7 plot because monocytes were CD3/CD7 dull-positive based on the nonspecific binding of the antibody.

A substantial subpopulation of T cells has been reported to be CD7-deficient under physiological[16,17] and certain pathological conditions, including autoimmune disorders and viral infection.[18–22] Consistent with these reports, the present study indicated that the proportion of $CD4^+CD7^-$ T cells in the peripheral blood of healthy adults is up to 10% (Fig. 1B,C). In ATL samples, the CD3 *vs* CD7 plot revealed various patterns, which may reflect the differences in clinical characteristics of each patient; however, the $CD3^{dim}CD7^{low}$ subpopulation, which was a minor population in the normal controls, was prominent in all ATL samples (Fig. 1B,C). These results prompted us to study this subpopulation in detail. Estimation of the HTLV-I proviral load by quantitative real-time PCR showed that the majority of cells in the $CD3^{dim}CD7^{low}$ subpopulation were infected with HTLV-I (Fig. 2). Immunophenotypic analysis revealed that the expression of CD25, a common ATL marker,[9,23] and CCR4, reported to be expressed in around 90% of cases of ATL,[24,25] were upregulated in the $CD3^{dim}CD7^{low}$ subpopulations of ATL samples in contrast to normal controls in which both markers were weakly expressed in the equivalent subpopulation (Fig. 3A,B). As several studies indicated that ATL cells originate from $CD4^+CD25^+FoxP3^+$ Treg cells,[26] we next analyzed FoxP3 expression in each subpopulation. In the $CD3^{dim}CD7^{low}$ subpopulation, the FoxP3 expression levels were variable, consistent with previous reports.[27] In one case, FoxP3 expression was upregulated in the $CD3^{high}CD7^{high}$ and $CD3^{dim}CD7^{dim}$ subpopulations suggesting that they were normal Treg cells.

The analysis of clonality is extremely important for determining whether cells are transformed and Southern blot analysis is usually used to confirm clonality. However, in the present study,



doi: 10.1111/j.1349-7006.2010.01833.x

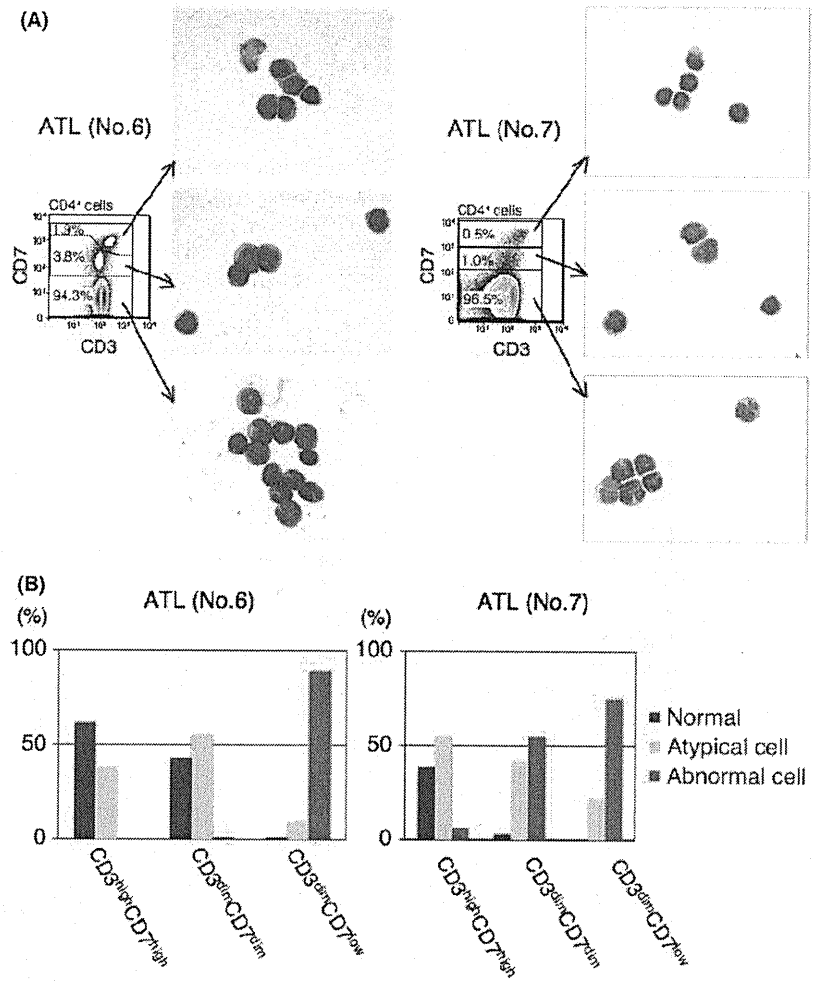


Fig. 6. Morphology of the $CD3^{high}CD7^{high}$, $CD3^{dim}CD7^{dim}$ and $CD3^{dim}CD7^{low}$ subpopulations in two representative adult T-cell leukemia (ATL) samples (patients no. 6 and 7). (A) May–Giemsa staining of FACS-sorted cells from each subpopulation from two patients with acute-type ATL. Top, $CD3^{high}CD7^{high}$ subpopulation; middle, $CD3^{dim}CD7^{dim}$ subpopulation; bottom, $CD3^{dim}CD7^{low}$ subpopulation. (B) Percentages of cells with different morphology in each subpopulation. Normal, lymphocytes with normal morphology; atypical, lymphocytes with notched nuclei and basophilic cytoplasm; abnormal, lymphocytes with convoluted, deeply indented or multilobulated flower cells.

the cell number following cell sorting was not sufficient for Southern blotting, and thus inverse long PCR for clonality analysis of HTLV-I-infected cells was used.[25] Studies of four ATL samples revealed clonal expansion of ATL cells in the $CD3^{dim}CD7^{low}$ subpopulations, although minor clones may exist in the population (Fig. 4). When PCR was performed in duplicate, we found that the major bands were consistently detected in all cases. However, the detection of multiple minor bands was not consistent. As reported previously, the inverse long PCR method stochastically amplifies the template originating from small clones.[28,29] The minor bands observed in the present study will contain small clones. However, the presence of non-specific bands cannot be eliminated.

The inverse long PCR method is commonly used for clonality analysis; however, it cannot quantify the size of major/minor clones and the degree of enrichment in each subpopulation. Therefore, we tested the FACS-based TCR-Vβ repertoire analysis combined with our multi-color FACS system (Fig. 5). In ATL patient no. 3, almost all cells in the $CD3^{dim}CD7^{low}$ subpopulations were clonal cells with TCR-Vβ9. Inverse long PCR analysis in the same patient showed multiple minor bands in the $CD3^{dim}CD7^{low}$ subpopulations (Fig. 4D). These results did not conflict with those of the TCR-Vβ repertoire analysis, as the inverse long PCR method is a more sensitive method for detecting small clones compared with flow cytometry. Taken together, the series of analyses in the present study indicated that the $CD3^{dim}CD7^{low}$ subpopulations consist of highly purified ATL cells in patients with acute-type ATL.

A substantial proportion of cells in the $CD3^{dim}CD7^{dim}$ subpopulation consisted of morphologically abnormal lymphocytes (Fig. 6) that exhibited upregulation of CD25 and CCR4 expression (Fig. 3A,B). Using the inverse long PCR method, a similar band pattern between $CD3^{dim}CD7^{dim}$ and $CD3^{dim}CD7^{low}$ subpopulations was observed in patients no. 6 and 8, suggesting that these cells belonged to the same clone (Fig. 4A,B). However, not all of the cells in this subpopulation were infected with HTLV-I because the HTLV-I proviral load was less than that of the $CD3^{dim}CD7^{low}$ subpopulation (Fig. 2). Thus, at least a small number of the $CD3^{dim}CD7^{dim}$ cells were expected to be ATL cells. Those cells observed in the $CD3^{dim}CD7^{dim}$ subpopulation that were phenotypically different from the $CD3^{dim}CD7^{low}$ subpopulations were of particular interest. We detected a band of the same size on inverse long PCR in the $CD3^{dim}CD7^{dim}$ subpopulations as in the $CD3^{dim}CD7^{low}$ subpopulation. This may have been because the two subpopulations originated from the same clone that evolved from a $CD3^{dim}CD7^{dim}$ to a $CD3^{dim}CD7^{low}$ phenotype. Further studies are required to determine the characteristics of the $CD3^{dim}CD7^{dim}$ subpopulation in greater detail.

The results of the present study indicated that HTLV-I-infected cells distribute from a $CD7^{high}$ to a $CD7^{low}$ subpopulation, although the proportion of HTLV-I-infected cells was remarkably low in the $CD3^{high}CD7^{high}$ subpopulation (Fig. 2). A considerable proportion of cells in the $CD3^{high}CD7^{high}$ subpopulation consisted of morphologically atypical lymphocytes (Fig. 6), but the CD25 and CCR4 levels were not upregulated

(Fig. 3A,B). When analyzing the pattern of the inverse long PCR of the $CD3^{high}CD7^{high}$ subpopulations, we observed a difference from those of the $CD3^{dim}CD7^{dim}$ and $CD3^{dim}CD7^{low}$ subpopulations (Fig. 4). In patients no. 6 (Fig. 4B) and 7 (Fig. 4C), the band detected in the $CD3^{high}CD7^{high}$ subpopulation may represent an expanded clone that was not transformed. Most likely, these cells do not represent ATL cells, but oligoclonal HTLV-I-infected lymphocytes. Previous studies indicated that HTLV-I-infected cells undergo transformation through multi-step oncogenesis.[30] A detailed analysis of these three subpopulations may therefore provide some insight into the oncogenesis of HTLV-I-infected cells.

Accurate determination of ATL cells in peripheral blood is critical for estimating the response to chemotherapy. However, as discussed above, morphological studies (Fig. 6) have limitations in their ability to discriminate ATL from non-ATL cells.[31,32]. Recently, hematopoietic stem cell transplantation has been explored as a promising treatment to overcome the poor prognosis of this most incurable lymphoid malignancy,[33,34] and monitoring minimal residual disease following hematopoietic stem cell transplantation is more important. Our method of analyzing ATL cells may be particularly useful for monitoring minimal residual disease. Although the $CD3^{dim}CD7^{dim}$ subpopulation in our analysis may have included some ATL cells, this is a minor population in the peripheral blood of patients with acute-type ATL, and it is sufficient for practical use to monitor the $CD3^{dim}CD7^{low}$ subpopulation. Another possible use of our procedure is for the definitive classification of ATL subtypes according to Shimoyama's criteria.[8] A proportion of abnormal lymphocytes in peripheral blood comprise part of the criteria for ATL-subtype classification but it is sometimes confusing. Our multi-color FACS system may clearly quantify this proportion.

In conclusion, we have constructed a multi-color FACS system to purify ATL cells in the peripheral blood of patients with acute-type ATL. This system may be useful for precisely monitoring the disease during chemotherapy, detecting minimal residual disease and analyzing ATL cells. This system may be of great benefit in investigating oncogenesis in HTLV-I-infected cells.

## Acknowledgments

The authors thank Dr. Toshiki Watanabe, Dr. Kazumi Nakano and Dr. Tadanori Yamochi (The University of Tokyo) for providing the TL-Om1 cell line and plasmid containing the HTLV-I genome, which were used as standards for quantification of the proviral load. We are also grateful for their substantial technical assistance. We thank Dr. Naofumi Mastsuno (The University of Tokyo) for providing technical assistance regarding multi-color flow cytometry. We are grateful to the hospital staff that have made a commitment to providing high quality care for all of our patients. This study was supported by the Ministry of Education, Culture, Sports, Science and Technology and the Ministry of Health, Labor, and Welfare of Japan.

## Disclosure Statement

The authors declare no financial conflicts of interest.

## References

1 Yoshida M, Miyoshi I, Hinuma Y. Isolation and characterization of retrovirus from cell lines of human adult T-cell leukemia and its implication in the disease. *Proc Natl Acad Sci U S A* 1982; **79** (6): 2031–5.
2 Matsuoka M. Human T-cell leukemia virus type I (HTLV-I) infection and the onset of adult T-cell leukemia (ATL). *Retrovirology* 2005; **2**: 27.
3 Nicot C. Current views in HTLV-I-associated adult T-cell leukemia/lymphoma. *Am J Hematol* 2005; **78** (3): 232–9.
4 Ginaldi L, Matutes E, Farahat N, De Martinis M, Morilla R, Catovsky D. Differential expression of CD3 and CD7 in T-cell malignancies: a quantitative study by flow cytometry. *Br J Haematol* 1996; **93** (4): 921–7.
5 Campana D, Coustan-Smith E. Minimal residual disease studies by flow cytometry in acute leukemia. *Acta Haematol* 2004; **112** (1–2): 8–15.
6 Craig FE, Foon KA. Flow cytometric immunophenotyping for hematologic neoplasms. *Blood* 2008; **111** (8): 3941–67.
7 Akl H, Badran B, Dobirta G *et al.* Progressive loss of CD3 expression after HTLV-I infection results from chromatin remodeling affecting all the CD3 genes and persists despite early viral genes silencing. *Virol J* 2007; **4**: 85.
8 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.
9 Tsukasaki K, Hermine O, Bazarbachi A *et al.* Definition, prognostic factors, treatment, and response criteria of adult T-cell leukemia-lymphoma: a proposal from an international consensus meeting. *J Clin Oncol* 2009; **27** (3): 453–9.
10 Yokote T, Akioka T, Oka S *et al.* Flow cytometric immunophenotyping of adult T-cell leukemia/lymphoma using CD3 gating. *Am J Clin Pathol* 2005; **124** (2): 199–204.
11 Yamada Y, Tomonaga M, Fukuda H *et al.* A new G-CSF-supported combination chemotherapy, LSG15, for adult T-cell leukaemia-lymphoma: Japan Clinical Oncology Group Study 9303. *Br J Haematol* 2001; **113** (2): 375–82.
12 Roncador G, Brown PJ, Maestre L *et al.* Analysis of FOXP3 protein expression in human CD4+CD25+ regulatory T cells at the single-cell level. *Eur J Immunol* 2005; **35** (6): 1681–91.
13 Iwanaga M, Watanabe T, Utsunomiya A *et al.* Human T-cell leukemia virus type I (HTLV-I) proviral load and disease progression in asymptomatic HTLV-I carriers: a nationwide prospective study in Japan. *Blood* 2010; **116** (8): 1211–19.
14 Sakaguchi S. Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self. *Nat Immunol.* 2005; **6** (4): 345–52.
15 Morice WG, Kimlinger T, Katzmann JA *et al.* Flow cytometric assessment of TCR-Vbeta expression in the evaluation of peripheral blood involvement by T-cell lymphoproliferative disorders: a comparison with conventional T-cell immunophenotyping and molecular genetic techniques. *Am J Clin Pathol* 2004; **121** (3): 373–83.
16 Reinhold U, Abken H. CD4+ CD7- T cells: a separate subpopulation of memory T cells? *J Clin Immunol* 1997; **17** (4): 265–71.
17 Reinhold U, Abken H, Kukel S *et al.* CD7- T cells represent a subset of normal human blood lymphocytes. *J Immunol* 1993; **150** (5): 2081–9.
18 Aandahl EM, Quigley MF, Moretto WJ *et al.* Expansion of CD7(low) and CD7(negative) CD8 T-cell effector subsets in HIV-1 infection: correlation with antigenic load and reversion by antiretroviral treatment. *Blood* 2004; **104** (12): 3672–8.
19 Autran B, Legac E, Blanc C, Debre P. A Th0/Th2-like function of CD4 + CD7- T helper cells from normal donors and HIV-infected patients. *J Immunol* 1995; **154** (3): 1408–17.
20 Legac E, Autran B, Merle-Beral H, Katlama C, Debre P. CD4+CD7-CD57+ T cells: a new T-lymphocyte subset expanded during human immunodeficiency virus infection. *Blood* 1992; **79** (7): 1746–53.
21 Schmidt D, Goronzy JJ, Weyand CM. CD4+ CD7- CD28- T cells are expanded in rheumatoid arthritis and are characterized by autoreactivity. *J Clin Invest* 1996; **97** (9): 2027–37.
22 Willard-Gallo KE, Van de Keere F, Kettmann R. A specific defect in CD3 gamma-chain gene transcription results in loss of T-cell receptor/CD3 expression late after human immunodeficiency virus infection of a CD4+ T-cell line. *Proc Natl Acad Sci U S A* 1990; **87** (17): 6713–17.
23 Matsuoka M, Jeang KT. Human T-cell leukaemia virus type 1 (HTLV-I) infectivity and cellular transformation. *Nat Rev Cancer* 2007; **7** (4): 270–80.
24 Ishida T, Utsunomiya A, Iida S *et al.* Clinical significance of CCR4 expression in adult T-cell leukemia/lymphoma: its close association with skin involvement and unfavorable outcome. *Clin Cancer Res* 2003; **9** (10 Pt 1): 3625–34.
25 Yoshie O, Fujisawa R, Nakayama T *et al.* Frequent expression of CCR4 in adult T-cell leukemia and human T-cell leukemia virus type 1-transformed T cells. *Blood* 2002; **99** (5): 1505–11.
26 Yano H, Ishida T, Inagaki A *et al.* Regulatory T-cell function of adult T-cell leukemia/lymphoma cells. *Int J Cancer* 2007; **120** (9): 2052–7.
27 Abe M, Uchihashi K, Kazuto T *et al.* Foxp3 expression on normal and leukemic CD4+CD25+ T cells implicated in human T-cell leukemia virus type-1 is inconsistent with Treg cells. *Eur J Haematol* 2008; **81** (3): 209–17.



doi: 10.1111/j.1349-7006.2010.01833.x

28 Tanaka G, Okayama A, Watanabe T *et al.* 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; **191** (7): 1140–7.
29 Cavrois M, Wain-Hobson S, Wattel E. Stochastic events in the amplification of HTLV-I integration sites by linker-mediated PCR. *Res Virol* 1995; **146** (3): 179–84.
30 Verdonck K, Gonzalez E, Van Dooren S, Vandamme AM, Vanham G, Gotuzzo E. Human T-lymphotropic virus 1: recent knowledge about an ancient infection. *Lancet Infect Dis* 2007; **7** (4): 266–81.
31 Sakamoto Y, Kawachi Y, Uchida T *et al.* Adult T-cell leukaemia/lymphoma featuring a large granular lymphocyte leukaemia morphologically. *Br J Haematol* 1994; **86** (2): 383–5.
32 Tsukasaki K, Imaizumi Y, Tawara M *et al.* Diversity of leukaemic cell morphology in ATL correlates with prognostic factors, aberrant immunophenotype and defective HTLV-I genotype. *Br J Haematol* 1999; **105** (2): 369–75.
33 Okamura J, Utsunomiya A, Tanosaki R *et al.* Allogeneic stem-cell transplantation with reduced conditioning intensity as a novel immunotherapy and antiviral therapy for adult T-cell leukemia/lymphoma. *Blood* 2005; **105** (10): 4143–5.
34 Utsunomiya A, Miyazaki Y, Takatsuka Y *et al.* Improved outcome of adult T-cell leukemia/lymphoma with allogeneic hematopoietic stem cell transplantation. *Bone Marrow Transplant.* 2001; **27** (1): 15–20.

Int J Hematol (2011) 94:443–452
DOI 10.1007/s12185-011-0941-5



# Antibody therapy for Adult T-cell leukemia–lymphoma

Takashi Ishida · Ryuzo Ueda

Received: 28 July 2011 / Revised: 19 September 2011 / Accepted: 20 September 2011 / Published online: 13 October 2011


**Abstract** Adult T-cell leukemia–lymphoma (ATL) has a very poor prognosis. Since there currently are limited treatment options for ATL patients, several novel agents are being developed and tested clinically. Antibody therapy against ATL was initially started with interleukin-2 receptor α-subunit, CD25, as a target molecule in the late 1980s, and is currently ongoing. CC chemokine receptor 4 (CCR4) was postulated as a novel molecular target in ATL antibody therapy, and humanized anti-CCR4 mAb (KW-0761), whose Fc region was defucosylated to enhance antibody-dependent cellular cytotoxicity, was developed. A phase I study of KW-0761 in relapsed ATL and peripheral T-cell lymphoma was started in 2006, and a subsequent phase II study was completed in 2010. KW-0761 showed a clinically meaningful antitumor activity in patients with relapsed ATL, with an acceptable toxicity profile. The prognosis of ATL patients should be improved in the near future with clinical applications of novel treatment strategies, including those involving KW-0761 and other promising antibody therapies targeting CD25 or CD30.

**Keywords** CCR4 · CD25 · ADCC · KW-0761

T. Ishida (✉) · R. Ueda
Department of Medical Oncology and Immunology,
Nagoya City University Graduate School of Medical Sciences,
1 Kawasumi, Mizuho-chou, Mizuho-ku,
Nagoya, Aichi 467-8601, Japan
e-mail: itakashi@med.nagoya-cu.ac.jp

## 1 Introduction

Adult T-cell leukemia–lymphoma (ATL) is a peripheral T-cell neoplasm caused by human T-cell leukemia virus type-1 (HTLV-1) [1–3] that has a very poor prognosis. The clinical features of 818 ATL patients with a mean age of 57 years, newly diagnosed from 1983 to 1987, were analyzed by Shimoyama [4] who demonstrated that the median overall survival (OS) was 6.2 and 10.2 months for acute and lymphoma type ATL, respectively.

## 2 Conventional chemotherapy for ATL

A most recent phase III trial for previously untreated aggressive ATL (acute, lymphoma, or unfavorable chronic type), with enrollment between 1998 and 2003, demonstrated that dose-intensified multidrug chemotherapy, including vincristine, cyclophosphamide, doxorubicin, prednisone, ranimustine, vindesine, etoposide, and carboplatin (VCAP-AMP-VECP), was superior to CHOP-14 and resulted in a median OS of 12.7 months, and an OS at 3 years of 24% [5]. Comparing two reports of Shimoyama [4] and Tsukasaki et al. [5], chemotherapy for ATL seems to have been improved. However, some of the ATL patients who were in good physical condition, relatively young and with preserved organ functions, could be enrolled into the later VCAP-AMP-VECP study, which likely might lead to a prolonged OS. A vast knowledge in molecular biology and oncogenesis of ATL has been accumulated since its recognition as a distinct disease in 1977 [1], and discovery of its causative virus in 1980–1982 [2, 3, 6], however, this knowledge does not seem to have contributed extensively to an improved clinical prognosis of ATL patients. Conventional chemotherapy seems to have reached its attainable limits of efficacy in ATL.

## 3 Antiviral therapy for ATL

Antiviral therapy, consisting of a combination of zidovudine (AZT) and interferon (IFN), was initially reported with a median OS of 3.0 months in 19 patients with acute or lymphoma type ATL [7]. Recently, Bazarbachi et al. proposed—based on a meta-analysis—that AZT/IFN should be considered the gold standard of first-line therapy for patients with acute, chronic, or smoldering types of ATL. They reported a median OS of 9 months for acute type ATL treated with AZT/IFN [8]. Considering this report together with the results of conventional chemotherapy, such as VCAP-AMP-VECP [5], it is difficult to conclude that AZT/IFN therapy should be considered as a standard first-line therapy in acute ATL [9]. As for chronic and smoldering types of ATL, Bazarbachi et al. reported 100% OS beyond 5 years, even though the number of patients in their study ($n = 17$) was small. This OS is surprisingly good, considering the report from Takasaki et al. that a 5-year OS was 47.2% in patients with chronic and smoldering types of ATL who were mainly observed by a watchful waiting policy until disease progression ($n = 90$) [10]. Considering the promising but preliminary nature of the results by Bazarbachi et al., the Japan Clinical Oncology Group is now planning a randomized phase III study that will compare the outcome of AZT/IFN versus watchful waiting in patients with favorable chronic and smoldering types of ATL. This study will establish the standard care for patients with non-aggressive ATL. On the other hand, it appears that antiviral therapy alone is likely to have the same limited efficacy in aggressive ATL as does conventional chemotherapy.

## 4 Allogeneic hematopoietic stem cell transplantation in ATL

Allogeneic hematopoietic stem cell transplantation in ATL has evolved over the last decade into a potential approach to bring complete cure to ATL patients [11–13]. Recently, Hishizawa et al. [14] reported a Japanese nationwide retrospective study of allogeneic stem cell transplantation in ATL, in which the unadjusted 3-year probability of OS was 33% (95% CI 28–38%), which seems promising. The study revealed that an age higher than 50 years was significantly associated with lower survival rates by a multivariate analysis, even though ATL has a long latency and occurs in elderly individuals with a median age of 60 years. The study also revealed that a status other than complete remission (CR) was significantly associated with lower survival rates by multivariate analysis, even though the CR rates of patients treated with VCAP-AMP-VECP and CHOP-14 were only 40% (95% CI 27.6–54.2%) and 25% (95% CI 14.5–37.3%), respectively [5]. These results indicate that only a small fraction of ATL patients currently benefit from allogeneic stem cell transplantation. Collectively, the development of alternative treatment strategies for ATL patients is an urgent issue.

## 5 Development of novel agents in the treatment of ATL

There are currently limited treatment options for ATL patients, as mentioned above, although several novel agents are currently being developed and tested clinically. Bortezomib, which inhibits the proteasome, is now widely used for relapsed or refractory multiple myeloma [15]. This agent induces cell death in HTLV-1-associated cell lines and in primary ATL cells in vitro via multiple pathways, including NF-kB inhibition [16, 17]. A phase II study of bortezomib in relapsed/refractory ATL is currently ongoing (UMIN000004061). The immunomodulatory agent lenalidomide is active in a variety of hematological malignancies, including multiple myeloma [15] and B-cell lymphomas. A dose-escalation study to assess the safety of lenalidomide in patients with advanced ATL and peripheral T-cell lymphoma (PTCL) is also currently ongoing (ClinicalTrials.gov: NCT01169298). In addition, other agents, including the histone deacetylase inhibitor vorinostat and the folate analog pralatrexate, are now being considered for clinical trials in ATL patients, whereas the present paper is focused on the development of antibody therapy for ATL.

## 6 Antibody therapy for hematological malignancies

The use of therapeutic monoclonal antibodies (mAb) in the treatment of cancer has evolved into a promising approach over the last decade. In the clinical field of hematological malignancies, development of the chimeric anti-CD20 mAb rituximab has changed the standard therapy in patients with B-cell lymphomas and has markedly improved their prognoses [18, 19]. The success of anti-CD20 therapy in B-cell lymphoma is prompting investigators to search for a similarly efficacious mAb in T-cell lymphoma.

## 7 CD25 targeting therapy in ATL

### 7.1 Unmodified anti-CD25 antibody therapy

Antibody therapy against ATL was initially started by Waldmann et al. Because ATL cells constitutively express interleukin-2 (IL-2) receptor $\alpha$-subunit, CD25 [20, 21], they assigned this molecule as a target of antibody therapy.

They developed a mouse IgG2a mAb, termed anti-Tac, that recognized CD25 [22]. Since this antibody blocks the binding of IL-2 to its receptor on ATL cells, they postulated that anti-Tac mAb prevented ATL cell proliferation. Among 9 ATL patients treated with anti-Tac mAb, objective responses were observed in two patients (22%), including one CR [22]. Further evaluation of this agent in 19 ATL patients was performed, and objective responses were observed in six patients (32%), including 2 CRs and 4 partial remissions (PRs) [23]. Although the use of unmodified anti-Tac mAb in the treatment of ATL seems encouraging, there are several problems. One obstacle to the success of this antibody was provided by the observation that the ATL cells of most patients in the aggressive phase no longer produce IL-2 or require IL-2 for their proliferation and survival [24]. Another obstacle was the appearance of antibodies against anti-Tac mAb, i.e. human anti-mouse antibody (HAMA). HAMA was observed in 11.1% (1/9) of patients in the former [22] and in 15.8% (3/19) of patients in the latter study [23]. The appearance of HAMA is a common obstacle to success of therapeutic mouse mAb such as anti-Tac [25]. Rodent mAbs have short half-lives in humans and induce an immune response that neutralizes their therapeutic effect. Furthermore, the murine antibodies only weakly recruit human effector cells or elements, leading to insufficient ADCC (antibody-dependent cellular cytotoxicity) or CDC (complement-dependent cytotoxicity). These limitations of mouse antibodies have largely been overcome by their chimerization, or humanization, or by the production of fully human antibodies [26], and such advances have ushered in the current era of antibody therapeutics. In the context of this scenario, Queen et al. also generated a humanized version of the anti-Tac mAb (daclizumab) [27]. In cell lines, it was thought that daclizumab blocked the interaction of IL-2 and its receptor, resulting in apoptotic cell death. Clinical efficacy in ATL was achieved predominantly in patients with smoldering or chronic ATL. Responding patients were hypothesized to have a blockage of the IL-2–IL-2 receptor autocrine pathway. Unlike other therapeutic mAb used in the treatment of human malignancies, daclizumab did not seem to have a strong cytocidal activity [24].

### 7.2 Radionuclide/toxin-conjugated CD25 targeting therapy

The limited efficacy of unmodified anti-CD25 antibody therapy prompted investigators to develop an alternative approach to enhance antitumor activity, i.e., using this agent as a carrier of radionuclides or toxins. Waldmann et al. [28] administered 5, 10, 15 mCi Yttrium-90-conjugated anti-Tac to 18 ATL patients, and 9 (7 PR and 2 CR) of 16 (56%) evaluable patients responded. Grade 3 and more severe therapy-related adverse events, such as granulocytopenia and thrombocytopenia, were limited to the hematopoietic system, however, a significant fraction of patients also developed HAMA, thus preventing the ability to administer repeated treatments. A Pseudomonas exotoxin A-single chain anti-CD25 (sc) Fv-fragment fusion protein (LMB-2) showed promising results in IL-2 receptor α-expressing lymphoid malignancies, including ATL [29]. A phase II trial of LMB-2 combined with fludarabine and cyclophosphamide for ATL is currently ongoing (ClinicalTrials.gov: NCT00924170). Other approaches to CD25 receptor-targeted therapy use ligand–toxin fusion molecules. Denileukin diftitox (Ontak) is a recombinant fusion protein composed of diphtheria toxin-A and a human IL-2 fragment [30]. It is able to direct its cytotoxic action to cells expressing a functional IL-2 receptor, and when it is internalized, active diphtheria toxin is released to kill tumor cells. In a pivotal trial in 71 patients with advanced refractory cutaneous T-cell lymphoma (CTCL), Ontak produced a 30% response rate with 10% CRs [31], while this study did not include ATL patients. Now, a phase II study of the efficacy and toxicity of Ontak in the therapy of ATL is ongoing (ClinicalTrials.gov: NCT00117845).

## 8 CC chemokine receptor 4 (CCR4)-targeting antibody therapy for ATL

### 8.1 ATL and CCR4

Chemokines are a group of structurally related small cytokines (8–14 kDa) that play essential roles in migration and homing of lymphocytes. Individual T-cell subsets express specific sets of chemokine receptors that impart unique migration and tissue homing properties to the cells [32], so manipulating a specific chemokine network can lead to altered behavior of the corresponding functional T-cell subset. CCR4 is known to be expressed selectively on regulatory T (Treg) and type 2 helper T (Th) cells [33–37] (Fig. 1). Yoshie et al. first carried out RT-PCR analysis of CCR4 in peripheral blood mononuclear cells (PBMCs) from ATL patients, and observed strong signals in 22 out of 24 patients (92%). They also showed that most ATL cases were positive for CCR4 by flow cytometric analysis (8/10, 80%) and immunologic staining of blood smears (6/7, 86%) [38]. We also performed an extensive immunohistochemical study of CCR4 on tissue biopsy samples from 103 ATL patients, and then reported that tumor cells from a large majority of patients with ATL expressed CCR4 (91/103, 88.3%) [39].

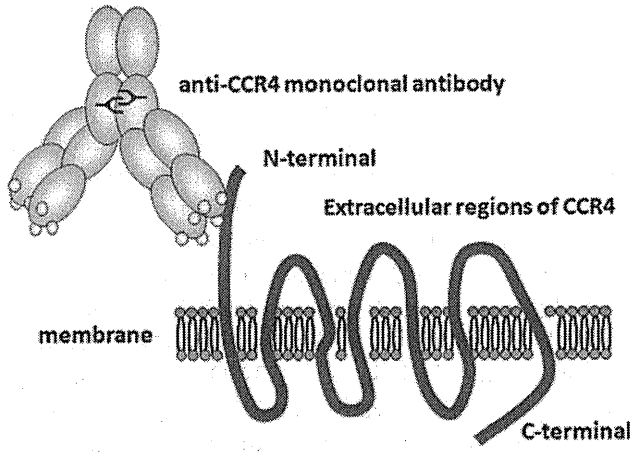


**Fig. 1** CC chemokine receptor 4 (CCR4) and humanized anti-CCR4 monoclonal antibody, KW-0761. The *CCR4 gene* is located on chromosome 3p24. CCR4 is a seven-domain transmembrane G protein-coupled receptor, and TARC/CCL17 and MDC/CCL22 are ligands of CCR4. The anti-CCR4 monoclonal antibody recognizes the N-terminal portion of the CCR4 molecule

### 8.2 Importance of ADCC in antibody therapy

ADCC is triggered following binding of the antibody Fc region to the Fcγ-receptor (FcγR) on effector cells, and is believed to represent the major in vivo antitumor mechanism of the antitumor therapeutic mAbs such as rituximab, trastuzumab, and alemtuzumab [40–42]. In particular, Carton et al. [42] found that follicular lymphoma patients homozygous for FcγRIIIa-158VV, which has the highest affinity for the IgG1 Fc region, have the best clinical and molecular responses to rituximab. The clear role for FcγR-bearing effectors in mediating the response to rituximab in clinical settings further demonstrates the importance of ADCC.

### 8.3 ADCC-enhancing defucosylated mAb

ADCC requires the presence of oligosaccharides in the Fc region and is sensitive to change in the oligosaccharide structure [43]. Of all the sugar components in the oligosaccharide, galactose, bisecting-GlcNAc, and fucose [44, 45] have been reported to affect ADCC. Shinkawa et al. [45] reported that fucose was the most important of these sugar components, and that defucosylation of IgG1 enhanced their ADCC >50-fold. The influence of non-fucosylated oligosaccharide on ADCC has also been reported by Shields et al. [44]. Subsequently, we revealed that fucose depletion of IgG1 could reduce the antigen amount on target cells required for the same level of ADCC induction by 3- to 10-fold [46]. Recently, it has been generally accepted that endogenous plasma IgG inhibits ADCC mediated by the therapeutic mAb by a competition for binding to FcγIIIa on effector cells, especially NK cells [47]. Iida et al. [48] reported that defucosylated IgG1 can evade the inhibitory effect of plasma IgG on ADCC through its high level of FcγRIIIa binding. Defucosylated IgG1 also has increased binding affinity to FcγRIIIb on neutrophils, leading to enhanced antibody-dependent neutrophil phagocytosis [49].

### 8.4 Preclinical study of the therapeutic defucosylated anti-CCR4 mAb

The work demonstrating that CCR4 was expressed on tumor cells from most ATL patients prompted us to postulate that this molecule might represent a novel molecular target for mAb therapy in ATL. Together with the ADCC-enhancing defucosylation technology, the therapeutic chimeric (KM2760) and humanized (KW-0761) anti-CCR4 mAbs were developed [50, 51] (Fig. 2). The therapeutic defucosylated anti-CCR4 mAb induced a robust ADCC activity against not only CCR4-positive human ATL cells, but also against other types of CCR4-positive human leukemia/lymphoma lines in the presence of PBMC from healthy individuals in a dose-dependent manner in vitro. In addition, a robust ADCC of the therapeutic defucosylated anti-CCR4 mAb mediated by autologous effector cells was triggered in some ATL cells as well as cells from other types of PTCL patients in vitro [52, 53].

Next, we evaluated antitumor activity of this antibody in mice in vivo, and this mAb demonstrated a significant antitumor effect in disseminated and non-disseminated CCR4-positive lymphoma models in SCID mice [54, 55]. The defucosylated anti-CCR4 mAb can induce a highly enhanced ADCC activity, but it does not mediate complement-dependent cytotoxicity or possess direct antitumor activities [51, 52]. ADCC depends on the cytotoxic activity of immune effector cells, so to evaluate the antitumor effects of therapeutic mAb in a small animal model in vivo, the species incompatibility of the immune systems in humans and animals is a critical issue. Indeed, we have previously reported that the mouse effector system mediating the antitumor action of therapeutic mAb does differ from that of the human [54, 55]. Thus, a current crucial problem in the field of human ADCC research is the lack of suitable small animal models. To overcome this, we have recently established "humanized mice," in which human immune cells mediate the antitumor action of the therapeutic mAb, using NOD/Shi-*scid*, IL-2Rγnull (NOG) mice [56, 57] as recipients. In this model, we showed that human PBMC from healthy individuals functioned as ADCC effector cells against allogeneic tumor cell lines engrafted in the mice [58]. Using this humanized mouse model, we had the opportunity to perform more appropriate preclinical evaluations of many types of Ab-based immunotherapy, although in the initial study we could not completely

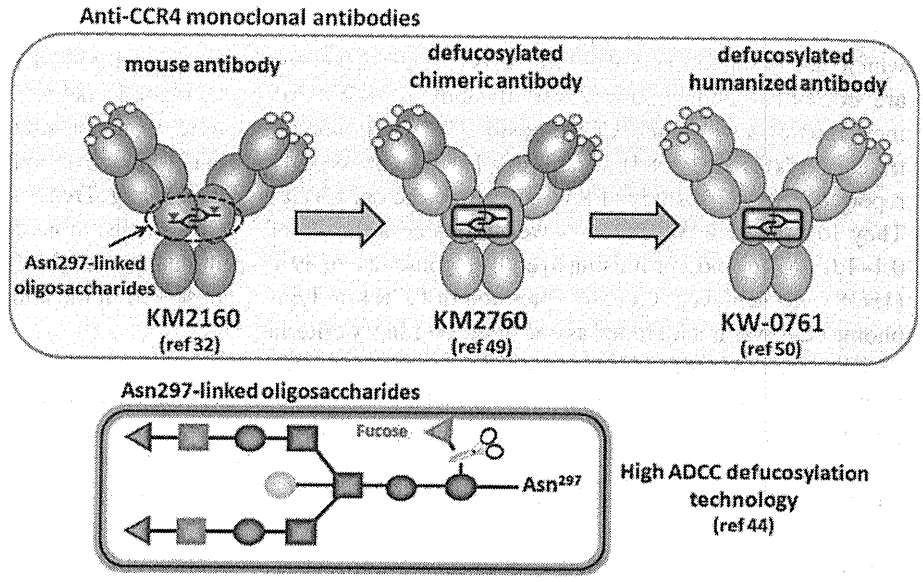


**Fig. 2** Development of the therapeutic anti-CCR4 monoclonal antibodies. The structure of oligosaccharide in the Fc region of human IgG1 is shown. Antibody-dependent cellular cytotoxicity (ADCC) requires the presence of oligosaccharides in the Fc region and is sensitive to change in the oligosaccharide structure. Of all the sugar components in the oligosaccharide, fucose has the most important influence on ADCC. The therapeutic anti-CCR4 mAbs, KM2760 and KW-0761, whose Fc regions are defucosylated were developed to enhance ADCC activity by increasing its binding affinity to the Fc receptor on effector cells

exclude nonspecific allogeneic immune responses because target and effector cells were obtained from different individuals. To overcome these problems, we have established a primary human-tumor-bearing NOG mouse model, in which autologous human immune cells are engrafted and mediate ADCC, and endogenous murine cells are unable to mediate ADCC. In that study, we used primary ATL cells-bearing NOG mice. The therapeutic anti-CCR4 mAb showed significant antitumor activity against the primary ATL cells in the NOG mice in vivo by a robust ADCC mediated by autologous effector cells [59]. That study was the first to report on a mouse model in which a potent antitumor effect of the therapeutic mAb against primary tumor cells was mediated by autologous human immune cells. This approach should make it possible to model a human immune system that is active in mAb-based immunotherapy in vivo, and thus to carry out more appropriate preclinical evaluations of novel therapeutic mAbs.

### 8.5 Clinical studies of KW-0761

Based on the promising results of this preclinical work, KW-0761 moved to the clinical phase. Yamamoto et al. [60] reported the results of a multicenter phase I study of KW-0761 in Japan (Clinicaltrials.gov: NCT00355472) in patients with relapsed CCR4-positive T-cell leukemia/lymphoma, including 13 ATL, 2 PTCL-not otherwise specified, and a mycosis fungoides. This phase I study was the first clinical trial to examine the safety and efficacy of a next-generation defucosylated therapeutic antibody against cancer. The study demonstrated good tolerability and predictable pharmacokinetics, and resulted in a recommended dose of 1.0 mg/kg for subsequent clinical trials. This study also demonstrated preliminary evidence of potent antitumor activity, thus, objective responses were observed in 31% of patients, with 13% CRs. Among 13 ATL patients, objective responses were observed in 31% of patients, with 15% CRs. In this study, clinical responses were observed even at 0.01 mg/kg, which is approximately 1/1,000 of the rituximab dose of about 10 mg/kg (375 mg/m$^2$) [18, 19]. The clinical effect observed at the 0.01 mg/kg dose of KW-0761 would be consistent with the concept of using defucosylation of therapeutic mAbs to enhance ADCC [45].

We subsequently carried out a multicenter phase II study of KW-0761 in patients with relapsed, aggressive CCR4-positive ATL in order to evaluate efficacy, pharmacokinetic profiles, and safety. The patients received 8 weekly intravenous infusions of KW-0761 at doses of 1.0 mg/kg. Objective responses were noted in 13 of 26 evaluable patients, including 8 CRs, with an overall response rate of 50% (95% CI 30–70%) (Ishida et al., ASH Annual Meeting Abstracts 2010 116: Abstract 285). The median progression-free and OS were 5.2 and 13.7 months, respectively. These results suggested an improvement compared to the results achieved with other agents in relapsed ATL [61]. The most common adverse events were infusion reactions and skin rashes, which were manageable and reversible in all cases. Collectively, this phase II study demonstrated that KW-0761 showed clinically meaningful antitumor activity in patients with relapsed ATL, with an acceptable toxicity profile. Based on this phase II study, an application has been submitted for approval of KW-0761 use in relapsed CCR4-positive ATL to Pharmaceuticals and Medical Devices Agency (PMDA), Japan, in April, 2011.

Following upon a phase II study, JCOG9801, on untreated aggressive ATL [5], the present promising results of KW-0761 monotherapy prompted us to conduct a subsequent randomized trial of VCAP-AMP-VECP chemotherapy with or without KW-0761 for previously untreated ATL (Clinicaltrials.gov:NCT01173887). CCR4 is also expressed on

tumor cells from a subgroup of PTCL, other than ATL, which also has an unfavorable prognosis [62, 63]. Thus, we are currently conducting a phase II study of KW-0761 monotherapy in relapsed CCR4-positive PTCL (Clinicaltrials.gov: NCT01192984). In addition, Duvic et al. recently reported a phase I/II study of KW-0761 in refractory CTCL. They found that KW-0761 was well tolerated at doses of 0.1–1.0 mg/kg, and a promising overall response rate of 39% (15/38) was achieved, though expression of CCR4 on lymphoma cells was not included as one of the eligibility criteria (ASH Annual Meeting Abstracts 2010 116: Abstract 962, Clinicaltrials.gov: NCT00888927). The history of translational research on the anti-CCR4 mAb is shown in Fig. 3.

### 8.6 Possible future direction of KW-0761

It has been generally accepted that an increased concentration of Treg cells in the tumor microenvironment plays an important role in tumor escape from host immunity in several different types of cancer. Moreover, Treg cells infiltrating the tumor may represent one of the main obstacles to successful tumor immunotherapy. Therefore, depletion of Treg cells in the vicinity of tumors is a potentially promising strategy for boosting tumor-specific immunity [64–66]. We showed that the therapeutic anti-CCR4 mAb actually depleted Treg cells in vitro [36, 52], and, furthermore, that it also had this activity in vivo in humanized mice [58]. Collectively, these data suggest that therapeutic defucosylated anti-CCR4 mAb could also be used in novel strategies for treatment of many types of cancer to overcome the suppressive effect of CCR4-expressing Treg cells on the host's immune response to tumor cells. Simultaneously, attention should be paid to the immune-related adverse events caused by KW-0761, as observed in the anti-CTLA4 mAb, ipilimumab, study [67].

## 9 Other antibody therapies for ATL

A clinical trial of anti-CD52 antibody (alemtuzumab) for ATL has been conducted, but the obtained information is currently limited (Clinicaltrials.gov: NCT00061048). The antitubulin agent monomethyl auristatin E (MMAE) was attached to a CD30-specific mAb by an enzyme-cleavable linker, thus producing the antibody–drug conjugate brentuximab vedotin (SGN-35). Brentuximab vedotin induced durable objective responses and resulted in tumor regression in most patients with relapsed or refractory CD30-positive lymphomas [68]. Although ATL patients were not included in this study, a subgroup of ATL is positive for CD30 [39], thus brentuximab vedotin might be worth trying in such subgroups of ATL.

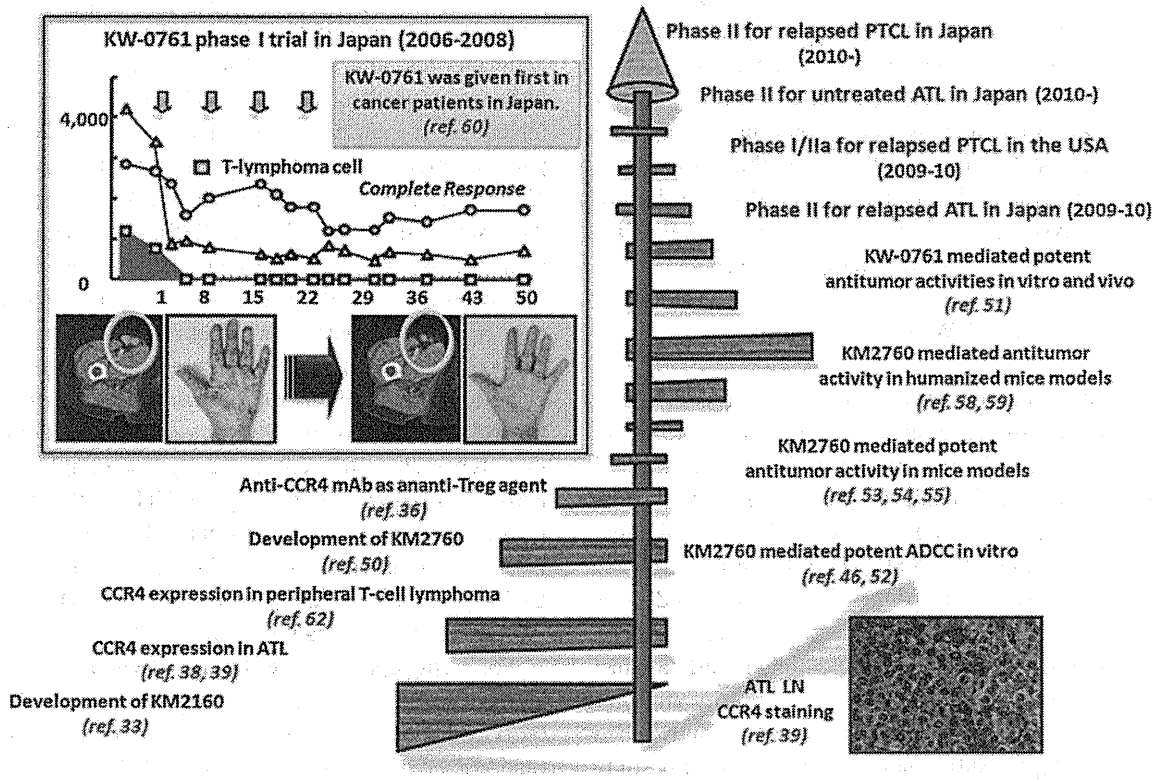


**Fig. 3** Translational research of the anti-CCR4 mAb. Based on the preclinical studies—such as CCR4 expression in primary ATL cells, potent antitumor activity of the anti-CCR4 mAb against ATL cells in vitro and in mice in vivo—clinical trials of KW-0761 in ATL/PTCL were started

## 10 Future directions for care-giving of HTLV-1 carriers and treatments of ATL patients

Finally, we would like to discuss the possible future directions for care-giving of HTLV-1 carriers and treatments of ATL patients. HTLV-1 is transmitted mainly from mother to infant through breast milk, and then only a small subpopulation of the virus-infected individuals (approximately 5% of subjects) progresses to ATL after a long latency period of about 50–70 years [69]. Although we now have only few ideas of what kind of HTLV-1-infected individuals develop ATL, a recent cohort study of 1,218 asymptomatic HTLV-1 carriers demonstrated that high proviral load levels (especially >4 copies/100 PBMCs), advanced age, family history of ATL, and having first learned about the HTLV-1 infection during treatment of other diseases were independent risk factors for progression from carrier status to ATL [70]. In the context of this scenario, the establishment of prevention strategies in ATL development in such high risk HTLV-1 carriers is expected (Fig. 4, ①). Although, at the present time, smoldering or favorable chronic type ATL patients should be considered for watchful waiting or AZT/IFN therapy [71], the establishment of more effective treatment options leading to inhibition of the development of aggressive ATL in such indolent ATL patients is also expected (Fig. 4, ②③). It is now generally accepted that conventional chemotherapy alone seems to have reached the limits of efficacy and

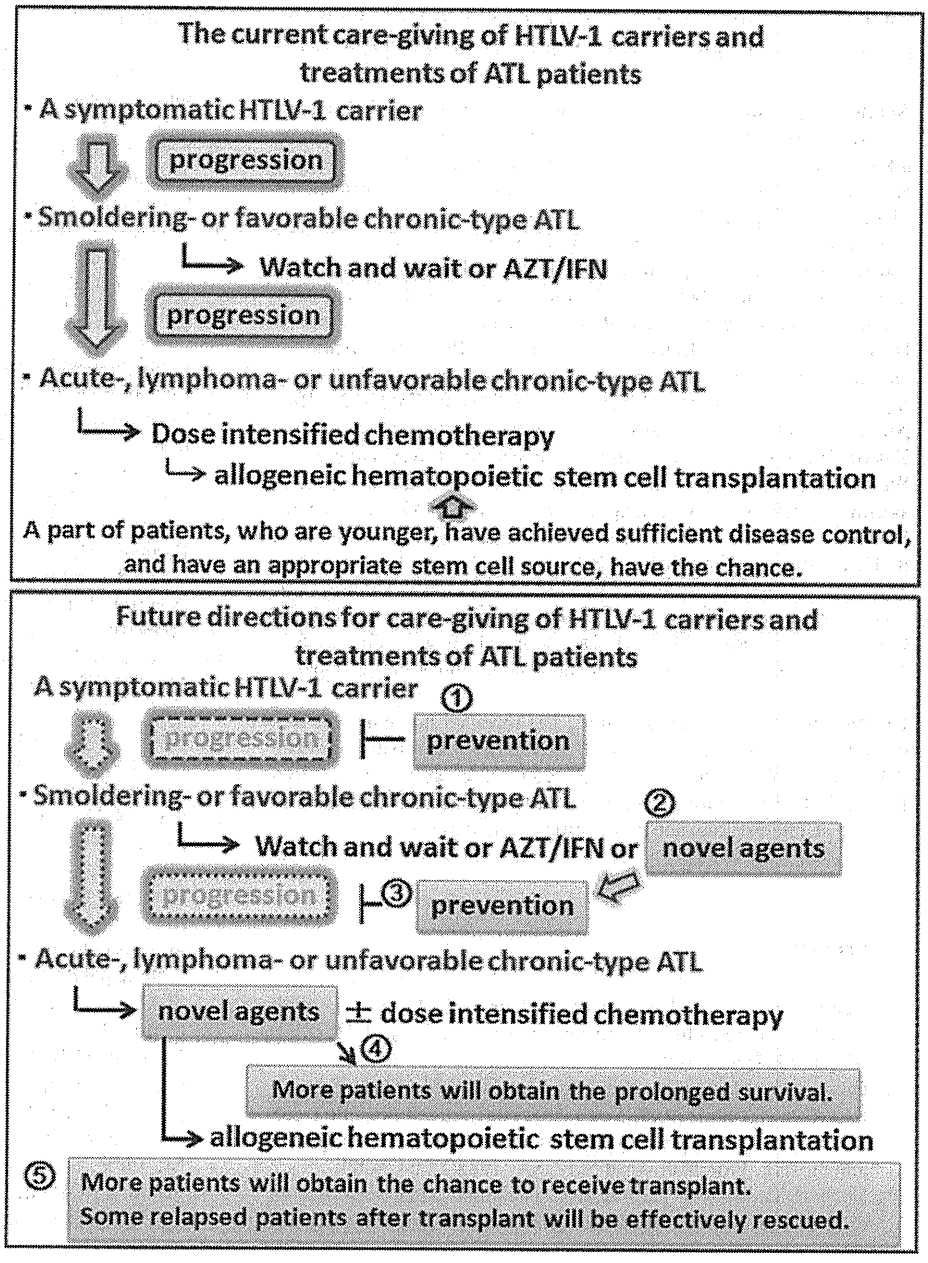


**Fig. 4** Future directions for care-giving of HTLV-1 carriers and treatments of ATL patients. The current and future directions for care-giving of HTLV-1 carriers and treatments of ATL patients are described

presents an extremely low prospect for cure in patients with aggressive ATL [68]. Therefore, there is an urgent need for development of ideal treatment strategies—applied as monotherapy or combined with the current conventional chemotherapy—which will offer a more prolonged survival and lead to a complete cure of some patients with aggressive ATL (Fig. 4, ④). These novel treatments should simultaneously increase the percentage of ATL patients who are in sufficient disease control—possibly leading to an improvement in allogeneic hematopoietic stem cell transplantation for ATL—and rescue some patients who have relapsed after transplantation (Fig. 4, ⑤). Clinical applications of novel treatment strategies, including those involving KW-0761 and other promising antibody therapies mentioned in this paper, will make a contribution at each stage of the disease, from HTLV-1 carrier status to aggressive ATL, and provide a more favorable prognosis.

**Acknowledgments** We are grateful to all the members of the CCR4 research group, Drs. Asahi Ito, Fumiko Mori, Ayako masaki, Fumihiko Sato, Toshihiko Ishii, Atsushi Inagaki, Hiroki Yano, Shigeru Kusumoto, Hirokazu Komatsu, and Shinsuke Iida (Department of Medical Oncology and Immunology, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan), Dr. Hiroshi Inagaki and Hisashi Takino (Department of Clinical Pathology, Nagoya City University Graduate School of Medical Sciences), Dr. Atae Utsunomiya (Department of Hematology, Imamura Bun-in Hospital), Dr. Kouji Matsushima (Department of Molecular Preventive Medicine, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan), Drs. Kenya Shitara, Shiro Akinaga, Takeshi Takahashi, Rinpei Niwa, and Nobuo Hanai (Tokyo Research Laboratories, Kyowa Hakko Kirin), for continuing fruitful collaborations over many years. We wish to thank Ms. Chiori Fukuyama for her skillful technical assistance. This work was supported by Grants-in-Aid for General Scientific Research (No. 22689029, T. Ishida and No 22300333, R. Ueda) and Scientific Support Programs for Cancer Research (No. 221S0001, T. Ishida) from the Ministry of Education, Culture, Science, Sports, and Technology, Japan; Grants-in-Aid for National Cancer Center Research and Development Fund (No. 21-6-3, T. Ishida) from the Ministry of Health, Labor, and Welfare, Japan.

**Conflict of interest** Takashi Ishida has received honoraria for lectures from Kyowa Hakko Kirin. Nagoya City University Graduate School of Medical Sciences has received research grant support from Kyowa Hakko Kirin for works provided by Takashi Ishida. No other conflict of interest relevant to this article was reported.

## References

1. Uchiyama T, Yodoi J, Sagawa K, Takatsuki K, Uchino H. Adult T cell leukemia: clinical and hematologic features of 16 cases. Blood. 1977;50:481–92.
2. Hinuma Y, Nagata K, Hanaoka M, Nakai M, Matsumoto T, Kinoshita KI, et al. Adult T cell leukemia: antigen in adult T-cell leukemia cell line and detection of antibodies to the antigen in human sera. Proc Natl Acad Sci USA. 1981;78:6476–80.
3. Yoshida M, Miyoshi I, Hinuma Y. Isolation and characterization of retrovirus from cell lines of human adult T cell leukemia and its implication in the disease. Proc Natl Acad Sci USA. 1982;79:2031–5.
4. 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;79:428–37.
5. Tsukasaki K, Utsunomiya A, Fukuda H, Shibata T, Fukushima T, Takatsuka Y, et al. VCAP-AMP-VECP compared with biweekly CHOP for adult T-cell leukemia-lymphoma: Japan Clinical Oncology Group Study JCOG9801. J Clin Oncol. 2007;25:5458–64.
6. 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;77:7415–9.
7. Gill PS, Harrington W Jr, Kaplan MH, Ribeiro RC, Bennett JM, Liebman HA, et al. Treatment of adult T-cell leukemia-lymphoma with a combination of interferon alfa and zidovudine. N Engl J Med. 1995;332:1744–8.
8. Bazarbachi A, Plumelle Y, Carlos Ramos J, Tortevoye P, Otrock Z, Taylor G, et al. Meta-analysis on the use of zidovudine and interferon-alfa in adult T-cell leukemia/lymphoma showing improved survival in the leukemic subtypes. J Clin Oncol. 2010;28:4177–83.
9. Ishitsuka K, Fukushima T, Tsukasaki K, Tobinai K. Is zidovudine and interferon-alfa the gold standard for adult T-cell leukemia-lymphoma? J Clin Oncol. 2010;28:e765.
10. 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;115:4337–43.
11. Utsunomiya A, Miyazaki Y, Takatsuka Y, Hanada S, Uozumi K, Yashiki S, et al. Improved outcome of adult T cell leukemia/lymphoma with allogeneic hematopoietic stem cell transplantation. Bone Marrow Transplant. 2001;27:15–20.
12. Fukushima T, Miyazaki Y, Honda S, Kawano F, Moriuchi Y, Masuda M, et al. Allogeneic hematopoietic stem cell transplantation provides sustained long-term survival for patients with adult T-cell leukemia/lymphoma. Leukemia. 2005;19:829–34.
13. Okamura J, Utsunomiya A, Tanosaki R, Uike N, Sonoda S, Kannagi M, et al. Allogeneic stem-cell transplantation with reduced conditioning intensity as a novel immunotherapy and antiviral therapy for adult T-cell leukemia/lymphoma. Blood. 2005;105:4143–5.
14. Hishizawa M, Kanda J, Utsunomiya A, Taniguchi S, Eto T, Moriuchi Y, et al. Transplantation of allogeneic hematopoietic stem cells for adult T-cell leukemia: a nationwide retrospective study. Blood. 2010;116:1369–76.
15. Kyle RA, Rajkumar SV. Multiple myeloma. N Engl J Med. 2004;351:1860–73.
16. Satou Y, Nosaka K, Koya Y, Yasunaga JI, Toyokuni S, Matsuoka M. Proteasome inhibitor, bortezomib, potently inhibits the growth of adult T-cell leukemia cells both in vivo and in vitro. Leukemia. 2004;18:1357–63.
17. Ri M, Iida S, Ishida T, Ito A, Yano H, Inagaki A, et al. Bortezomib-induced apoptosis in mature T-cell lymphoma cells partially depends on upregulation of Noxa and functional repression of Mcl-1. Cancer Sci. 2009;100:341–8.
18. Coiffier B, Lepage E, Briere J, Herbrecht R, Tilly H, Bouabdallah R, et al. CHOP chemotherapy plus rituximab compared with CHOP alone in elderly patients with diffuse large-B-cell lymphoma. N Engl J Med. 2001;346:235–42.
19. Pfreundschuh M, Trümper L, Osterborg A, Pettengell R, Trneny M, Imrie K, et al. CHOP-like chemotherapy plus rituximab versus CHOP-like chemotherapy alone in young patients with good-prognosis diffuse large-B-cell lymphoma: A randomised

controlled trial by the MabThera International Trial (MInT) Group. Lancet Oncol. 2006;7:379–91.
20. Waldmann TA, Greene WC, Sarin PS, Saxinger C, Blayney DW, Blattner WA, et al. Functional and phenotypic comparison of human T cell leukemia/lymphoma virus positive adult T cell leukemia with human T cell leukemia/lymphoma virus negative Sézary leukemia, and their distinction using anti-Tac. Monoclonal antibody identifying the human receptor for T cell growth factor. J Clin Invest. 1984;73:1711–8.
21. Uchiyama T, Hori T, Tsudo M, Wano Y, Umadome H, Tamori S, et al. Interleukin-2 receptor (Tac antigen) expressed on adult T cell leukemia cells. J Clin Invest. 1985;76:446–53.
22. Waldmann TA, Goldman CK, Bongiovanni KF, Sharrow SO, Davey MP, Cease KB, et al. Therapy of patients with human T-cell lymphotrophic virus I-induced adult T-cell leukemia with anti-Tac, a monoclonal antibody to the receptor for interleukin-2. Blood. 1988;72:1805–16.
23. Waldmann TA, White JD, Goldman CK, Top L, Grant A, Bamford R, et al. The interleukin-2 receptor: a target for monoclonal antibody treatment of human T-cell lymphotrophic virus I-induced adult T-cell leukemia. Blood. 1993;82:1701–12.
24. Waldmann TA. Daclizumab (anti-Tac, Zenapax) in the treatment of leukemia/lymphoma. Oncogene. 2007;26:3699–703.
25. Dickman S. Antibodies stage a comeback in cancer treatment. Science. 1998;280:1196–7.
26. Carter PJ. Potent antibody therapeutics by design. Nat Rev Immunol. 2006;6:343–57.
27. Queen C, Schneider WP, Selick HE, Payne PW, Landolfi NF, Duncan JF, et al. A humanized antibody that binds to the interleukin 2 receptor. Proc Natl Acad Sci USA. 1989;86:10029–33.
28. Waldmann TA, White JD, Carrasquillo JA, Reynolds JC, Paik CH, Gansow OA, et al. Radioimmunotherapy of interleukin-2R alpha-expressing adult T-cell leukemia with Yttrium-90-labeled anti-Tac. Blood. 1995;86:4063–75.
29. Kreitman RJ, Wilson WH, White JD, Stetler-Stevenson M, Jaffe ES, Giardina S, et al. Phase I trial of recombinant immunotoxin anti-Tac(Fv)-PE38 (LMB-2) in patients with hematologic malignancies. J Clin Oncol. 2000;18:1622–36.
30. Foss FM, Saleh MN, Krueger JG, Nichols JC, Murphy JR. Diphtheria toxin fusion proteins. Curr Top Microbiol Immunol. 1998;234:63–81.
31. Olsen E, Duvic M, Frankel A, Kim Y, Martin A, Vonderheid E, et al. Pivotal Phase III trial of two dose levels of denileukin diftitox for the treatment of cutaneous T-cell lymphoma. J Clin Oncol. 2001;19:376–88.
32. Yoshie O, Imai T, Nomiyama H. Chemokines in immunity. Adv Immunol. 2001;78:57–110.
33. Imai T, Nagira M, Takagi S, Kakizaki M, Nishimura M, Wang J, et al. Selective recruitment of CCR4-bearing Th2 cells toward antigen-presenting cells by the CC chemokines thymus and activation-regulated chemokine and macrophage-derived chemokine. Int Immunol. 1999;11:81–8.
34. Iellem A, Mariani M, Lang R, Recalde H, Panina-Bordignon P, Sinigaglia F, et al. Unique chemotactic response profile and specific expression of chemokine receptors CCR4 and CCR8 by CD4+CD25+ regulatory T cells. J Exp Med. 2001;194:847–53.
35. Yagi H, Nomura T, Nakamura K, Yamazaki S, Kitawaki T, Hori S, et al. Crucial role of FOXP3 in the development and function of human CD25+CD4+ regulatory T cells. Int Immunol. 2005;16:1643–56.
36. Ishida T, Ishii T, Inagaki A, Yano H, Komatsu H, Iida S, et al. Specific recruitment of CC chemokine receptor 4-positive regulatory T cells in Hodgkin lymphoma fosters immune privilege. Cancer Res. 2006;66:5716–22.
37. Ishida T, Ueda R. CCR4 as a novel molecular target for immunotherapy of cancer. Cancer Sci. 2006;97:1139–46.
38. Yoshie O, Fujisawa R, Nakayama T, Harasawa H, Tago H, Izawa D, et al. Frequent expression of CCR4 in adult T-cell leukemia and human T-cell leukemia virus type 1-transformed T cells. Blood. 2002;99:1505–11.
39. Ishida T, Utsunomiya A, Iida S, Inagaki H, Takatsuka Y, Kusumoto S, et al. Clinical significance of CCR4 expression in adult T-cell leukemia/lymphoma: its close association with skin involvement and unfavorable outcome. Clin Cancer Res. 2003;9:3625–34.
40. Voso MT, Pantel G, Rutella S, Weis M, D'Alò F, Urbano R, et al. Rituximab reduces the number of peripheral blood B-cells in vitro mainly by effector cell-mediated mechanisms. Haematologica. 2002;87:918–25.
41. Clynes RA, Towers TL, Presta LG, Ravetch JV. Inhibitory Fc receptors modulate in vivo cytotoxicity against tumor targets. Nat Med. 2000;6:443–6.
42. Cartron G, Dacheux L, Salles G, Solal-Celigny P, Bardos P, Colombat P, et al. Therapeutic activity of humanized anti-CD20 monoclonal antibody and polymorphism in IgG Fc receptor FcgammaRIIIa gene. Blood. 2002;99:754–8.
43. Jefferis R, Lund J, Pound JD. IgG-Fc-mediated effector functions: molecular definition of interaction sites for effector ligands and the role of glycosylation. Immunol Rev. 1998;163:59–76.
44. Shields RL, Lai J, Keck R, O'Connell LY, Hong K, Meng YG, et al. Lack of fucose on human IgG1 N-linked oligosaccharide improves binding to human FcRIII and antibody-dependent cellular toxicity. J Biol Chem. 2002;277(26):733–40.
45. Shinkawa T, Nakamura K, Yamane N, Shoji-Hosaka E, Kanda Y, Sakurada M, et al. The absence of fucose but not the presence of galactose or bisecting N-acetylglucosamine of human IgG1 complex-type oligosaccharides shows the critical role of enhancing antibody-dependent cellular cytotoxicity. J Biol Chem. 2003;278:3466–73.
46. Niwa R, Sakurada M, Kobayashi Y, Uehara A, Matsushima K, Ueda R, et al. Enhanced natural killer cell binding and activation by low-fucose IgG1 antibody results in potent antibody-dependent cellular cytotoxicity induction at lower antigen density. Clin Cancer Res. 2005;11:2327–36.
47. Preithner S, Elm S, Lippold S, Locher M, Wolf A, da Silva AJ, et al. High concentrations of therapeutic IgG1 antibodies are needed to compensate for inhibition of antibody-dependent cellular cytotoxicity by excess endogenous immunoglobulin G. Mol Immunol. 2006;43:1183–93.
48. Iida S, Misaka H, Inoue M, Shibata M, Nakano R, Yamane-Ohnuki N, et al. Nonfucosylated therapeutic IgG1 antibody can evade the inhibitory effect of serum immunoglobulin G on antibody-dependent cellular cytotoxicity through its high binding to FcgammaRIIIa. Clin Cancer Res. 2006;12:2879–87.
49. Shibata-Koyama M, Iida S, Misaka H, Mori K, Yano K, Shitara K, et al. Nonfucosylated rituximab potentiates human neutrophil phagocytosis through its high binding for FcgammaRIIIb and MHC class II expression on the phagocytotic neutrophils. Exp Hematol. 2009;37:309–21.
50. Niwa R, Shoji-Hosaka E, Sakurada M, Shinkawa T, Uchida K, Nakamura K, et al. Defucosylated chimeric anti-CC chemokine receptor 4 IgG1 with enhanced antibody-dependent cellular cytotoxicity shows potent therapeutic activity to T-cell leukemia and lymphoma. Cancer Res. 2004;64:2127–33.
51. Ishii T, Ishida T, Utsunomiya A, Inagaki A, Yano H, Komatsu H, et al. Defucosylated humanized anti-CCR4 monoclonal antibody KW-0761 as a novel immunotherapeutic agent for adult T-cell leukemia/lymphoma. Clin Cancer Res. 2010;16:1520–31.
52. Ishida T, Iida S, Akatsuka Y, Ishii T, Miyazaki M, Komatsu H, et al. The CC chemokine receptor 4 as a novel specific molecular target for immunotherapy in adult T-Cell leukemia/lymphoma. Clin Cancer Res. 2004;10:7529–39.

53. Yano H, Ishida T, Inagaki A, Ishii T, Ding J, Kusumoto S, et al. Defucosylated anti CC chemokine receptor 4 monoclonal antibody combined with immunomodulatory cytokines: a novel immunotherapy for aggressive/refractory mycosis fungoides and Sezary syndrome. Clin Cancer Res. 2007;13:6494–500.
54. Ishida T, Ishii T, Inagaki A, Yano H, Kusumoto S, Ri M, et al. The CCR4 as a novel-specific molecular target for immunotherapy in Hodgkin lymphoma. Leukemia. 2006;20:2162–8.
55. Yano H, Ishida T, Imada K, Sakai T, Ishii T, Inagaki A, et al. Augmentation of antitumour activity of defucosylated chimeric anti-CCR4 monoclonal antibody in SCID mouse model of adult T-cell leukaemia / lymphoma using G-CSF. Br J Haematol. 2008;140:586–9.
56. Ito M, Hiramatsu H, Kobayashi K, Suzue K, Kawahata M, Hioki K, et al. NOD/SCID/gamma(c)(null) mouse: an excellent recipient mouse model for engraftment of human cells. Blood. 2002;100:3175–82.
57. Ito M, Kobayashi K, Nakahata T. NOD/Shi-scid IL2rcnull (NOG) mice more appropriate for humanized mouse models. Curr Top Microbiol Immunol. 2008;324:53–76.
58. Ito A, Ishida T, Yano H, Inagaki A, Suzuki S, Sato F, et al. Defucosylated anti-CCR4 monoclonal antibody exercises potent ADCC-mediated antitumor effect in the novel tumor-bearing humanized NOD/Shi-scid, IL-2Rgamma(null) mouse model. Cancer Immunol Immunother. 2009;58:1195–206.
59. Ito A, Ishida T, Utsunomiya A, Sato F, Mori F, Yano H, et al. Defucosylated anti-CCR4 monoclonal antibody exerts potent ADCC against primary ATLL cells mediated by autologous human immune cells in NOD/Shi-scid, IL-2R gamma(null) mice in vivo. J Immunol. 2009;183:4782–91.
60. Yamamoto K, Utsunomiya A, Tobinai K, Tsukasaki K, Uike N, Uozumi K, et al. Phase I study of KW-0761, a defucosylated humanized anti-CCR4 antibody, in relapsed patients with adult T-cell leukemia-lymphoma and peripheral T-cell lymphoma. J Clin Oncol. 2010;28:1591–8.
61. Tobinai K. Current management of adult T-cell leukemia/lymphoma. Oncology (Williston Park). 2009;14:1250–6.
62. Ishida T, Inagaki H, Utsunomiya A, Takatsuka Y, Komatsu H, Iida S, et al. CXCR3 and CCR4 expression in T-cell and NK-cell lymphomas with special reference to clinicopathological significance for peripheral T-cell lymphoma, unspecified. Clin Cancer Res. 2004;10:5494–500.
63. Ohshima K, Karube K, Kawano R, Tsuchiya T, Suefuji H, Yamaguchi T, et al. Classification of distinct subtypes of peripheral T-cell lymphoma unspecified, identified by chemokine and chemokine receptor expression: Analysis of prognosis. Int J Oncol. 2004;25:605–13.
64. Zou W. Regulatory T cells, tumor immunity and immunotherapy. Nat Rev Immunol. 2006;6:295–307.
65. Zou W. Immunosuppressive networks in the tumor environment and their therapeutic relevance. Nat Rev Cancer. 2005;5:263–74.
66. Ishida T, Ueda R. Immunopathogenesis of lymphoma: focus on CCR4. Cancer Sci. 2011;102:44–50.
67. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, et al. Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med. 2010;363:711–23.
68. Younes A, Bartlett NL, Leonard JP, Kennedy DA, Lynch CM, Sievers EL, et al. Brentuximab vedotin (SGN-35) for relapsed CD30-positive lymphomas. N Engl J Med. 2010;363:1812–21.
69. Matsuoka M, Jeang KT. Human T-cell leukaemia virus type 1 (HTLV-1) infectivity and cellular transformation. Nat Rev Cancer. 2007;7:270–80.
70. Iwanaga M, Watanabe T, Utsunomiya A, Okayama A, Uchimaru K, Koh KR, et al. Human T-cell leukemia virus type I (HTLV-1) proviral load and disease progression in asymptomatic HTLV-1 carriers: a nationwide prospective study in Japan. Blood. 2010; 116:1211–9.
71. Tsukasaki K, Hermine O, Bazarbachi A, Ratner L, Ramos JC, Harrington W Jr, et al. Definition, prognostic factors, treatment, and response criteria of adult T-cell leukemia-lymphoma: a proposal from an international consensus meeting. J Clin Oncol. 2009;27:453–9.

# Type of skin eruption is an independent prognostic indicator for adult T-cell leukemia/lymphoma

Yu Sawada,[1] Ryosuke Hino,[1] Kayo Hama,[1] Shun Ohmori,[1] Haruna Fueki,[1] Shigenori Yamada,[1] Shoko Fukamachi,[1] Makiko Tajiri,[1] Rieko Kubo,[1] Manabu Yoshioka,[1] Daiki Nakashima,[1] Kazunari Sugita,[1] Ryutaro Yoshiki,[1] Takatoshi Shimauchi,[1] Tomoko Mori,[1] Kunio Izu,[2] Miwa Kobayashi,[1] Motonobu Nakamura,[1] and Yoshiki Tokura[1,3]

[1]Department of Dermatology, University of Occupational and Environmental Health, Kitakyushu, Fukuoka, Japan; [2]Department of Dermatology, Kyushu Kosei Nenkin Hospital, Kitakyushu, Fukuoka, Japan; and [3]Department of Dermatology, Hamamatsu University School of Medicine, Hamamatsu, Shizuoka, Japan

**Cutaneous involvement is seen in ~ 50% of adult T-cell leukemia/lymphoma (ATLL) patients. We investigated the association between skin eruption type and prognosis in 119 ATLL patients. ATLL eruptions were categorized into patch (6.7%), plaque (26.9%), multipapular (19.3%), nodulotumoral (38.7%), erythrodermic (4.2%), and purpuric (4.2%) types. When the T stage of the tumor-node-metastasis-blood (TNMB) classification of mycosis fungoides/Sézary syndrome was applied to ATLL staging, 16.0% were T1, 17.7% T2, 38.7% T3, and 4.2% T4, and the remaining 23.5% were of the multipapular and purpuric types. For the patch type, the mean survival time (median survival time could not be estimated) was 188.4 months. The median survival times (in months) for the remaining types were as follows: plaque, 114.9; multipapular, 17.3; nodulotumoral, 17.3; erythrodermic, 3.0; and purpuric, 4.4. Kaplan-Meier curves of overall survival showed that the erythrodermic type had the poorest prognosis, followed by the nodulotumoral and multipapular types. The patch and plaque types were associated with better survival rates. Multivariate analysis demonstrated that the hazard ratios of the erythrodermic and nodulotumoral types were significantly higher than that of the patch type, and that the eruption type is an independent prognostic factor for ATLL. The overall survival was worse as the T stage became more advanced: the multipapular type and T2 were comparable, and the purpuric type had a significantly poorer prognosis than T1. (*Blood*. 2011;117(15): 3961-3967)**

## Introduction

Adult T-cell leukemia/lymphoma (ATLL) is a malignancy of mature $CD4^+$ T cells caused by the human T-cell lymphotropic virus type I (HTLV-1).[1-3] HTLV-1 infection is prevalent in southern Japan, especially in Kyushu,[4,5] and in the Caribbean region and Africa.[6,7] Based on the number of abnormal lymphocytes, organ involvement, and severity, ATLL is divided into 4 clinical categories: acute, lymphoma, chronic, and smoldering (Shimoyama classification).[8] This classification is the most common tool used for estimating the prognosis of ATLL patients. The smoldering type has the best prognosis, followed by the chronic type, lymphoma type, and acute type. The median survival times (MSTs) of the acute, lymphoma, and chronic types are 6.2, 10.2, and 24.3 months, respectively.[8] Thus, the acute and lymphoma types of ATLL are associated with remarkably poor prognoses despite advances in chemotherapy and allogeneic hematopoietic stem cell transplantation.[9-11] In contrast, the chronic and smoldering types are relatively indolent and can usually be managed with "watchful waiting" until the disease progresses to acute crisis, just as smoldering (asymptomatic) myeloma is managed.[12]

Studies have attempted to identify other prognostic factors for survival of ATLL patients. Advanced performance status, high blood lactate dehydrogenase (LDH) level, age of 40 years or more, more than 3 involved lesions, and hypercalcemia have all been associated with shortened survival.[13] The existence of hepatosplenomegaly and lymphadenopathy also indicates poor prognosis.[8,14] However, there has been no large study on the correlation between the type and spread of skin eruptions and the prognosis of ATLL.

Because cutaneous involvement can be recognized in approximately 50% of ATLL patients,[15,16] the evaluation of skin lesions in relation to prognosis is important. Tumor cells infiltrating the skin exhibit several differences in phenotype and function.[17,18] ATLL patients can develop various types of eruptions, including nodules, tumors, plaques, erythrodermas, and even purpuric lesions,[19,20] and the categorization of these eruption types remains unclear. In this study, we retrospectively analyzed the prognosis of ATLL on the basis of the skin manifestations. We classified the skin eruptions and applied the T stage of the tumor-node-metastasis-blood (TNMB) classification for mycosis fungoides (MF) and Sézary syndrome (SS) to the type of skin lesions of ATLL. Our results indicate that eruption type is a predictor for prognosis.

## Methods

### Patients

We analyzed 119 patients with newly diagnosed, untreated ATLL who had skin eruptions and were seen at the University of Occupational and Environmental Health and Kyushu Kosei Nenkin Hospital from April 1979 to December 2009. The cutoff date for analysis was June 2010. The diagnosis of ATLL was based on clinical features, histopathologically and cytologically proven mature T-cell malignancy, presence of anti–HTLV-1 antibody, and monoclonal integration of HTLV-1 proviral DNA into the blood and/or skin tumor cells, as described previously.[2,8,21,22] The subtypes

Submitted November 5, 2010; accepted January 28, 2011. Prepublished online as *Blood* First Edition paper, February 16, 2011; DOI 10.1182/blood-2010-11-316794.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

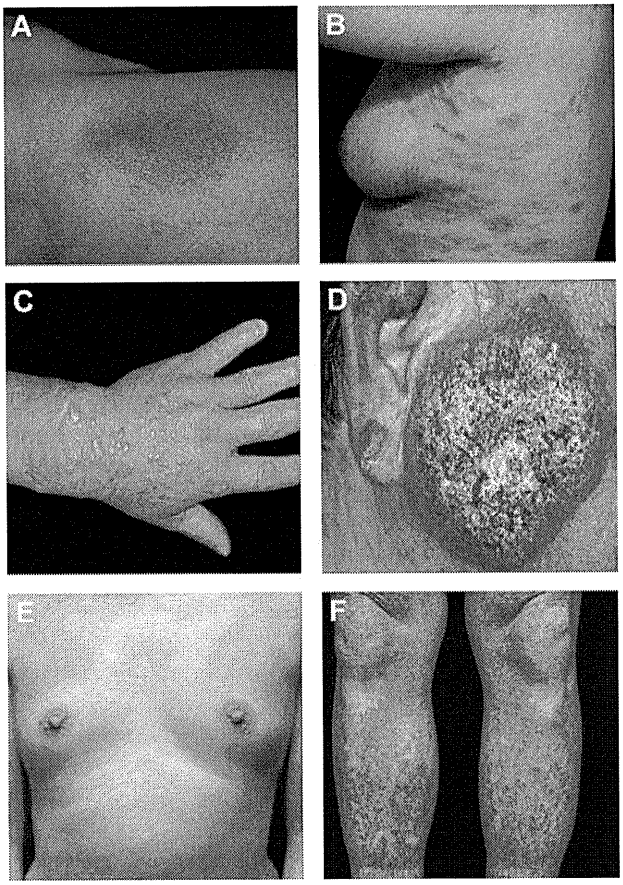


**Figure 1. Clinical features of ATLL with skin eruptions.** (A) Patch type, (B) plaque type, (C) multipapular type, (D) nodulotumoral type, (E) erythrodermic type, and (F) purpuric type.

of ATLL were classified according to the criteria established by the Lymphoma Study Group of Japan Clinical Oncology Group (Shimoyama classification).[8] Our retrospective, nonrandomized, observational study using existing data was granted an exemption from the institutional review board and was exempt from the requirement for written informed consent in accordance with the Declaration of Helsinki.

### Clinical evaluation and definitions

The patients were categorized into 2 age groups: younger than 60 years and 60 years or older. Complications at diagnosis were classified into present and absent. Leukocytosis and lymphocytosis were defined as white blood cell count more than $12 \times 10^9$/L, and total lymphocyte count more than $6.5 \times 10^9$/L, respectively. LDH and calcium levels were classified into 2 groups according to a standard index.[13] We categorized skin eruptions of ATLL into 6 different types: patch, plaque, multipapular, nodulotumoral, erythrodermic, and purpuric (Figure 1). We defined the criteria for categorizing ATLL-related skin involvement into the patch type as no infiltrated erythema, the plaque type as infiltrated erythema, the multipapular type as multiple papules with diameter less than 1 cm, the nodulotumoral type as nodules or tumors with diameters more than 1 cm, the erythrodermic type as generalized erythema involving 80% or more of the patient's skin, and the purpuric type as red or purple discolorations that did not change with diascopy.

### Statistical analyses

Overall survival (OS) was defined as the time from the date of first diagnosis to the date of death or the latest contact with the patient. Survival curves were drawn using the Kaplan-Meier method and were compared with the log-rank test. *P* values were calculated using the generalized Wilcoxon test. MST was defined as the time point at which the Kaplan-Meier survival curves crossed 50%. Mean survival time was provided when MST could not be calculated. To examine the multiple comparisons of the factors and of the pairs of groups, univariate and multivariate Cox regression analyses were applied to evaluate prognosis factors for survival. The effects of clinical parameters were evaluated as hazard ratios (HRs) and their 95% confidence intervals. All statistical analyses were performed using Dr SPSS II software (SPSS). A $P$ value $< .05$ was considered statistically significant.

## Results

### Patient clinical characteristics

The clinical data of 119 patients with skin eruptions (ratio of male: female = 1.2:1) are summarized in Table 1. The mean age of the patients was 64.0 years (range, 23-91 years; SD, 12.00 years). According to Shimoyama classification, 40 (33.6%) patients were diagnosed with the acute type of ATLL, 6 (5.0%) with the chronic type, 17 (14.3%) with the lymphoma type, and 56 (47.1%) with the smoldering type. Twenty-three patients had complications at the time of diagnosis, including 7 patients with diabetes mellitus, 10 with hypertension, 3 with stroke, and 9 with opportunistic infections. Blood examination revealed that 36 patients (30.3%) had leukocytosis, 26 (21.9%) had lymphocytosis, and 49 (41.2%) had high LDH levels. Hypercalcemia was found in 70 patients (58.8%).

### Patient skin lesions

We categorized the skin eruptions into the patch, plaque, multipapular, nodulotumoral, erythrodermic, and purpuric types (Figure 1). The most highly incident was the nodulotumoral type in 46 patients (38.7%), followed by the plaque type in 32 patients (26.9%), the multipapular type in 23 patients (19.3%), the patch type in 8 patients (6.7%), the erythrodermic type in 5 patients (4.2%), and the purpuric type in 5 patients (4.2%). Because the categorized skin eruptions of ATLL have similarities to those of MF/SS (with the exception of the multipapular and purpuric types), and because the TNMB classification for MF/SS[16] has been widely used, we attempted to apply the T stage of the TNMB classification to ATLL skin lesions. According to the MF/SS classification,[16] eruptions are classified into: T1 (patch/plaque, less than 10% of body surface), T2 (patch/plaque, more than 10% of body surface area), T3 (nodulotumoral type), and T4 (erythrodermic type). Ninety-one (76.5%) of our 119 patients could be classified using this system: 19 patients (16.0%) belonged to T1, 21 (17.7%) to T2, 46 (38.7%) to T3, and 5 (4.2%) to T4. The remaining 28 patients (23.5%) had multipapular (19.3%) and purpuric (4.2%) types, which are peculiar for ATLL and are not described in the T classification of MF/SS. We also evaluated these 2 types to investigate whether they are comparable with either the T1 or T4 category of the MF/SS classification system.

We examined the frequencies of the clinical subtypes of Shimoyama classification in each of the eruption types and T stages (Table 2). All patients with the erythrodermic type belonged to the acute type, whereas most of the patients with the patch type were grouped into the smoldering subtype. As the T stage advanced, the frequencies of the aggressive types (the acute and lymphoma types) increased, whereas those of the smoldering type decreased.

### Survival by baseline clinical factors

Sixty-nine of our 119 patients died during the observation period, with a median follow-up duration of 3.0 years (range, 30 days-20.3 years). The MSTs of the acute, lymphoma, chronic, and

**Table 1. Survival by baseline clinical factors**

| Factor | No. of evaluated cases | No. of deaths | MST, mos | P |
|---|---|---|---|---|
| Total | 119 | 69 | | |
| **Clinical subtype** | | | | < .001 |
| Acute type | 40 | 30 | 7.7 | |
| Lymphoma type | 17 | 12 | 15.0 | |
| Chronic type | 6 | 5 | 16.6 | |
| Smoldering type | 56 | 22 | 154.0 | |
| **Patient-related factors** | | | | |
| Sex | | | | .956 |
| Male | 66 | 38 | 20.3 | |
| Female | 53 | 31 | 24.9 | |
| Age, y | | | | .702 |
| ≥ 60 | 81 | 46 | 24.5 | |
| < 60 | 38 | 23 | 18.4 | |
| **Complications at diagnosis** | | | | .114 |
| Absent | 96 | 59 | 21.0 | |
| Present | | | | |
| Diabetes mellitus | 7 | 3 | 14.8 | |
| Hypertension | 10 | 4 | 141.4* | |
| Stroke | 3 | 2 | 17.2 | |
| Opportunistic infections | 9 | 4 | 49.3 | |
| **Hematologic factors** | | | | |
| WBC count, × $10^9$/L | | | | < .001 |
| ≥ 12.0 | 36 | 25 | 9.5 | |
| < 12.0 | 83 | 44 | 47.8 | |
| Total lymphocyte count, × $10^9$/L | | | | < .001 |
| ≥ 6.5 | 26 | 20 | 10.4 | |
| < 6.5 | 93 | 49 | 47.8 | |
| **Laboratory factors** | | | | |
| LDH | | | | < .001 |
| ≤ NI | 70 | 37 | 47.9 | |
| > NI | 49 | 32 | 9.5 | |
| Calcium | | | | .420 |
| ≤ NI | 49 | 28 | 27.8 | |
| > NI | 70 | 41 | 18.6 | |
| **Skin lesions** | | | | < .001 |
| Patch type | 8 | 2 | 188.4* | |
| Plaque type | 32 | 9 | 114.9 | |
| Multipapular type | 23 | 12 | 17.3 | |
| Nodulotumoral type | 46 | 38 | 17.3 | |
| Erythrodermic type | 5 | 5 | 3.0 | |
| Purpuric type | 5 | 3 | 4.4 | |
| **T stage** | | | | < .001 |
| T1 | 19 | 3 | 192.6* | |
| T2 | 21 | 8 | 47.9 | |
| T3 | 46 | 38 | 17.3 | |
| T4 | 5 | 5 | 3.0 | |

The cumulative probability of the survival rate was estimated using the Kaplan-Meier method and the *P* value was calculated using the generalized Wilcoxon test.

MST indicates median survival time; and NI, normal index.

*Mean survival time is given because the MST cannot be calculated.

smoldering types were 7.7, 15.0, 16.6, and 154.0 months, respectively (Table 1). Of the 69 fatal cases during the observation, 45 patients died of acute ATLL, 17 of acute crisis from the other subtypes, 5 of other diseases (3 of chronic pulmonary diseases and 2 of acute respiratory disease syndrome [ARDS]), and 2 patients of unknown causes.

The effects of various clinical factors on prognosis in the 119 patients were analyzed using the Kaplan-Meier method (Table 1). There was no statistically significant difference in survival rates between the absence and presence of any complication ($P = .114$), between the ≥ 60 years and < 60 years age groups ($P = .702$), or between males and females ($P = .956$). The survival rate was poor in patients with leukocytosis ($P < .001$), lymphocytosis ($P < .001$), and higher LDH levels ($P < .001$). Blood calcium level did not significantly affect survival in this study.

### Survival and multivariate analyses in each eruption type

The MSTs were different between the types of skin eruptions. In the erythrodermic type, all 5 patients died of the disease with 3.0 months of MST. In the nodulotumoral type, the MST was 17.3 months, and 38 of 46 patients died, 17 of acute ATLL, 16 of acute crisis, 1 of ARDS, 2 of chronic pulmonary disease, and 2 of unknown causes. In the plaque type, the MST was 114.9 months, and 9 of 32 died of the disease. The multipapular type showed the same MST (17.3 months) as the nodulotumoral type, and 9 died of acute ATLL, 1 of acute crisis, 1 of ARDS, and 1 of chronic pulmonary disease. The patch type exhibited a good prognosis, with 188.4 months of mean survival time (the MST was not estimable). The purpuric type was found to have a poor prognosis, with an MST of 4.4 months and 3 of 5 patients dying of the disease.

Kaplan-Meier curves of the OS for each eruption type are shown in Figure 2A. The OS rate of the erythrodermic type was significantly lower than those of the other eruption types ($P < .001$, erythrodermic type vs the nodulotumoral, multipapular, plaque, or patch types). The OS rate of the nodulotumoral type was significantly lower than those of the multipapular, plaque, or patch types ($P = .010$, nodulotumoral type vs multipapular type; $P < .001$, nodulotumoral type vs plaque or patch type). The OS rate of the multipapular type was significantly lower than that of the patch type ($P = .045$). Therefore, the erythrodermic type of ATLL is associated with the poorest prognosis, followed by the nodulotumoral and multipapular types. The patch and plaque types showed better survival rates.

We performed univariate and multivariate analyses of the eruption types in a comparison with Shimoyama classification, sex, age, complications, leukocyte counts, lymphocyte counts, LDH level, and calcium level (Table 3). In the multivariate analysis, the smoldering type proved to be a good prognostic factor. We fixed the HR of the patch type to be 1, and then compared it with those of the other eruption types. In the univariate analysis, the HRs of the other eruption types were significantly higher than that of the patch type. In the multivariate analysis, the HRs of the nodulotumoral and erythrodermic types were significantly higher than that of the patch type. The purpuric type also showed such a tendency; however, this result provided limited power for tests against the other groups. The analysis demonstrated that the eruption type is an independent prognostic factor for ATLL.

### Survival and univariate and multivariate analyses in each T stage

We also performed the univariate and multivariate analyses of T stage and other clinical and laboratory parameters for OS. Of 19 patients in the T1 stage, 3 died of the disease, and the mean survival time (the MST was not estimable) was 192.6 months (Table 1). In the T2 stage, 8 of 21 died of the disease and the MST was 47.9 months. In the T3 stage, the MST was 17.3 months and 38 of 46 patients died: 17 of acute ATLL, 16 of acute crisis, 1 of ARDS, 2 of chronic pulmonary disease, and 2 of unknown etiology. In the T4 stage, 5 patients died of the disease with 3.0 months of MST. The OS of the patients was worse as the T stage became more advanced (Figure 2B). Patients in the T1 stage had the longest OS, followed by patients in the T2-T4 stages ($P = .034$, T1 vs T2; $P < .001$, T1 vs T3 or T4; $P < .001$ T2 vs T3 or T4; and $P < .001$, T3 vs T4).

The multipapular and purpuric types are missing in the T stage of the MF/SS system due to their peculiarity. We therefore compared the OS of

**Table 2. Frequencies of the clinical types of Shimoyama classification in each eruption type and T stage**

| | Acute type | Lymphoma type | Chronic type | Smoldering type | *P* |
|---|---|---|---|---|---|
| **Eruption type** | | | | | .015 |
| Patch type | 0 | 0 | 1 (12.8%) | 7 (87.2%) | |
| Plaque type | 9 (28.1%) | 4 (12.5%) | 0 | 19 (59.4%) | |
| Multipapular type | 10 (43.5%) | 2 (8.7%) | 0 | 11 (47.8%) | |
| Nodulotumoral type | 14 (30.4%) | 10 (21.7%) | 5 (10.9%) | 17 (37.0%) | |
| Erythrodermic type | 5 (100%) | 0 | 0 | 0 | |
| Purpuric type | 2 (40.0%) | 1 (20.0%) | 0 | 2 (40.0%) | |
| **T stage** | | | | | .004 |
| T1 | 2 (10.5%) | 1 (5.3%) | 0 | 16 (84.2%) | |
| T2 | 7 (33.3%) | 3 (14.3%) | 1 (4.8%) | 10 (47.6%) | |
| T3 | 14 (30.4%) | 10 (21.7%) | 5 (10.9%) | 17 (37.0%) | |
| T4 | 5 (100%) | 0 | 0 | 0 | |

these 2 eruption types with those of the T stages. Patients with the multipapular type and T2 had a similar outcome (Figure 2C), and there was no statistical significance ($P = .415$). Patients with the purpuric type had a significantly poorer prognosis than those with T1 ($P = .001$); Figure 2D). The differences in OS between the purpuric type and the other T stages were not statistically significant ($P = .412$, purpuric type vs T2; $P = .257$; purpuric type vs T3; $P = .099$, purpuric type vs T4).

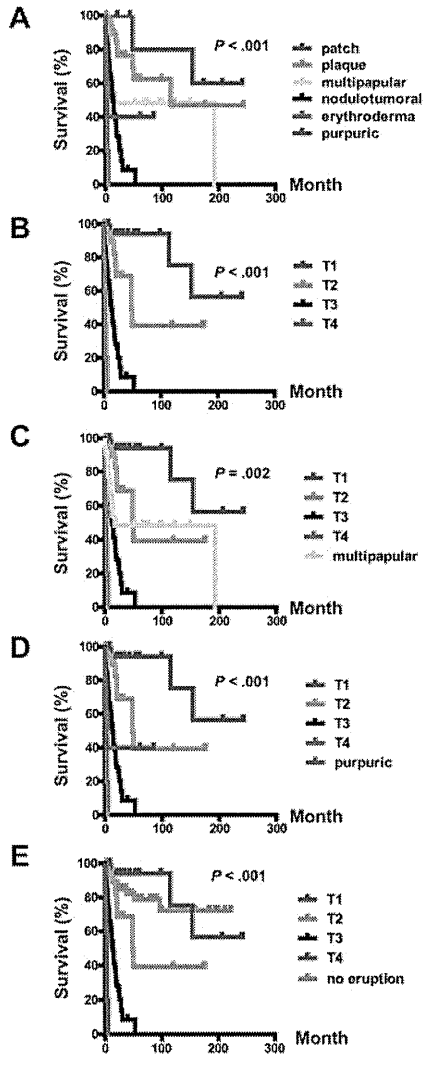


**Figure 2. OS of ATLL patients with skin eruptions.** (A) OS rates of skin eruption types. (B) OS rate of T stage. (C) OS rate of the T stage and the multipapular type. (D) OS rate of the T stage and the purpuric type. (E) OS rate of the T stage and the no-eruption type.

We performed univariate and multivariate analyses of T stage and clinical and laboratory parameters with the HR of T1 set as 1 (Table 3). The univariate analysis revealed that the prognoses of T2, T3, T4, and the multipapular and purpuric types were significantly higher than that of T1. In the multivariate analysis, the HR of T3 and T4 and the multipapular and purpuric types were significantly higher than that of T1.

### Survival and univariate and multivariate analyses in each T stage and in the no-eruption group

We performed univariate and multivariate analyses of T stage by comparing them with the no-eruption group and other clinical and laboratory parameters for OS. Of 51 patients without skin eruptions, 10 died of the disease and the mean survival time (the MST was not estimable) was 66.5 months. When classifying the no-eruption patients into each clinical Shimoyama subtype, 7 patients (13.7%) belonged to the acute type, 5 (9.8%) to the lymphoma type, 12 (23.5%) to the chronic type, and 27 (52.9%) to the smoldering type. The OS of the patients without eruption was better than those at T2-T4 (Figure 2E; no-eruption group vs T2, $P = .033$; no eruption group vs T3 or T4, $P < .001$). There was no statistically significant difference in OS between the no-eruption group and T1.

We performed univariate and multivariate analyses of T stage, including the no-eruption group and clinical and laboratory parameters, by assigning a value of 1 to the HR of T1 (Tables 4 and 5). The univariate and multivariate analyses revealed that the prognoses of T3 and T4 were significantly worse than that of T1.

## Discussion

In the present study, we investigated the association of each type of skin eruption with prognosis in ATLL patients and attempted to apply the T stage of MF/SS classification to the assessment of ATLL skin lesions. We classified ATLL skin eruptions into 6 categories: patch, plaque, multipapular, nodulotumoral, erythrodermic, and purpuric. Table 2 shows that the frequencies of the clinical subtypes of Shimoyama classification were different for each eruption type and T stage. All erythrodermic patients belonged to the acute type, whereas most of patients with the patch type were of the smoldering type. This raised the possibility that prognosis is different among the individual eruption types. Our results revealed the poorest prognosis in the erythrodermic type, followed by the nodulotumoral and multipapular types. The patch and plaque types exhibited better survival rates. Moreover, our multivariate analysis demonstrated that the HRs of the erythrodermic and nodulotumoral