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The side population, as a precursor of Hodgkin and Reed-Sternberg cells and a target for nuclear factor-κB inhibitors in Hodgkin's lymphoma

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Although disturbed cytokinesis of mononuclear Hodgkin (H) cells is thought to generate Reed-Sternberg (RS) cells, differentiation of Hodgkin's lymphoma (HL) cells is not fully understood. Recent studies indicate that cells found in a side population (SP) share characteristics of cancer stem cells. In this study we identified an SP in the HL cell lines, KMH2 and L428. This SP almost entirely consists of distinct small mononuclear cells, whereas the non-SP is a mixture of relatively large cells with H or RS cell-like morphology. Culture of the small mononuclear cells in the SP from KMH2 generated a non-SP. Single cell culture of the SP cells generated large cells with H or RS cell-like morphology. We found that CD30 overexpression and constitutive nuclear factor-κB (NF-κB) activity, both of which are characteristics of HL cells, are shared between the SP and non-SP cells for both KMH2 and L428. Inhibition of NF-κB induced apoptosis in both fractions, whereas the SP cells were resistant to a conventional chemotherapeutic agent doxorubicin. The results show that HL cell lines contain an SP, that is enriched for distinct small mononuclear cells and generates larger cells with H and RS cell-like morphology. The results also stress the significance of NF-κB inhibition for eradication of HL cells. (*Cancer Sci* 2010; 101: 2490–2496)

Hodgkin's lymphoma (HL) is a malignant lymphoma characterized by a low frequency of giant malignant cells, known as mononucleated Hodgkin (H) cells, and by multinucleated Reed-Sternberg (RS) cells in a background of reactive cells.[1] Most HL cells are B cells that originate from a germinal center B cell.[2] Previous studies using HL cell lines showed that mononuclear H cells are the precursors of multinucleated RS cells and that generation of RS cells may be associated with abortive mitosis rather than with cell fusion.[3]

CD30 is a member of the tumor necrosis factor receptor superfamily that was first identified as a cell surface molecule characteristic to H and RS cells of HL.[4] It was found that constitutively activated nuclear factor-κB (NF-κB) is a hallmark of H and RS cells and is considered to be the molecular basis of their aberrant growth and abnormal cytokine gene expression.[5] We have previously reported that overexpression of CD30 results in ligand-independent constitutive signaling that activates the transcription factors NF-κB and activator protein-1 (AP-1) in HL cells.[6,7]

Recent research indicates that most cancer cells originate from a small number of cancer cells with stem cell-like properties, called cancer stem cells (CSCs).[8] These cells are found in multiple tumor types.[9] They frequently possess MDR1 or ATP-binding cassette (ABC) transporter activities and show chemoresistance and a side population (SP) phenotype.[10] The presence of surface markers selectively expressed on CSCs are used to isolate these cells, but no marker or pattern of markers is known to prospectively identify CSCs in many tumor types. In such cases, exploitation of stem cell characteristics can be used to identify CSCs, and one such characteristic is the capacity to extrude dyes such as Hoechst 33342. Cells that exclude this dye are referred to as SP cells. These cells share characteristics of CSCs, and, specifically, they are enriched for tumor initiating capacity.[11,12]

We observed that cells present in HL cell lines and in HL patients that were previously described as "mononuclear H cells" are heterogeneous in their morphology. This observation led us to question if HL cells might contain SP cells. In fact, differentiation of "mononuclear H cells" cells was reported more than 20 years ago but was not further investigated.[13] In this study, we attempted to isolate SP cells from HL cell lines using Hoechst 33342 dye efflux as an SP marker, then we investigated the properties of these SP cells. We further discuss the significance of our findings from the viewpoint of the differentiation and the treatment of HL.

Materials and Methods

Cell culture. The HL cell lines, KMH2 and L428, were purchased from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). The cell lines were cultured under standard conditions in RPMI-1640 supplemented with 10% FBS.

Chemicals. Dehydroxymethylepoxyquinomicin (DHMEQ), an NF-κB inhibitor, was dissolved in DMSO.[14] Bisbenzimide H 33342 fluorochrome (Hoechst 33342) and the topoisomerase inhibitor doxorubicin were purchased from Calbiochem (Bad Soden, Germany) and Sigma-Aldrich (St. Louis, MO, USA), respectively.

Flow cytometry and SP analysis. The samples were incubated with 5 μg/mL Hoechst 33342 dye (Calbiochem) at 37°C for 60 min, with or without 30 μg/mL verapamil (Sigma-Aldrich). The cells were counterstained with 0.5 μg/mL propidium iodide (PI) (Sigma-Aldrich) to label dead cells and were analyzed with FACSAria (Becton-Dickinson, Franklin Lakes, NJ, USA) by dual-wavelength analysis using 450/20BP nm (blue) and 670/50BP nm (red) after excitation with 350-nm UV light. For sorting and culture assays of SP cells and non-SP cells, the cells were gated to exclude PI-positive cells and were sorted from the regions indicated. For phenotypic analysis, the cells were also stained with antibodies for 1 h at 4°C. When unlabeled antibodies were used, fluorescein-conjugated secondary antibody was used. Finally, the cells were washed and resuspended in PBS

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containing 2% FBS and PI, then analyzed by FACSAria (Becton-Dickinson) using the FlowJo analysis program (Tree Star, Ashland, OR, USA).

Antibodies used were obtained from the following companies. CD19, CD34, CD44, CD133, and CD20 (all from Becton-Dickinson), CD30 (Dako, Kyoto, Japan), CD40 (Beckman Coulter, Brea, CA, USA), CD15 (EXBIO Praha, Vestec, Czech Republic), CD117, HLA-DR (Cell Signaling Technology, Danvers, MA, USA). Isotype matched control antibody was obtained from Becton-Dickinson.

Immunohistochemistry. Fluorescence immunostaining was carried out on cultured cells as previously described.[15] Primary antibodies used were: anti-Ki-67 (DAKO), anti-JunB (C-11), anti-Bcl-xL (H-62), anti-cellular FLICE-like inhibitory protein (c-FLIP) (H-202; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-activated NF-κB p65 (Chemicon International, Temecula, CA, USA). Signals were detected using a fluorescence microscope (BX50F; Olympus, Tokyo, Japan).

Immunostaining of CD30 on paraffin-embedded specimens of primary HL samples with informed consent was carried out as previously described.[7]

Analysis of cell viability and apoptosis. Cell viability was analyzed using a Trypan blue dye exclusion test as described.[16] The experiment was carried out in triplicate, and at least 100

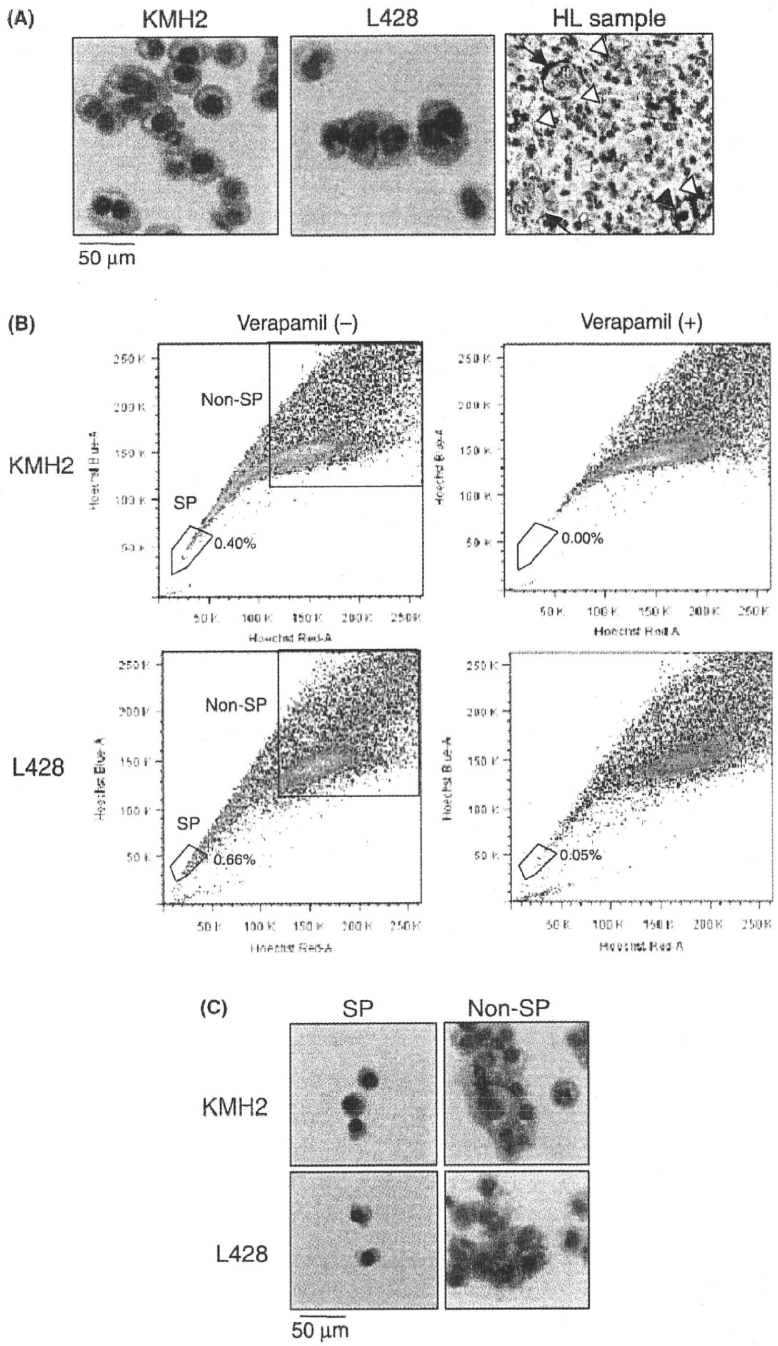

Fig. 1. Morphological appearance of mononuclear Hodgkin's lymphoma (HL) cells and side population (SP) analysis of HL cell lines KMH2 and L428. (A) The HL cell lines were stained with Giemsa (left and middle panels). Hodgkin's lymphoma cells in primary HL samples (n = 5) were detected by anti-CD30 antibody and a representative result is shown (right panel). The black arrows and arrowhead indicate Reed-Sternberg cells and a Hodgkin cell, respectively. The white arrowheads indicate smaller HL cells. (B) Analysis of SP cells in HL cell lines. The SP fractions of both cell lines were reduced by treatment with verapamil. The percentage of SP cells is indicated in each graph. (C) Morphology of SP and non-SP cells, both stained with Giemsa.

cells per experiment were counted. To quantify apoptosis, cells were stained using an annexin V–Azami-Green apoptosis detection kit (MBL, Nagoya, Japan). The cells were photographed through a UV filter on a fluorescence microscope.

Statistical analysis. Differences between mean values were assessed using a two-tailed *t*-test. A *P*-value of <0.05 was considered to be statistically significant.

Results

Identification of an SP consisting of distinct small mononuclear cells in HL cell lines. Large mononuclear cells observed in HL are usually called H cells. However, mononuclear cells of the HL cell lines consist of cells showing heterogeneous morphology (Fig. 1A, left and middle). Hodgkin's lymphoma cells can be identified by overexpression of CD30. We identified CD30-overexpressing smaller mononuclear cells among multinucleated RS cells and large mononucleated H cells in lymph nodes of HL patients (a representative photograph is shown in the right panel of Fig. 1A). Control experiments using isotype-matched primary antibody showed negative staining (data not shown). These observations led us to hypothesize the existence of precursor cells from which neoplastic component characteristics for HL, that is, H and RS cells, might be derived. Based on these observations, we carried out SP analysis of the HL cell lines and identified a minor population of SP cells (Fig. 1B). These SPs were enriched for small mononuclear cells with distinct morphology, whereas the non-SPs consisted of larger heterogeneous cells, which included cells with H or RS cell-like morphology (Fig. 1C).

Side population cells generate cells with H or RS cell-like morphology. We next separated the SP from KMH2 and determined if cells with H or RS cell-like morphology could be derived from this SP fraction. Culture of the SP for 14 days generated a non-SP and reconstituted the original cell line pattern with both an SP and a non-SP (Fig. 2A). The morphology of the

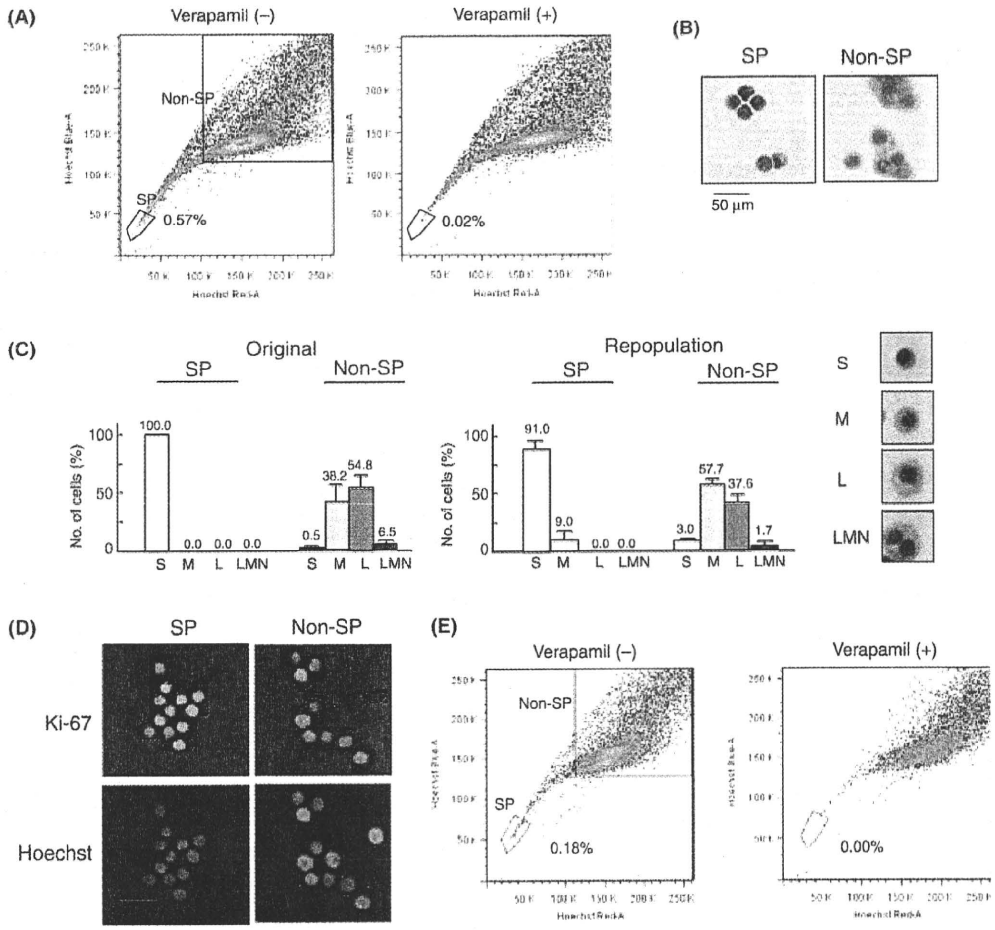

Fig. 2. Side population (SP) analyses of cultured SP and non-SP cells. (A) Side population analysis of the SP fraction cultured for 14 days. The SP fraction of KMH2 cells (5×10^3) was cultured for 14 days, then the presence of SP and non-SP fractions were analyzed. (B) Morphology of SP and non-SP cells derived from the SP fraction cultured for 14 days. Both SP and non-SP cells were stained with Giemsa. (C) Morphology of SP and non-SP cells in the original KMH2 cells (Original) and those derived from 2×10^3 cells of the SP fraction cultured for 14 days (Repopulation). The absolute number of the cells in the repopulation was 8×10^6. The morphology was classified as small (S), medium (M), large (L), or large multinucleated (LMN). Representative morphologies of each classification are indicated on the right. More than one hundred cells were classified for each fraction at least three times and representative results are presented as bar graphs. Data are the means ± SD. The mean values are indicated above each bar graph. (D) Ki-67 staining of SP and non-SP cells from the original KMH2 using an anti-Ki-67 antibody. The cells were counterstained with Hoechst 33342. Detection of Hoechst was varied between the SP and non-SP cells, because of the very weak signal in the SP cells. (E) Side population analysis of the non-SP fraction cultured for 14 days. The non-SP fraction of KMH2 cells (5×10^3) was cultured for 14 days, then the presence of SP and non-SP fractions were analyzed.

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SP and non-SP cells was very similar to that observed in Figure 1(C) (Fig. 2B). To clarify the cell morphology in these populations, we morphologically classified and counted SP and non-SP cells of the original and the reconstituted samples. The results indicate that the original SP almost entirely consists of typical small mononuclear cells, and the repopulated non-SP contains abundant large cells and a small fraction of large multinucleated cells. The repopulation of SP cells was accompanied with the increase in cell number, suggesting that the proliferation and differentiation of SP cells occurred during repopulation (Fig. 2C).

We examined whether SP cells were replicating as actively as non-SP cells by anti-Ki-67 antibody. Ki-67 reacts with nuclei of cells in G_1, S, and G_2 and mitosis but not with G_0 cells, and has been shown to be generally positive in H and RS cells.[3] Ki-67 was more strongly expressed in SP cells than in non-SP cells. In addition, the SP cells showed almost all Ki-67 positive, whereas a part of the non-SP cells were negative, indicating that SP cells are more actively replicating than non-SP cells (Fig. 2D). Control experiments showed negative staining (data not shown). Collectively, these results support the notion that small mononuclear cells found in the SP are actively replicating and generate cells with H or RS cell-like morphology.

However, non-SP cells also reconstituted the original cell line pattern with both an SP and a non-SP (Fig. 2E). This result appears to conflict with the result of reconstitution by SP cells, however, the reconstitution by non-SP cells has already been reported by several published works, supporting the notion that CSC-like precursor cells are partly, but not exclusively, enriched in an SP.[17,18] The SP has been hypothesized to be linked by a very small population of cells in the non-SP, although contamination of a small amount of SP cells in the non-SP cannot be ruled out.[19]

Single SP cell generates larger mononuclear cells and large multinucleated cells. To confirm the notion that the small mononuclear cells in the SP generate cells with H or RS cell-like morphology, single cell analysis was carried out. The results of single cell culture for 7 days showed that both fractions could proliferate, although the SP cells were prone to form mulberry-formed colonies (Fig. 3A). However, polynucleated cells failed to proliferate, as reported previously.[13] The SP cells generated larger cells, whereas the non-SP cells proliferated as large cells (Fig. 3B,C). This indicates that the small mononuclear cells in the SP are a major pool for generation of large cells in the non-SP. These results further support the notion that the small cells found in the SP generate cells with H or RS cell-like morphology.

Side population cells share biological characteristics of HL with non-SP cells. To compare the phenotype of SP and non-SP cells, we carried out extensive cell marker analysis. We focused on markers related to stem cells,[20] HL,[1] and B cells. The results indicate that the expression pattern of both markers is very similar between the SP and non-SP cells (Fig. 4A). Overlaid histograms of each marker are shown in Figure S1. Although it was not possible to show markers that discriminate between the SP and non-SP cells, the conservation of HL markers, especially the same level of expression of CD30 in both fractions, indicates that the biological basis previously reported in HL may be also applicable to the SP cells.

Analysis of CD30 expression in SPs and non-SPs of KMH2 and L428, using FACS, showed a very similar CD30 mean fluorescence intensity and histogram, suggesting that CD30 is also overexpressed in SP cells (Fig. 4B). We also determined the level of activation of NF-κB and AP-1 using immunofluorescence. The results showed the same pattern of expression of activated RelA and JunB in both fractions[7,15] (Fig. 4C). Control experiments showed negative staining (data not shown). These results indicate that the biological bases characteristic for HL appear to be shared between the SP and non-SP cells.

Side population cells are resistant to doxorubicin, but are sensitive to NF-κB inhibition. We and others have reported that constitutive NF-κB activation is a molecular target of HL cells.[5] However, it is entirely unknown how effective NF-κB inhibition is against the SP of HL. Therefore, we determined the effect of NF-κB inhibition on the viability and apoptosis of the SP cells using the NF-κB inhibitor DHMEQ.[21] Treatment with DHMEQ effectively abrogated constitutive NF-κB activation by inhibiting nuclear localization of activated RelA (Fig. 5A). Treatment with DHMEQ reduced the viable cell number of both fractions, suggesting DHMEQ induced the death of these cells (Fig. 5B). In contrast, treatment with doxorubicin, a key conventional chemotherapeutic agent for HL, potently reduced the viable cell number of the non-SP, but not the SP cells (Fig. 5B). Both the non-SP and SP cells treated with DHMEQ stained positive for annexin V (Fig. 5C). These results show that the reduction in viability of the SP cells with DHMEQ is accompanied by apoptosis and indicate that inhibition of NF-κB may be effective for eradication of HL cells by inducing apoptosis in their precursor cells, which appear to be resistant to conventional chemotherapeutic agents.

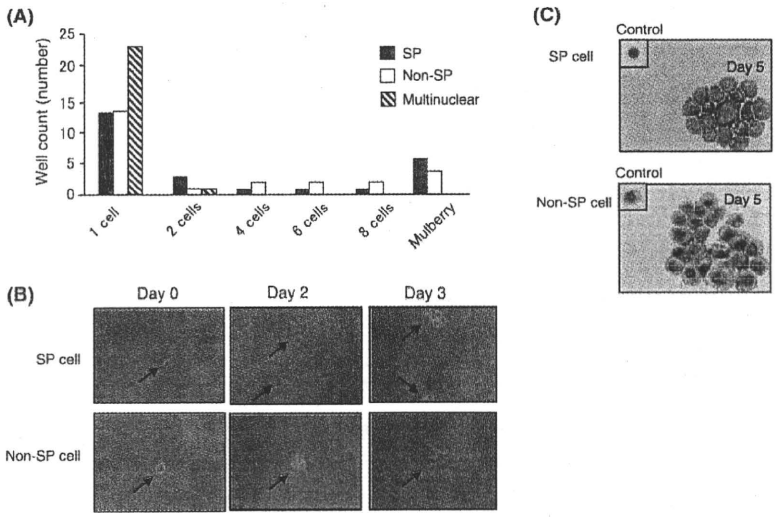

Fig. 3. Single cell culture of side population (SP) cells. Twenty-four cells sorted by FACSAria in each fraction were served for single cell culture in 96-well plates for 7 days and their proliferation was analyzed by microscopic observation. Data represent three independent experiments. (A) The number of cells in each well was counted at 7 days, and the results are shown as bar graphs. (B) Microphotographs of representative proliferation of a single cell at the indicated time points. Cells are indicated by arrows. (C) The SP or non-SP cells harvested from a single well at day 5 were cytospined and stained with Giemsa. Representative cases are shown. Side population and non-SP cells at day 0 were stained and a representative cell for each fraction, although not identical to the original cell of the harvested cells, is shown as an inset and served as control presentation.

(A)

KMH2		SP positive (%)	Non-SP positive (%)
Stem cell-related marker	CD34	0.00	0.01
	CD44	98.88	99.25
	CD117	0.00	0.00
	CD133	2.98	1.77
	HLA-DR	73.20	65.08
Hodgkin-related marker	CD30	100.00	99.70
	CD40	100.00	100.00
	CD15	99.70	97.07
B cell-related marker	CD19	0.00	0.00
	CD20	0.00	0.00

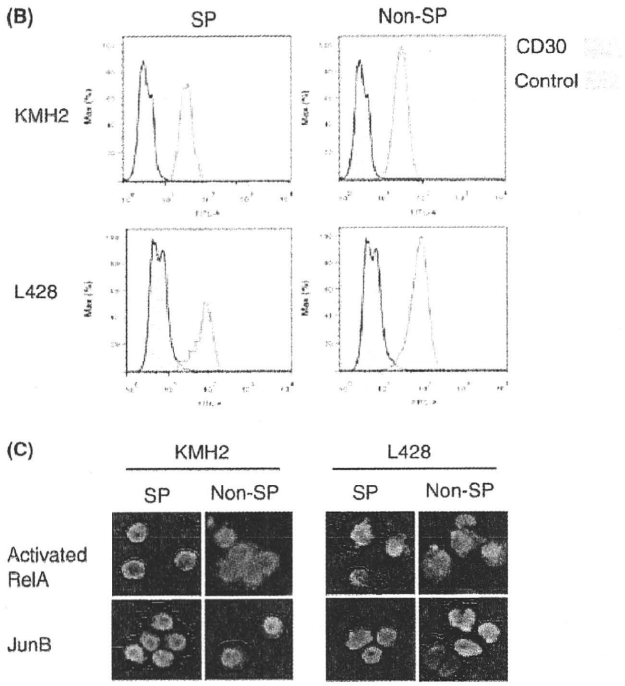

Fig. 4. Expression of key Hodgkin's lymphoma cell molecules in side population (SP) and non-SP cells. (A) Summary of the phenotype. The positive percentage for each marker is shown. (B) CD30 expression analyzed by FACS analysis. Stain of the total population by isotype-matched antibody served as control. (C) Activation of nuclear factor-κB (RelA) and activator protein-1 (JunB). Cells were immunostained with anti-activated RelA or anti-JunB as described.

Previous reports indicated frequent expression of anti-apoptotic molecules Bcl-xL and c-FLIP and involvement of their activity in the survival of HL cells.[22,23] We previously showed the downregulation of Bcl-xL and c-FLIP upon blocking of NF-κB activity and indicated the involvement of these molecules in the survival of HL cells dependent on NF-κB signaling.[15] Based on these backgrounds, we examined the effect of an NF-κB inhibitor DHMEQ on SP and non-SP cells of HL cell line, KMH2. The results clearly showed the downregulation of Bcl-xL and c-FLIP by treatment with DHMEQ in both SP and non-SP cells, indicating Bcl-xL and c-FLIP are potential key molecules, which support the survival of not only non-SP cells, but also SP cells (Fig. 5D).

To obtain an insight into the mechanism of doxorubicin resistance of SP cells of HL cell lines, the SP and non-SP cells were treated under the same conditions as shown in Figure 5(B). The cells were cultured with doxorubicin for 1 h then the cellular doxorubicin distribution was analyzed. The results showed that both cellular fractions incorporated doxorubicin (Fig. 5E).

Discussion

We have identified SPs in HL cell lines. These SPs are enriched for small mononuclear cells with distinct morphology, whereas the non-SPs consist of larger heterogeneous cells, which include cells with characteristic H or RS cell-like morphology. Culture of SP cells of KMH2 reconstituted the original cell line pattern with both an SP and a non-SP. As the isolated SP cells were highly pure and almost entirely consisted of typical small mononuclear cells, the small mononuclear cells were thought to be responsible for the reconstitution. This is further supported by the result that the single cell analysis of the cells sorted from the SP generates larger heterogeneous cells. The SP cells showed strongly positive for proliferation marker Ki-67 compared with the non-SP cells. Previous reports that stated that SP cells retain high proliferation property support this notion.[24,25] SP cells have been reported to share characteristics of CSCs, in particular, SPs are enriched for tumor initiating capacity[12]. Generally CSCs are considered to be resistant to conventional chemotherapeutic agents. The SPs identified in HL cell lines showed resistance to a conventional agent, doxorubicin, compared with the non-SPs, indicating the SPs appear to share important CSC-like properties. These results indicate the possibility that the SP cells identified here may represent "precursor cells" of the characteristic neoplastic H and RS cells of HL.

A previous report hypothesized that a very small population of slow cycling CSC-like cells without ABCA2 expression exist in a non-SP. After acquisition of ABCA2 expression, these cells, which are identified as SP cells, have been thought to act as fast cycling tumor progenitors.[19] Culture of the non-SP of KMH2 cells for 14 days also reconstituted the SP and non-SP pattern, indicating the possibility that the small mononuclear cells found in the SP of HL cell lines represent the fast cycling tumor progenitors. The single cell culture of the non-SP cells generally did not generate small mononuclear cells. Therefore, we prefer the notion that a very small fraction of non-SP cells, which share their morphology with SP cells, are candidates for slow cycling CSC-like cells. In fact, we found small mononuclear cells in the non-SP of KMH2 (Fig. 2C). However, we could not characterize these cells in this study, because no pattern of markers is known to prospectively identify these cells.

A previous study indicated the existence of clonogenic H cells in L428 and the relationship of the continuum of malignant mononuclear H and multinuclear RS cells. This study showed that single cloned mononuclear H cells were clonogenic and H and RS cells arose in a 10% cloning efficiency, whereas small abortive colonies sometimes arose from RS cells. This study was reported more than 20 years ago but was not further investigated.[13] The result appears to indicate that H cells retain proliferative potential and generate RS cells, although the morphology of those clonogenic mononuclear cells was not described in detail. Our results provide a new insight to the above study and indicate the existence of a very small population of "precursor cells" with distinct morphology and the SP phenotype among mononuclear cells of HL cell lines. A recent report indicated that aldehyde dehydrogenase $(ALDH)^+$ $CD20^+$ $CD30^-$ $Ig\lambda^+$ clonotypic B cells exist in the blood of HL patients and that these cells in the KMH2 and L428 cell lines can function as precursor cells for H and RS cells,[26] however, this notion is currently controversial.[27] The SP cells that we identified in HL cell lines do not represent the above described cell fraction, because they are $CD20^-$ $CD30^+$. $ALDH^+$ $CD20^+$ $CD30^-$ $Ig\lambda^+$ clonotypic B cells were isolated

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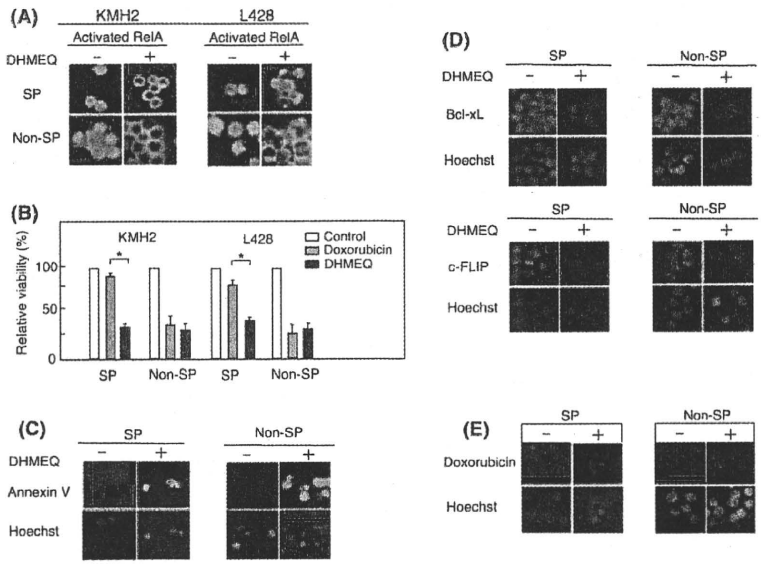

Fig. 5. Effect of dehydroxymethylepoxyquinomicin (DHMEQ) and doxorubicin on side population (SP) and non-SP cells. (A) DHMEQ inhibits constitutive nuclear factor-κB activity in both SP and non-SP cells. The SP and non-SP cells were treated with 20 μg/mL DHMEQ for 6 h. The treated cells were stained for activated nuclear factor-κB (RelA). (B) DHMEQ reduces the viability of SP cells resistant to doxorubicin. Two thousand of cells were treated with 20 μg/mL DHMEQ or 1 μg/mL doxorubicin for 24 h. Viable cells were measured using the Trypan blue exclusion test and expressed as a percentage relative to the DMSO-treated control, which assigned a value of 100%. Data are the means ± SD of three independent experiments. $*P < 0.05$. (C) The DHMEQ-mediated reduction in cell viability is accompanied by an increase in the number of annexin V-reactive cells. Cells were separated then treated with 20 μg/mL DHMEQ for 18 h, stained with annexin V, and observed. Detection of Hoechst varied between SP and non-SP cells, because of the very weak signal in the SP cells. (D) DHMEQ inhibits the expression of Bcl-xL and c-FLIP in both SP and non-SP cells. The SP and non-SP cells were treated with 20 μg/mL DHMEQ or DMSO alone for 7 h. The treated cells and their control were stained for Bcl-xL and c-FLIP. Isotype-matched control antibody showed negative staining (data not shown). Detection of Hoechst was varied. (E) Incorporation of doxorubicin into SP and non-SP cells. Cells were cultured for 1 h with 1 μg/mL doxorubicin under the same conditions as Figure (5B). The cells were observed using a fluorescence microscope with a WIG filter (Olympus) on an Olympus BX50F fluorescence microscope. Detection of Hoechst was varied.

from the $CD30^-$ fraction in KMH2 and L428 cell lines and the $CD30^+$ fraction is not yet analyzed. Our study indicates the presence of precursor cells within the $CD30^+$ fraction and emphasizes the requirement of further study of this fraction.

We have shown that CD30 is overexpressed in HL and that overexpressed CD30 induces ligand-independent signals.[6] These signals drive constitutive NF-κB activation that is critical for the survival of HL cells, and also induce the expression of JunB, which is responsible for positive regulation of CD30 overexpression through AP-1 activation.[6,7] Although chemotherapy and radiotherapy successfully contribute to a better prognosis of HL, problems of late side-effects and cases resistant to the treatment with poor prognosis, are also important issues that remain to be solved.[28] As SP cells, which share characteristics of CSCs, are considered to be associated with resistance to chemotherapy,[29] successful targeting of this fraction appears to be critical for eradication of HL cells. We showed that the SP cells are resistant to doxorubicin, a key conventional chemotherapeutic agent for HL, whereas the SP cells are sensitive to NF-κB inhibitor DHMEQ. We indicated that DHMEQ induced apoptosis in not only non-SP cells, but also in SP cells by downregulation of anti-apoptotic molecules, Bcl-xL and c-FLIP. Although the SP cells showed resistance for doxorubicin, doxorubicin was incorporated into their nucleus. The results indicated that the resistance of the SP to doxorubicin was not due to increased efflux by transporters. Taking into account the fact that the SP of KMH2 is actively replicating, the resistance to doxorubicin does not relate to the quiescent nature of cells, but might therefore be due to altered intracellular metabolism or signaling, which may be distinct compared to non-SP cells.[30,31] Taken collectively, these results underscore the significance of NF-κB inhibition to eradicate HL cells.

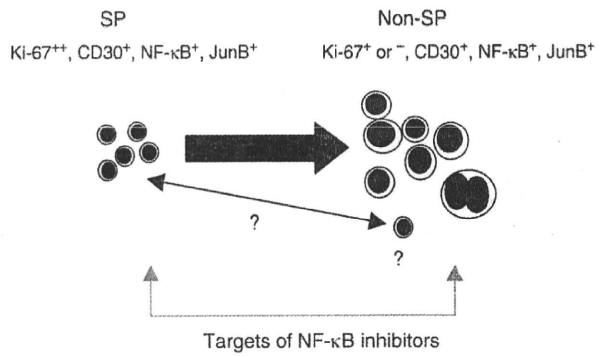

Fig. 6. Proposed model for the roles of the small mononuclear cells found in the side population (SP). The small mononuclear cells in the SP proliferate actively and generate the non-SP, consisting of larger cells with Hodgkin or Reed-Sternberg cell-like morphology. Part of the non-SP cells is not actively replicating. The SP may be linked with the non-SP by a very small fraction of cells that exist in the non-SP. The biological bases of Hodgkin's lymphoma previously reported appear to be conserved and nuclear factor (NF)-κB is a molecular target for both populations.

In conclusion, our results indicate that HL cell lines contain an SP and typical small mononuclear cells enriched in this SP appear to be a certain stage of precursor cells for H and RS cells. A population of non-SP cells, which link the non-SP and SP, may exist. The biological bases characteristic for HL appear to be shared between the SP and non-SP cells. The results also present an example of NF-κB as a key signaling pathway for the SP cells and stress the significance of NF-κB inhibition for

eradication of HL cells. A schematic representation of the conclusion is presented as Figure 6.

Acknowledgments

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Fig. S1. Overlaid histograms of each marker. The expression was analyzed by FACSAria using each antibody (light blue, right peak of each plot) or a control antibody (light grey, left peak of each plot). The horizontal axis indicates fluorescence intensity, the vertical axis represents cell count (% of maximum).

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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

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Definitive risk factors for the development of adult T-cell leukemia (ATL) among asymptomatic human T-cell leukemia virus type I (HTLV-1) carriers remain unclear. Recently, HTLV-1 proviral loads have been evaluated as important predictors of ATL, but a few small prospective studies have been conducted. We prospectively evaluated 1218 asymptomatic HTLV-1 carriers (426 males and 792 females) who were enrolled during 2002 to 2008. The proviral load at enrollment was significantly higher in males than females (median, 2.10 vs 1.39 copies/100 peripheral blood mononuclear cells [PBMCs]; $P < .001$), in those 40 to 49 and 50 to 59 years of age than that of those 40 years of age and younger ($P = .02$ and .007, respectively), and in those with a family history of ATL than those without the history (median, 2.32 vs 1.33 copies/100 PBMCs; $P = .005$). During follow-up, 14 participants progressed to overt ATL. Their baseline proviral load was high (range, 4.17-28.58 copies/100 PBMCs). None developed ATL among those with a baseline proviral load lower than approximately 4 copies. Multivariate Cox analyses indicated that not only a higher proviral load, advanced age, family history of ATL, and first opportunity for HTLV-1 testing during treatment for other diseases were independent risk factors for progression of ATL. (*Blood*. 2010;116(8): 1211-1219)

Introduction

Human T-cell leukemia virus type I (HTLV-1), the first human retrovirus to be identified, is etiologically associated with adult T-cell leukemia (ATL), HTLV-1–associated myelopathy/tropical spastic paraparesis (HAM/TSP), and HTLV-1 uveitis/HTLV-1–associated uveitis (HU/HAU).[1-3] Worldwide, endemic areas for the virus are unevenly distributed, which include southwest Japan, the Caribbean islands, South America, and a part of Central Africa.[4] In Japan, the number of HTLV-1 carriers was estimated to be approximately 1.2 million people during the late 1980s.[5] The majority of HTLV-1 carriers remain asymptomatic throughout their lives. The lifetime risks of developing ATL and HAM/TSP are estimated to be approximately 2.5% to 5%[6,7] and 0.3% to 2%,[8,9] respectively.

Several molecular biologic studies have reported that various cellular dysfunctions induced by viral genes (eg, *tax* and *HBZ*), genetic and epigenetic alterations, and the host immune system may be involved in the leukemogenesis of ATL.[10-12] Clinical and epidemiologic studies have also reported a variety of possible risk factors for ATL, including vertical transmission of HTLV-1 infection, male gender, a long latent period, increased leukocyte counts or abnormal lymphocyte counts, and higher levels of anti–HTLV-1 antibody titers and soluble interleukin-2 receptor-α.[13-19] However, there are no clear determinants that separate those who develop ATL from those who remain healthy carriers.

Recently, HTLV-1 proviral load levels have been evaluated as important predictors of development of ATL and HAM/TSP. Some cross-sectional studies showed that HTLV-1 proviral load levels were higher in ATL and HAM/TSP compared with asymptomatic HTLV-1 carriers.[20,21] However, the proviral load levels of asymptomatic HTLV-1 carriers exhibited a very wide range,[20,22,23] and these levels may vary by sex, race, habitats, and comorbidities.[24] The proviral load levels of asymptomatic HTLV-1 carriers were also examined serially in some prospective studies; however, the

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number of reported cases was very small.[25-28] Although these previous studies suggest a possible important role for HTLV-1 proviral load in the development of ATL and HAM/TSP, the association between HTLV-1 proviral load and diseases development remains unclear.

The identification of risk factors for developing ATL among virus carriers is necessary to prevent these diseases in HTLV-1 endemic areas. To investigate detailed viral- and host-specific determinants of disease development, larger and longer prospective studies are warranted. In 2002, we established a nationwide cohort study for asymptomatic HTLV-1 carriers in Japan named the Joint Study on Predisposing Factors of ATL Development (JSPFAD).[29] The main objective of this project is to establish reliable predisposing factors for developing ATL by prospectively following a large number of asymptomatic HTLV-1 carriers. Here, for the first-time, we report the study method, baseline demographic characteristics, and distribution characteristics of baseline HTLV-1 proviral load of asymptomatic HTLV-1 carriers. We have also evaluated progression to ATL and its risk predictors.

Methods

Participants and study design

The JSPFAD is a nationwide prospective study of HTLV-1 carriers, which was approved by the Ministry of Education, Culture, Sports, Science and Technology of Japan. The project was established in August 2002 by Japanese clinicians and basic researchers of 41 institutions composed of 14 university hospitals and 27 educational hospitals located in various areas of Japan (supplemental Appendix, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). Objectives of the project are to establish reliable predisposing factors for development of ATL by prospectively following a large number of asymptomatic HTLV-1 carriers. This includes performing clinical examinations and biomarker assays, as well as establishing a biomaterial resource bank of plasma, viable peripheral blood mononuclear cells (PBMCs), frozen PBMCs pellets, and genomic DNA from PBMCs of HTLV-1–infected persons for the future evaluations with new molecular biology techniques.

Hematologists at the collaborating institutions were responsible for enrolling participants after receiving approval from their Institutional Review Boards. The study protocol was approved by the Ministry of Education, Culture, Sports, Science and Technology of Japan. Eligible participants were those who had known of their HTLV-1 infection and had confirmed the HTLV-1–positive serology at any of the medical institutions. Potential participants visited any of the collaborating institutions directly or via the website of the JSPFAD (www.htlv1.org/). They received adequate explanations for the enrollment procedure from the hematologists at the collaborating institutions. Enrollment was conditional on participants giving written informed consent in accordance with the Declaration of Helsinki. The primary participants were asymptomatic HTLV-1 carriers. A small number of patients with definite ATL, HAM/TSP, and HU/HAU were also enrolled as controls.

Data collection and sample storage

After providing written informed consent, participants were expected to fill out a questionnaire regarding demographic information, to provide peripheral blood samples, and to periodically visit the institution for follow-up. After reconfirming the asymptomatic HTLV-1 carrier status of the participants, hematologists at the collaborating institutions assigned a unique identification number to each participant and subsequently sent all materials (individual questionnaire sheets, clinical data, and blood samples drawn into ethylenediaminetetraacetic acid and heparin tubes) to the JSPFAD office (Department of Medical Genome Sciences, Laboratory of Tumor Cell Biology, Graduate School of Frontier Sciences, University of Tokyo, Japan).

The self-administered questionnaire included items on demographic characteristics, birthplaces of the participants and their mothers, family history regarding HTLV-1 status and HTLV-1–associated diseases, length of marriage, partner's HTLV-1 status, first opportunity for HTLV-1 testing, and histories of disease manifestations other than HTLV-1–associated diseases. Additional questionnaire items, information on prior blood transfusion, and smoking habits (present, past, or nonsmoking) were also included after April 2008.

Clinical data included information on the date of visit, complete blood cell count, differential cell counts (including abnormal lymphocytes per 100 leukocytes), lactate dehydrogenase, HTLV-1 serologic test, comorbidities other than HTLV-1–associated diseases, and the development of any HTLV-1–associated diseases during follow-up. Blood samples were collected at enrollment, annually thereafter (in principal), and as needed. Blood samples sent to the study office at the University of Tokyo were separated into plasma, PBMCs, and genomic DNA and then used for viral marker assays at the University of Tokyo or stored for the biomaterial bank at the Japanese Red Cross Fukuoka Blood Center.

Viral marker assays

HTLV-1 proviral load of PBMC samples was measured by real-time polymerase chain reaction (PCR) using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems Japan), as previously described with minor modifications.[30,31] Genomic DNA from PBMCs was isolated using a QIAGEN Blood Kit (QIAGEN). Quantitative real-time PCR was performed using multiplex PCR with 2 sets of primers specific for the HTLV-1 provirus and the human gene encoding the RNase P enzyme. The primers and the probe for the gene encoding RNase P were purchased from Applied Biosystems; those for the pX region of the HTLV-1 provirus were described previously.[30,31] Genomic DNA of normal control PBMCs mixed with a plasmid DNA, which contained almost the whole genome of the HTLV-1 provirus (*SacI* site of 5′-LTR to *SacI* site of 3′-LTR), was used as control template. The copy number of the plasmid DNA was calculated based on the size and weight of the plasmid DNA, as measured by spectrophotometry. The proviral loads were expressed as copy numbers per 100 PBMCs, based on the assumption that infected cells harbored 1 copy of the integrated HTLV-1 provirus per cell. Samples with a higher proviral load (> 20 copies/100 PBMCs) were subjected to Southern blot analysis to examine the clonality of the infected cells. Assays to detect the integrated band of HTLV-1 provirus genome were described previously.[32] Genomic DNA samples (10 mg) were digested with *PstI* or *Eco*RI restriction enzymes and were size-fractionated on 0.7% agarose gels. They were then transferred onto a nylon membrane by the Southern blot technique. Hybridization to randomly primed ^{32}P-labeled DNA probes for the whole proviral genome (*SacI* to *SacI* fragment of the HTLV-1 proviral genome) was performed, followed by appropriate stringency washing steps and autoradiography. Soluble interleukin-2 receptor was measured by a commercial laboratory (SRL Inc) using an enzyme-linked immunosorbent assay (Endogen) and reported as units per milliliter.

Statistical analysis

Analyses were performed for participants who enrolled as of December 2008. Age at enrollment was categorized into 5 groups: younger than 40, 40 to 49, 50 to 59, 60 to 69, and 70 years or older. Geographic location was divided into 4 areas: northern (Hokkaido and Tohoku), metropolitan (Tokyo, Osaka, and Nagoya), southern (Kyushu and Okinawa), and others (supplemental Figure 1). First opportunity for HTLV-1 testing was divided into 3 categories: by screening for HTLV-1 (regional-mass, multiphasic, blood donor, and maternal screenings), by the presence of HTLV-1–infected family members (including spouse), and by the patient status under treatment for diseases unrelated to HTLV-1. A positive family history was considered to be present when participants had information on first-degree relatives (parents, siblings, or offspring) who were HTLV-1 carriers or had HTLV-1–associated diseases (ie, ATL, HAM/TSP, and HU/HAU). Any leukemia and/or lymphoma other than ATL were also taken into consideration. A positive comorbidity at enrollment was considered to be present when any information on diseases other than HTLV-1–associated diseases

was available at enrollment. HTLV-1 proviral loads (copy numbers/100 PBMCs) were used as a continuous variable (raw and the power-transformed data) or by categorizing them into quartiles. We applied a square-root transformation to the raw data of proviral loads to reduce the skewness. Continuous data were presented as median (range) values and compared using a Mann-Whitney test. Categorical data were compared using a χ^2 test or Fisher exact test. We calculated person-years of follow-up for each participant from the date of enrollment to the date of ATL diagnosis, the date of last follow-up, or September 30, 2009, whichever came first. Cumulative progression to ATL was estimated using Kaplan-Meier curves. To estimate the effect of baseline HTLV-1 proviral load and selected demographic factors on ATL development, we performed Cox proportional hazards analyses, and expressed as hazard ratios (HR) and 95% confidence intervals (CI), which were calculated by robust sandwich variance estimates. To check for possible incompleteness in the multivariate model, we also performed analyses using sub-datasets. All statistical analyses were performed using SAS Version 9.1 (SAS Institute Japan) with a 2-tailed significance level of .05.

Results

Baseline demographic characteristics

From August 2002 to December 2008, 1259 participants of asymptomatic HTLV-1 carriers were enrolled in this study. However, HTLV-1 proviral load was not measured for 41 participants. Thus, a total of 1218 participants (426 males and 792 females) were included in this analysis. Demographic characteristics of the participants at enrollment are shown in Table 1. The median ages at enrollment in the cohort were 59.6 years (range, 6.9-92.8 years) for males or 58.3 years (range, 17.8-90.3 years) for females. The largest percentage of study participants was from the southern area, which is a well-known HTLV-1 endemic area in Japan, followed by the metropolitan area. The southern area also had the largest percentage for birthplaces for most participants and their mothers.

One-half of the participants came to know of their HTLV-1 infections through screening for HTLV-1, and one-fourth was informed of their infections while receiving treatments for diseases other than HTLV-1–associated diseases. More than half of the participants did not know their family status of HTLV-1 infection. Only 119 female participants knew about the HTLV-1 infection status of their husbands, of whom 53 (45%) of the husbands were positive for HTLV-1 (data not shown). However, we were not able to obtain reliable information on male-to-female transmission for the female participants. We obtained information on comorbidities at enrollment from 257 participants, of which 45 had comorbid infectious diseases (eg, strongyloidiasis, chronic bronchitis, hepatitis C virus infection, lymphadenitis), 29 had autoimmune diseases (rheumatoid arthritis, chronic thyroiditis, Sjögren syndrome, and other autoimmune or chronic inflammatory diseases), 80 had a variety of definite malignant diseases other than ATL (non-Hodgkin lymphoma, acute myeloid leukemia, gastric cancer, lung cancer, or other malignancies), 16 had skin diseases, and 87 had other common diseases (eg, hypertension, diabetes).

Distributions of baseline HTLV-1 proviral load

Figure 1 shows distribution of baseline HTLV-1 proviral load in 1218 participants. There was a wide range of skewness in the raw data, with a median of 1.60 copies/100 PBMCs (range, 0-55.8 copies/100 PBMCs; 25th-75th percentile, 0.29-4.54 copies/100 PBMCs; Figure 1A). The square-root transformation reduced the skew in the raw data, with a median of 1.26 copies/100

Table 1. Baseline demographic characteristics of asymptomatic HTLV-1 carriers

Variable	Male, no. (%)	Female, no. (%)
Total	426	792
Age, y		
Younger than 40	48 (11.3)	119 (15.0)
40-49	70 (16.4)	130 (16.4)
50-59	99 (23.2)	174 (22.0)
60-69	88 (20.7)	172 (21.7)
70 or older	121 (28.4)	197 (24.9)
Place of enrollment		
Northern area	10 (2.3)	32 (4.0)
Metropolitan area	75 (17.6)	144 (18.1)
Southern area	333 (78.2)	597 (75.4)
Other areas	8 (1.9)	19 (2.4)
Birthplace of participants		
Northern area	18 (4.2)	33 (4.2)
Metropolitan area	30 (7.0)	80 (10.1)
Southern area	240 (56.3)	400 (50.5)
Other areas	20 (4.7)	54 (6.8)
Unknown	118 (27.7)	225 (28.4)
Birthplace of participants' mothers		
Northern area	16 (3.8)	32 (4.0)
Metropolitan area	13 (3.1)	39 (4.9)
Southern area	247 (58.0)	426 (53.8)
Other areas	28 (6.6)	64 (8.1)
Unknown	122 (28.6)	231 (29.2)
First opportunity for HTLV-1 testing		
Screening for HTLV-1	209 (49.1)	452 (57.1)
Regional mass screening	77	164
Multiphasic screening	24	44
Blood donor screening	108	128
Maternal screening	0	116
Revelation of HTLV-1–positive family	33 (7.7)	101 (12.7)
During treatment of other diseases	117 (27.5)	148 (18.7)
Unknown	67 (15.7)	91 (11.5)
Family history of HTLV-1–associated diseases*		
Absent	98 (23.0)	154 (19.5)
Absent for a first-degree relative but having an infected spouse	6 (1.4)	23 (2.9)
Carrier only	27 (6.3)	74 (9.3)
HU/HAU only	2 (0.5)	1 (0.1)
HAM	2 (0.5)	7 (0.9)
ATL	34 (8.0)	74 (9.3)
Leukemia or lymphoma	9 (2.1)	26 (3.3)
Unknown family history	248 (58.2)	433 (54.7)
Comorbidity†		
Absent	331 (77.7)	630 (79.5)
Present	95 (22.3)	162 (20.5)
Infectious diseases	20	25
Autoimmune diseases	3	26
Malignant diseases	36	44
Skin diseases	8	8
Other disease	28	59

HTLV-1 indicates human T-cell leukemia virus type 1; HU, HTLV-1 uveitis; HAU, HTLV-1–associated uveitis; HAM, HTLV-1 myelopathy; and ATL, adult T-cell leukemia.

*Family history was restricted to a first-degree relative. "Present" indicates that participants have a parent, sibling, or offspring diagnosed with HTLV-1-associated diseases. Family members with HAM and HU/HAU were included into the category of "HAM." Family members with ATL and HAM and/or HU/HAU were included into the category of "ATL."

†Comorbidity indicates that participants have any diseases other than HTLV-1–associated diseases at enrollment.

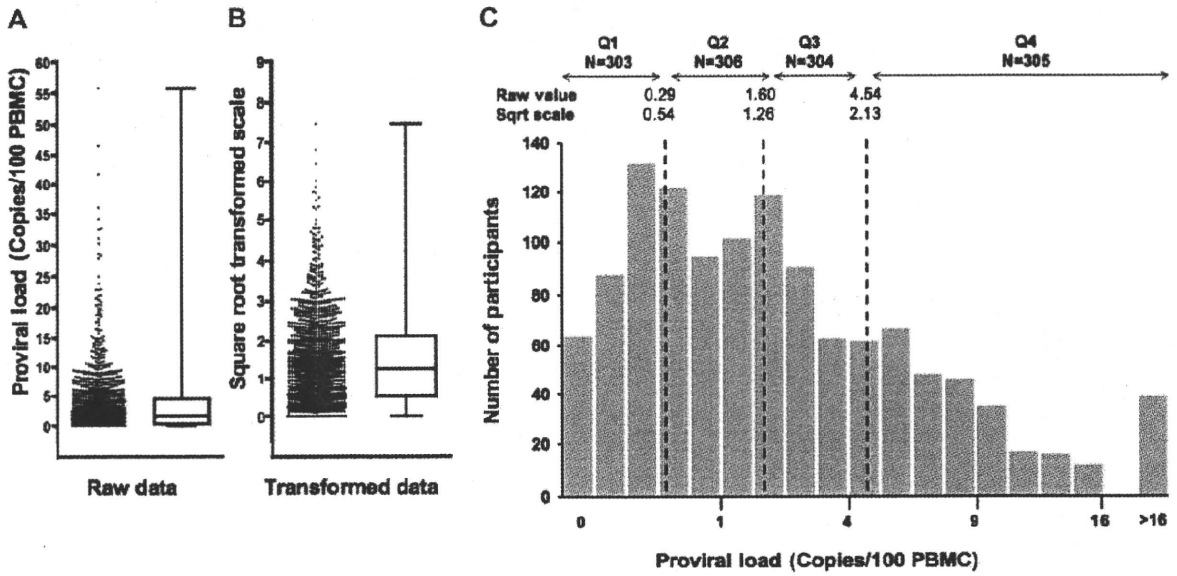

Figure 1. Distribution of baseline HTLV-1 proviral load levels among 1218 asymptomatic HTLV-1 carriers. (A) Scatter plot of raw data of proviral load (left) and the vertical box and whiskers plot (right): the box delineates 25th percentile (0.29 copies/100 periopheral blood mononuclear cells [PBMCs]), median (1.60 copies/100 PBMCs), and 75th percentiles (4.54 copies/100 PBMCs), and the whiskers delineate the minimum (0 copies/100 PBMCs) and maximum (55.8 copies/100 PBMCs). (B) Scatter plot of square-root transformed values of the raw proviral load (left) and the vertical box and whiskers plot (right): the box delineates 25th percentile (0.54 copies/100 PBMCs), median (1.26 copies/100 PBMCs), and 75th percentiles (2.13 copies/100 PBMCs), and the whiskers delineate the minimum (0 copies/100 PBMCs) and maximum (7.47 copies/100 PBMCs). (C) The frequency of participants in the quartile distributions of proviral load. Q1 indicates quartile 1 (< 25th percentile); Q2, quartile 2 (25th percentile to median); Q3, quartile 3 (median to 75th percentile); Q4: quartile 4 (> 75th percentile); Sqrt, square-root transformation; and N, number of participants.

PBMCs (range, 0-7.47 copies/100 PBMCs; 25th-75th percentile, 0.54-2.13 copies/100 PBMCs; Figure 1B). Figure 1C shows the frequency of participants in each quartile of proviral load.

The median proviral load and a frequency of subjects in each quartile of proviral load by demographic characteristics are shown in Table 2. Males and females were significantly different in proviral load levels, with a median value of 2.10 copies/100 PBMCs (range, 0-46.6 copies/100 PBMCs) for males and that of 1.39 copies/100 PBMCs (range, 0-55.8 copies/100 PBMCs) for females ($P < .001$). Males were probably distributed in the highest quartile of proviral load level than females.

Among age groups, the median proviral load of those 40 to 49 and 50 to 59 years of age was significantly higher than that of those less than or equal to 40 years ($P = .02$ and $P = .007$, respectively). Both age groups were probably distributed in the highest quartile of proviral load levels. Because we found a significantly different median proviral load by sex, we additionally evaluated the proviral load level by age group in each sex. The highest median value was found in those 50 to 59 years of age (2.89 copies/100 PBMCs) in males, but in 40 to 49 years of age (1.49 copies/100 PBMCs) in females, although there were no statistical differences by age group for both sexes (data not shown).

Among the categories for the first opportunity for HTLV-1 testing, the proviral load level was significantly higher ($P = .002$) in participants informed of their infection during treatment for diseases unrelated to HTLV-1 compared with those who came to know of their infection by screenings (Table 2). Participants informed of their infection during treatment for diseases unrelated to HTLV-1 were probably distributed in the highest quartile of proviral load levels. There was no difference in the proviral load level between those who came to know of their infection by the presence of HTLV-1–positive family members and those who came to know of their infection by screenings.

When we evaluated the proviral load level by family history status, participants who had no family history of HTLV-1 infection, who had only HTLV-1 carriers in the family, who had only an HTLV-1 carrier husband, and who had only HU/HAU in the family were grouped together as a reference category. The proviral load levels of those with a family history of HAM/TSP (median 3.85 copies/100 PBMCs) and ATL (median 2.32 copies/100 PBMCs) were significantly higher ($P = .01$ and $P = .005$, respectively) compared with those of the reference group (Table 2). Indeed, those with a family history of HAM/TSP and ATL were probably distributed in the third and fourth quartiles of proviral load levels. Of interest, the median proviral load level of those with a family history of leukemia or lymphoma was also significantly higher ($P = .009$) compared with those of the reference group.

Among the categories for comorbidity, there was no statistical difference in the proviral load levels when we simply compared between those with and without comorbidity at enrollment (data not shown). However, when we compared those without comorbidity and those with infectious diseases at enrollment, the median proviral load of the latter was significantly higher than that of the former ($P = .05$; Table 2).

Prognosis

During a median follow-up period of 1.0 year (range, 0-6.6 years) and a total of 1981.2 person-years, 14 (1.1%) participants (4 males and 10 females) progressed to overt ATL (2 acute, 2 lymphoma, and 10 smoldering types; Table 3). The incidence rate of ATL was 7.1 per 1000 person-years for all types of ATL and 2.0 per 1000 person-years for the aggressive types (acute and lymphoma) of ATL. The median duration from date of enrollment to date of diagnosis of ATL was 13.8 months (range, 2.8-64.4 months). The cumulative probability of progression to ATL was reached 4.8% (95% CI, 1.9%-11.8%) at 5.4 years (Figure 2).

Table 2. HTLV-1 VL levels by demographic characteristics

Demographic characteristics	No.	Median VL (range) (copies/100 PBMCs)	Frequency of subjects by VL level, n (% of row)			
			Quartile 1 (VL: < 0.29)‡	Quartile 2 (VL: 0.29-1.60)	Quartile 3 (VL: 1.60-4.54)	Quartile 4 (VL: ≥ 4.54)
Total		1.60 (0-55.8)	303	306	304	305
Sex						
Male	426	2.10 (0-46.6)*	84 (19.7)	100 (23.5)	93 (21.8)	149 (35.0)
Female	792	1.39 (0-55.8)†	219 (27.7)	206 (26.0)	211 (26.6)	156 (19.7)
Age, y						
Younger than 40	167	1.37 (0-16.4)†	49 (29.3)	43 (25.8)	50 (29.9)	25 (15.0)
40-49	200	1.77 (0-41.7)*	43 (21.5)	52 (26.0)	51 (25.5)	54 (27.0)
50-59	273	1.84 (0-36.1)*	64 (23.4)	64 (23.4)	63 (23.1)	82 (30.4)
60-69	260	1.56 (0-46.6)	66 (25.4)	66 (25.4)	61 (23.5)	67 (25.8)
70 or older	318	1.52 (0-55.8)	81 (25.5)	81 (25.5)	79 (24.8)	77 (24.2)
First opportunity for HTLV-1 testing						
Screening	661	1.46 (0-55.8)†	182 (27.5)	160 (24.2)	175 (26.5)	144 (21.8)
Revelation of HTLV-1–positive family	134	1.45 (0-46.6)	31 (23.1)	40 (29.9)	39 (29.1)	24 (17.9)
During treatment for other diseases	265	1.93 (0-41.7)*	56 (21.1)	66 (24.9)	57 (21.5)	86 (32.5)
Unknown	158	2.08 (0-30.3)*	34 (21.5)	40 (25.3)	33 (20.9)	51 (32.3)
Family history of HTLV-1–related diseases						
Absence or carrier/HU/HAU only	385	1.33 (0-32.4)†	100 (26.0)	105 (27.2)	100 (26.0)	80 (20.8)
HAM/TSP	9	3.85 (1.2-9.4)*	0	1 (11.1)	5 (55.6)	3 (33.3)
ATL	108	2.32 (0-46.6)*	18 (16.7)	26 (24.1)	33 (30.6)	31 (28.7)
Leukemia or lymphoma	35	2.47 (0-12.8)*	3 (8.6)	9 (25.7)	11 (31.4)	12 (34.3)
Unknown family history	681	1.55 (0-55.8)	182 (26.7)	165 (24.2)	155 (22.8)	179 (26.3)
Comorbidity						
Absence	961	1.65 (0-55.8)†	241 (25.1)	234 (24.4)	244 (25.4)	242 (25.2)
Infectious diseases	45	2.75 (0-28.6)*	7 (15.6)	8 (17.8)	13 (28.9)	17 (37.8)
Autoimmune diseases	29	1.33 (0-41.7)	10 (34.5)	7 (24.1)	4 (13.8)	8 (27.6)
Malignant diseases	80	1.57 (0-19.4)	19 (23.8)	21 (26.3)	23 (28.8)	17 (21.3)
Skin diseases	16	0.60 (0.07-14.6)	6 (37.5)	5 (31.3)	3 (18.8)	2 (12.5)
Other disease	87	1.17 (0-22.0)	20 (23.0)	31 (35.6)	17 (19.5)	19 (21.8)

HTLV-1 indicates human T-cell leukemia virus type 1; VL, HTLV-1 proviral load; PBMCs, peripheral blood mononuclear cells; HU, HTLV-1 uveitis; HAU, HTLV-1–associated uveitis; HAM, HTLV-1 myelopathy; TSP, tropical spastic paraparesis; and ATL, adult T-cell leukemia.

*Mann-Whitney test revealed a statistically significant difference in the VL level compared with the reference group.

†Reference group.

‡The VL was categorized based on quartile cutoff points (the 25th, 50th, and 75th percentiles of the VL distribution) in 1218 HTLV-1 carriers. The unit of VL was copies/100 PBMCs.

The median proviral load at enrollment for these 14 participants was 10.3 copies/100 PBMCs (range, 4.17-28.58 copies/100 PBMCs), which was significantly higher than those who did not develop ATL (1.56 copies/100 PBMCs; range, 0-55.8 copies/100 PBMCs; $P < .001$). Of interest, the median proviral load level at enrollment was significantly higher for those who developed smoldering types of ATL than for those who developed aggressive types of ATL (11.4 and 5.1 copies/100 PBMCs, respectively, $P = .02$), whereas the median entry age was significantly younger for the former than for the latter (59.8 and 73.9 years, respectively, $P = .02$). Distribution of the 14 participants who developed ATL by demographic characteristics and by quartile of proviral load levels is shown in Table 4. Among 14 ATLs, 13 occurred in the highest quartile of baseline proviral load (> 4.54 copies/100 PBMCs) and 1 occurred in the third quartile (1.60-4.54 copies/100 PBMCs), whereas no ATL developed in quartiles 1 and 2 (< 1.60 copies/100 PBMCs). A high frequency of ATL was also seen in older age group, those with first opportunity for HTLV-1 testing during treatment of other diseases and those with a family history of ATL. Therefore, we decided to include the baseline HTLV-1 proviral load (the square-root transformed continuous value), age, first opportunity for HTLV-1 testing, and family history into Cox hazard analyses as covariates to test the effects on the development of ATL.

We identified that baseline proviral load was strongly associated with the risk of progression to ATL on both univariate and multivariate Cox analyses. In the multivariate analysis, the adjusted HR for the square-root transformed proviral load per unit increase was 3.57 (95% CI, 2.25-5.68; Table 5). We also found that advanced age, family history of ATL, and first opportunity to learn of HTLV-1 infection during treatment of other diseases were independently associated with the development of ATL, after adjusting the effect of proviral load. The adjusted HR for developing ATL per 5-year increase of age from 40 years was 1.67 (95% CI, 1.12-2.50). HTLV-1 carriers having a family history of ATL had 12 times higher risk of developing ATL compared with those not having the history (adjusted HR = 12.1; 95% CI, 2.26-64.7), and those who came to know their HTLV-1 infection during treatment for other diseases had 4 times higher risk of developing ATL compared with references (adjusted HR = 4.16; 95% CI, 1.37-12.6), although the CIs were wide because of the smaller group sizes (Table 5). Of interest, male gender was not a significant risk factor for developing ATL, even though the median proviral load was significantly higher in males than in females (Table 2).

Because the distribution of proviral load was skewed even after the value was square-root transformed, it was possible that ATL events in subjects with skewed high proviral loads contributed to results. To check the possibility, we performed a multivariate analysis using a sub-dataset that excluded subjects with skewed proviral load (> 16 copies in Figure 1C; n = 39, including 3 who developed ATL). Nevertheless, we observed similar results as the original dataset, although age factor was no longer statistically

Table 3. Cases who developed ATL from HTLV-I carrier status

	Demographic characteristics						Baseline clinical and biologic values					ATL development	
Case no.	Sex	Age, y	Place of birth	First opportunity for HTLV-1 testing	Family history of HTLV-1–related disease	Comorbidity at enrollment	HTLV-1 VL, copies/100 PBMCs	sIL-2R, U/mL	Abnormal lymphocytes, percentage	LDH, IU/L	WBC, $\times 10^3/mm^3$	Clinical type	Duration from enrollment, mo
Progression to aggressive type of ATL													
1	M	79.9	Southern	ATL family	ATL	None	5.47	479	2	157	4200	Acute	7.4
2	F	70.3	Southern	ATL family	ATL	None	4.73	904	0	365	9130	Acute	38.6
3	M	71.9	Southern	Other disease	None	Skin disease	4.17	1450	0	351	5140	Lymph	4.6
4	F	75.8	Southern	Unknown	Unknown	None	10.52	2080	3	308	3600	Lymph	30.6
Progression to indolent type of ATL													
5	F	60.0	Southern	Other disease	Unknown	None	9.12	340	14	192	5100	Sm	6.0
6	F	71.9	Southern	Multiphasic screening	None	None	10.60	1320	2	199	4000	Sm	29.8
7	F	59.5	Southern	Multiphasic screening	None	None	21.90	635	4.5	188	4100	Sm	12.0
8	F	74.0	Southern	Other disease	Unknown	Gallbladder cancer	10.11	1110	2	240	2700	Sm	26.8
9	F	54.1	Southern	Other disease	Unknown	None	18.85	971	2	198	5660	Sm	29.0
10	F	43.3	Southern	Pregnancy	ATL	None	13.90	372	1	ND	5400	Sm	64.4
11	F	62.2	Southern	Other disease	Unknown	Eye disease	6.86	1560	ND	508	12100	Sm	6.0
12	M	57.6	Southern	Other disease	Unknown	None	7.67	ND	2	234	5500	Sm	15.4
13	F	41.0	Metropolitan	Pregnancy	None	None	12.14	349	2.5	189	7690	Sm	12.2
14	M	66.1	Southern	Other disease	None	Prostatitis	28.58	2660	0	158	8500	Sm	2.8

ATL indicates adult T-cell leukemia; HTLV-1, human T-cell leukemia virus type 1;VL, HTLV-1 proviral load; PBMCs, peripheral blood mononuclear cells; sIL-2R, soluble interleukin-2 receptor; LDH, lactate dehydrogenase; WBC, white blood cell count; Sm, smoldering type; and ND, not done.

significant ($P = .07$; supplemental Table 1). It is also possible that effects of some of the risk factors are weighted because of only 1 patient with an event because only 14 were analyzed as events in the multivariate analyses. To check the possibility, we performed 14 leave-one-out analyses, omitting 1 of 14 cases at a time from the original dataset. The Jackknifed coefficient of each parameter revealed the stability, which indicated that none of 14 cases affected the original model (data not shown).

Discussion

Previous studies reported no significant differences in the HTLV-1 proviral load by sex and age in asymptomatic HTLV-1 carriers.[21,22,24,33] In the present study, however, we found that there were significant differences in the proviral load by sex and age (Table 2). The median HTLV-1 proviral load was significantly higher in males than females. The median HTLV-1 proviral load for those 40 to 49 and 50 to 59 years of age was significantly higher than for those less than or equal to 40 years. The discrepancy between results of previous studies and those of the present study may be primarily explained by the differences in study population characteristics. We also found sex differences in age distributions of HTLV-1 proviral load; in male subjects, the median proviral load level was the highest at 50 to 59 years of age, whereas in female subjects it was highest at 40 to 49 years of age, although there were no statistical differences. These distribution characteristics of HTLV-1 proviral load are of interest when we consider the differences in sex and age at onset between ATL and HAM/TSP. ATL occurs predominantly in older males (~ 60 years), whereas HAM/TSP occurs predominantly in middle-aged females (~ 45-55 years). Thus, the proviral load levels of asymptomatic HTLV-1 carriers might be the highest in the age groups approximately 5 to 10 years before the average age at onset of ATL and HAM/TSP. These distribution characteristics may be related to differences in host immune responses to HTLV-1 and other unknown host factors.[34]

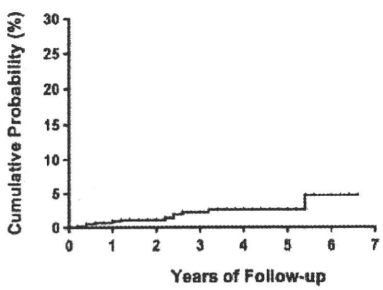

Figure 2. Probability of progression to ATL among 1218 asymptomatic HTLV-1 carriers.

The present study revealed that the median proviral load level of those with a family history of ATL or HAM/TSP was significantly higher than for those with no family history (Table 2). These results support previous studies indicating that HTLV-1–infected blood donors and asymptomatic carriers with familial HAM/TSP or ATL tend to have a higher HTLV-1 proviral load than those without family history.[21,33] In the present study, the proviral loads were also higher in those with a family history of leukemia or lymphoma than those without such history. We assume that the family history of leukemia or lymphoma may have included some ATL cases because some participants provided a diagnosis as just unknown leukemia or lymphoma. Although the present study was a large cohort, data collection regarding family history of HTLV-1–associated diseases was insufficient because one-half of the participants did not know their family HTLV-1 status. Further detailed data collection is needed to confirm the characteristics of HTLV-1 proviral load levels by family histories among asymptomatic HTLV-1 carriers, as this is necessary to determine genetic determinants of HTLV-1–associated diseases.

HTLV-1 carriers have various comorbidities, such as infectious, autoimmune, and malignant diseases.[4,25,35-38] In the present study, 45 participants had various infectious diseases at enrollment (Table

Table 4. Frequency of subjects who developed ATL by demographic characteristics and by VL level

Demographic characteristics	No. of subjects	No. of ATLs (% of subjects)	Frequency of ATL by VL level, n (% of subjects in each quartile in Table 2)		
			Quartiles 1 and 2 (VL: < 1.60)*	Quartile 3 (VL: 1.60-4.54)	Quartile 4 (VL: ≥ 4.54)
Total	1218	14 (1.1)	0	1 (0.3)	13 (4.3)
Sex					
Male	426	4 (0.9)	0	1 (1.1)	3 (2.0)
Female	792	10 (1.3)	0	0	10 (6.4)
Age, y					
Younger than 40	167	0	—	—	—
40-49	200	2 (1.0)	0	0	2 (3.7)
50-59	273	3 (1.1)	0	0	3 (3.7)
60-69	260	3 (1.2)	0	0	3 (4.5)
70 or older	318	6 (1.9)	0	1 (1.3)	5 (6.5)
First opportunity for HTLV-1 testing					
Screening	661	4 (0.6)	0	0	4 (2.8)
Revelation of HTLV-1-positive family	134	2 (1.5)	0	0	2 (8.3)
During treatment for other diseases	265	7 (2.6)	0	1 (1.8)	6 (7.0)
Unknown	158	1 (0.6)	0	0	1 (2.0)
Family history of HTLV-1–related diseases					
Absence or carrier/HU/HAU only	385	5 (1.3)	0	1 (1.0)	4 (5.0)
HAM/TSP	9	0	—	—	—
ATL	107	3 (2.8)	0	0	3 (9.7)
Leukemia or lymphoma	36	0	0	0	0
Unknown family history	681	6 (0.9)	0	0	6 (3.4)
Comorbidity					
Absence	961	10 (1.0)	0	0	10 (4.1)
Infectious diseases	45	1 (2.2)	0	0	1 (5.9)
Autoimmune diseases	29	0	—	—	—
Malignant diseases	80	1 (1.3)	0	0	1 (5.9)
Skin diseases	16	1 (6.3)	0	1 (33.3)	0
Other disease	87	1 (1.1)	0	0	1 (5.3)

ATL indicates adult T-cell leukemia; HTLV-1, human T-cell leukemia virus type 1; VL, HTLV-1 proviral load; HU, HTLV-1 uveitis; HAU, HTLV-1–associated uveitis; HAM, HTLV-1 myelopathy; TSP, tropical spastic paraparesis; and —, not applicable.

*The VL was categorized based on quartile cutoff points (the 25th, 50th, and 75th percentiles of the VL distribution) in 1218 HTLV-1 carriers. The unit of VL was copies/100 PBMCs.

1). We found that the median proviral load of these participants was significantly higher than that of those with no comorbidity (Table 2). The results of the present study support previous reports indicating higher HTLV-1 proviral loads in HTLV-1 carriers with comorbid *Strongyloides stercoralis* or bladder and kidney infections than those without such infections.[25,35,36] HTLV-1 carriers with rheumatoid arthritis or connective tissue disease and those with myelodysplastic syndromes carrying HLA-A26 were also reported to have higher HTLV-1 proviral loads compared with the median proviral load of those without such diseases.[37,38] In the present study, however, the median proviral load was not significantly high in those with autoimmune and malignant diseases. Further studies are required to find other predisposing factors affecting the proviral load level in each person.

A high HTLV-1 proviral load is currently considered as one of the main indicators for the progression to ATL.[20,28] In the present study, 14 participants of asymptomatic HTLV-1 carriers progressed to overt ATL as of 2009, all of whose baseline proviral load levels were high (range, 4.17-28.58 copies/100 PBMCs; Table 3). Therefore, we suggest that those with a high proviral load level (~ > 4 copies/100 PBMCs) are in a high-risk group for developing ATL (this group accounted for ~ 29% of the cohort). Multivariate Cox analyses confirmed that a higher proviral load level was a strong factor in the development of ATL (Table 5). This result strongly supports previous small-scale studies.[20,28] However, the role of the high proviral load level still remains unclear because the majority of asymptomatic carriers with a high HTLV-1 proviral load level in the present study remain carrier status. In the present study, male gender was not a significant risk factor for ATL, even though the median proviral load was significantly higher in males than in females. A high HTLV-1 proviral load is also reported to be associated with HAM/TSP.[20,21,27] These findings suggest that a high

Table 5. Cox proportional hazards modeling of risk factors for ATL development

	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P*	HR (95% CI)	*P*
Male sex (vs female)	0.74(0.23-2.37)	.61	0.38(0.12-1.18)	.09
Square-root transformed VL per unit increase	2.55(1.91-3.41)	< .001	3.57(2.25-5.68)	< .001
Age per 5-year increase from 40 y	1.20(0.94-1.53)	.15	1.67(1.12-2.50)	.012
Family history of ATL (vs others)	2.68(0.80-8.98)	.11	12.1 (2.26-64.7)	.004
First opportunity for HTLV-1 testing during treatment of other diseases (vs others)	3.40(1.12-10.28)	.03	4.16(1.37-12.6)	.012

Analyses were performed using robust sandwich variance estimates.

ATL indicates adult T-cell leukemia; HTLV-1, human T-cell leukemia virus type 1; VL, HTLV-1 proviral load; HR, hazard ratio; and CI, confidence interval.

proviral load alone is not a unique predictive marker for ATL. In addition, the present study showed that the median proviral load level at enrollment was lower in those who developed aggressive types of ATL (5.1 copies/100 PBMCs) than that in those who developed smoldering types of ATL (11.4 copies/100 PBMCs; $P = .02$). This also suggests that a high proviral load alone is not a predictive marker for aggressive types of ATL.

In the present study, multivariate Cox analysis indicated that increased age, family history of ATL, and first opportunity to learn of HTLV-1 infection during treatment of other diseases were also independent risk factors for the development of ATL, after adjusting for proviral load (Table 5). This suggests that multiple risk factors (including unknown factors) are related to the progression from HTLV-1 carrier status to ATL. The reason why "opportunity to learn of HTLV-1 infection during treatment of other diseases" was an independent risk factor is unknown. The findings that more advanced states of HTLV-1 carriers (ie, an intermediate state[6] and a preleukemic state[13]) tend to be complicated by various comorbid diseases and that HTLV-1 carriers with various comorbid diseases had higher HTLV-1 proviral loads[25,35-38] could in part explain the reason.

Some prospective studies serially evaluated HTLV-1 proviral loads in HTLV-1 carriers and reported that their proviral load level was relatively stable over time with a certain level of fluctuations for persons.[25,26,28] Taylor et al reported that proviral loads of 20 HTLV-1 carriers were stable over a mean of 27 months, even though 9 carriers with various comorbidities showed high proviral load levels.[25] Meanwhile, an increasing proviral load was observed before progression to HAM/TSP and ATL.[27,28] However, there remain more questions how much of the fluctuations in proviral load over time could predict disease progression over the natural fluctuations within persons. Factors other than the proviral load level might be influencing the development of HTLV-1–associated diseases. Future studies should perform serial evaluations of HTLV-1 proviral loads by considering risk factors that have been confirmed in the present study.

The present study has several limitations. The number of ATL events was very small to obtain a conclusive result. However, we have a confidence for our results because we used a robust variance estimate in the multivariate analysis and because 2 validity analyses confirmed the original results. Data collection was insufficient for some items in the questionnaire. To resolve this issue, we will need to administer the questionnaire repeatedly. Our study design did not include enough information for evaluating the development of HAM/TSP. The follow-up duration is too short with regard to the natural history of ATL that has a long latency. Further follow-up of this cohort and similar prospective investigations should provide data needed to support more detailed conclusions. We did not compare the proviral loads by place of enrollment because we realized that many HTLV-1 carriers have migrated from the southern area to the metropolitan area.[39] The migration of HTLV-1 carriers has raised some public health issues in Japan. Screening for HTLV-1 in pregnant women and prevention programs for mother-to-child transmission of HTLV-1 are conducted in endemic areas[40,41] but not in metropolitan areas, which could introduce a higher chance of new HTLV-1 infections in the metropolitan area. To date, there is no nationwide program for preventing new HTLV-1 infections in Japan. Further nationwide studies are needed to determine the precise numbers of HTLV-1 carriers and to prevent HTLV-1 infection.

In conclusion, the present cohort study of 1218 asymptomatic HTLV-1 carriers provided detailed distributions for HTLV-1 proviral loads regarding the host-specific characteristics and the associations with the development of ATL. We confirmed that a higher proviral load levels (especially $\sim > 4$ copies/100 PBMCs), advanced age, family history of ATL, and having the first opportunity to learn of HTLV-1 infection during treatment of other diseases were independent risk factors for progression from carrier status to ATL. Further large-scale epidemiologic studies are needed to clearly identify the determinants of ATL for early detection and rapid cure for HTLV-1–associated diseases.

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Authorship

Contribution: M.I. managed the study database, analyzed data, and wrote the manuscript; T.W. organized the study and managed processing of the samples and measurement of proviral loads; A.U., A.O., K. Uchimaru, K.-R.K., M.O., H.K., K. Uozumi, M.M., K.T., Y. Saburi, M.Y., J.T., and Y.M. were responsible for participant enrollment and data collection; Y. Sagara managed the biomaterial bank; S.H. organized the study and managed the database; S.K. and K.Y. established the study; and all authors critically reviewed the article and approved the final version.

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A complete list of JSPFAD participants is available online in the supplemental Appendix.

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Long-term study of indolent adult T-cell leukemia-lymphoma

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The long-term prognosis of indolent adult T-cell leukemia-lymphoma (ATL) is not clearly elucidated. From 1974 to 2003, newly diagnosed indolent ATL in 90 patients (65 chronic type and 25 smoldering type) was analyzed. The median survival time was 4.1 years; 12 patients remained alive for more than 10 years, 44 progressed to acute ATL, and 63 patients died. The estimated 5-, 10-, and 15-year survival rates were 47.2%, 25.4%, and 14.1%, respectively, with no plateau in the survival curve. Although most patients were treated with watchful waiting, 12 patients were treated with chemotherapy. Kaplan-Meier analyses showed that advanced performance status (PS), neutrophilia, high concentration of lactate dehydrogenase, more than 3 extranodal lesions, more than 4 total involved lesions, and receiving chemotherapy were unfavorable prognostic factors for survival. Multivariate Cox analysis showed that advanced PS was a borderline significant independent factor in poor survival (hazard ratio, 2.1, 95% confidence interval, 1.0-4.6; $P = .06$), but it was not a factor when analysis was limited to patients who had not received chemotherapy. The prognosis of indolent ATL in this study was poorer than expected. These findings suggest that even patients with indolent ATL should be carefully observed in clinical practice. Further studies are required to develop treatments for indolent ATL. (*Blood*. 2010;115(22):4337-4343)

Introduction

Adult T-cell leukemia-lymphoma (ATL) is a peripheral T-lymphocytic malignancy associated with human T-cell lymphotropic virus type I (HTLV-1).[1] ATL has been classified into 4 clinical subtypes: acute, lymphoma, chronic, and smoldering.[2] In general, acute and lymphoma types of ATL have a extremely poor prognosis despite advances in chemotherapy and allogenic hematopoietic stem cell transplantation[3-5] because of multidrug resistance, a large tumor burden with multiorgan failure, hypercalcemia, and/or frequent infectious complications associated with a T-cell immunodeficiency. A previous study, in which Japanese patients with ATL were followed for a maximum duration of 7 years, reported that the 4-year survival rates for acute, lymphoma, chronic, and smoldering type were 5.0%, 5.7%, 26.9%, and 62.8%, respectively, with the median survival time (MST) of 6.2 months, 10.2 months, 24.3 months, and not yet reached, respectively.[2] Therefore, the chronic and smoldering subtypes of ATL are considered indolent and are usually managed with watchful waiting until disease progression to acute crisis, similar to the management of chronic lymphoid leukemia or smoldering myeloma. However, the follow-up duration of the previous Japanese study was too short for indolent ATL to evaluate the overall risk of progression to acute or lymphoma types (ie, aggressive ATL). A recent Brazilian study, in which patients with ATL were followed for a maximum duration of 14 years, reported that the MST of chronic and smoldering types were 18 months and 58 months, respectively, and the overall survival (OS) rates were less than 20% in both types.[6] Their results suggest that the long-term prognosis of indolent ATL might be worse than expected.

The long-term prognosis of Japanese patients with indolent ATL has not been well evaluated so far. Prognostic factors for patients with indolent ATL are also unclear. In the present study, we investigated the long-term outcome of 90 patients with indolent ATL. The purposes of this study were to estimate the 5-, 10-, and 15-year survival rates for indolent ATL and to evaluate the prognostic factors.

Methods

Patients

We evaluated a total of 90 patients with indolent ATL (25 smoldering type and 65 chronic type) who were newly diagnosed at the Nagasaki University Hospital between July 1974 and December 2003. The distribution of patients by year of diagnosis in decades (1974-1983, 1984-1993, and 1994-2003) are presented in Table 1. The cutoff date for analysis was December 2008. The diagnosis of ATL was based on clinical features, histologically and/or cytologically proven mature T-cell malignancy, the presence of anti–HTLV-1 antibody, and monoclonal integration of HTLV-1 proviral DNA into tumor cells as described previously.[2,7-9] The subtypes of ATL were classified according to criteria established by the Lymphoma Study Group of Japan Clinical Oncology Group.[2] Clinical data included date of diagnosis, complications at diagnosis, therapy regimens if applicable, date of death, cause of death, and date of latest contact. This retrospective, nonrandomized, observational study that used existing data

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Table 1. Distribution of patients in 3 decades from 1974 to 2003

Year	Total no. of patients	No. of smoldering type (% of total)	No. of chronic type (% of total)
1974-1983	19	2 (10.5)	17 (89.5)
1984-1993	35	7 (20.0)	28 (80.0)
1994-2003	36	16 (44.4)	20 (55.6)
Total for all years	90	25 (27.8)	65 (72.2)

was granted an exemption from the institutional review board and waived the requirement for written informed consent.

Clinical factors and definitions

Age was dichotomized into 2 groups: 60 years or older and younger than 60 years. Performance status (PS) was based on the 5-grade scale of the World Health Organization. Complications at diagnosis were dichotomized into present and absent. Leukocytosis was defined as white blood cell count of 12×10^9/L or greater with the median value as cutoff level. Lymphocytosis was defined as a total lymphocyte count of 6.5×10^9/L or greater with the median value as cutoff level. Neutrophilia was defined as a neutrophil count of 7.5×10^9/L or greater.[10] Eosinophilia was defined as an eosinophil count of 0.4×10^9/L greater.[11] Lactate dehydrogenase (LDH) and blood urea nitrogen (BUN) were dichotomized into normal and elevated concentrations.[12] Albumin was dichotomized into concentrations of 40.0 g/L (4.0 g/dL) or greater and less than 40.0 g/L (4.0 g/dL).[2] Potential prognostic factors (PPFs) for chronic ATL were defined as those with at least one of the following 3 factors: low serum albumin, high LDH, or high BUN according to previous reports.[13,14] Tumor lesions were evaluated as the number of lymph node lesions, number of extranodal lesions, and number of total involved lesions. Extranodal lesions were defined as follows: bone marrow (BM) involvement as the presence of more than 5% typical ATL cells on a BM smear or detection of their infiltration in a BM biopsy specimen; skin involvement as the presence of ATL infiltration in a skin biopsy specimen or as the clinically presence of typical types of skin lesions such as tumors, nodules, erythema, and papules, if biopsy was impossible; lung involvement as lesions with ATL cell infiltration in a transbronchial lung biopsy specimen or in bronchoalveolar lavage fluid; liver involvement as hepatomegaly determined by any imaging tests or liver biopsy if done; spleen involvement as splenomegaly on any imaging test. All patients had peripheral blood involvement. Both lymph node and extranodal tumor lesions were determined according to Ann Arbor classification.[2] The number of total involved lesions was defined as the sum of lymph node lesions and extranodal lesions.[2] Factors used in analyses were listed in Table 2.

Statistical analysis

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 estimated using the Kaplan-Meier method and were compared using the generalized Wilcoxon test. MST was estimated as the time point at which the Kaplan-Meier survival curves crossed 50%. Time to transformation was calculated as the time from the date of the first diagnosis to the date of transformation into the aggressive type (acute or lymphoma type). Univariate and multivariate Cox regression analyses were applied to evaluate prognostic factors for survival. The effects of clinical parameters were evaluated as hazard ratios (HRs) and their 95% confidence intervals (95% CIs). All statistical analyses were performed using SAS software (Version 9.1; SAS Japan Institute). All tests were 2-tailed, and the statistical significance level was set at .05.

Results

Baseline characteristics

The median value of white blood cell count, lymphocyte count, neutrophil count, and eosinophil count was 11.5×10^9/L (range, 3.9-94.4 $\times 10^9$/L), 6.5×10^9/L (range, 0.9-80.2 $\times 10^9$/L), 4.9×10^9/L (range, 1.5-25.5 $\times 10^9$/L), and 0.06×10^9/L (range, 0-3.0 $\times 10^9$/L), respectively. Frequencies of the patients at baseline are summarized in Table 2. Fifty-eight percent of the patients were male, 52% were 60 years or older, and 22% had an advanced PS (2 or more grade). Regarding complications, 35 patients (39%) had some complications at the time of diagnosis, including 13 with chronic pulmonary diseases (10 chronic bronchitis, 2 diffuse panbronchiolitis, and 1 bronchial asthma with chronic bronchitis), 9 with opportunistic infections (3 pneumocystis pneumonia, 2 cryptococcal meningitis, 2 aspergillus pneumonia, 1 cytomegalovirus infection, and 1 pulmonary tuberculosis), 7 with malignancies other than ATL (2 lung cancer, 1 larynx cancer, 1 pharynx cancer, 1 colon cancer, 1 hepatic cell carcinoma, and 1 lip cancer), and 6 with autoimmune diseases (2 infective dermatitis, 1 primary biliary cirrhosis, 1 autoimmune hemolytic anemia, 1 dermatomyositis, and 1 ulcerative colitis). The 6 patients with autoimmune diseases had received a variety of medications as follows: antibiotics for infective dermatitis, ursodeoxycholic acid for primary biliary cirrhosis, prednisolone for autoimmune hemolytic anemia and dermatomyositis, and sulfasalazine for ulcerative colitis. Concerning the hematologic factors, 43 patients (48%) had leukocytosis, 45 (50%) had lymphocytosis, 17 (19%) had neutrophilia, and 17 (19%) had eosinophilia. Regarding the laboratory factors, 28 patients (31%) had a high LDH level (greater than the normal limit). Only 5 of 87 patients (6%) had an abnormal BUN level; 34 of 88 patients (39%) had a low albumin level. Forty-seven patients (55%) had more than 1 of the 3 unfavorable prognostic factors.

Twenty-four patients (27%) had more than 2 involved lymph node lesions. Regarding the extranodal lesions, skin involvement was observed in 46 patients (51%), liver involvement in 15 (17%), spleen involvement in 6 (7%), and pulmonary involvement in 1 (1%). Of the 64 patients who had BM examined, the involvement was observed in 16 patients (25%; data not shown). Twenty percent of the patients ($n = 18$) had more than 3 extranodal lesions. Regarding the number of total involved lesions (extranodal lesions plus lymph node lesions), more than 4 involved lesions were observed in 24 patients (27%), 2 or 3 involved lesions in 42 patients (46%), and only 1 involved lesion in 24 patients (27%).

Prognosis

Among 90 patients with indolent ATL, 63 (70%) died, with a median duration of follow-up of 4.1 years (range, 8 days to 17.6 years). The estimated 5-, 10-, and 15-year survival rates were 47.2% (95% CI, 36.1%-57.5%), 25.4% (95% CI, 15.3%-36.8%), and 14.1% (95% CI, 6.2%-25.3%), respectively, with an MST of 4.1 years (95% CI, 2.9-6.3 years; Figure 1A). No plateaus were observed in the survival curves for OS. Of the 27 survivors, 12 were alive for more than 10 years. Of the 63 patients who died, 41 (65.1%) died of acute ATL after transformation, 5 (7.9%) died of severe chronic ATL, 11 (17.5%) died of other diseases (3 malignancies other than ATL, 2 chronic pulmonary diseases, 2 opportunistic infections, 2 autoimmune diseases, 1 cardiac failure, and 1 myocardial infarction), 2 died of transplantation-related complications, and 4 died of unknown cause. No significant difference in OS was observed between patients who died of ATL and patients who died of other causes (data not shown). Among 90 patients, 44 (49%) progressed to aggressive ATL (all were acute types), among those, 41 (93%) died. The median time to transformation was 18.8 months (range, 0.3 months to 17.6 years).

Table 2. Survival by baseline clinical factors

Factors	No. of evaluated (% of total)	No. of deaths (%)*	MST, y	Cumulative probability of survival†		
				5-y survival, % (95% CI)	10-y survival, % (95% CI)	P†
Total	90	63 (70)	4.1	47.2 (36.1-57.5)	25.4 (15.3-36.8)	
Clinical subtype						
Smoldering	25 (28)	17 (68)	2.9	39.4 (19.8-58.6)	25.3 (8.2-47.0)	.36
Chronic	65 (72)	46 (71)	5.3	50.2 (37.0-62.0)	26.3 (14.6-39.5)	
Patient-related factors (n = 90)						
Sex						
Male	52 (58)	34 (65)	4.3	48.1 (33.4-61.3)	24.9 (11.8-40.5)	.99
Female	38 (42)	29 (76)	4.1	46.4 (29.5-61.6)	26.5 (12.0-43.4)	
Age						
60 y or older	46 (52)	32 (70)	3.7	45.5 (30.4-59.4)	29.5 (14.8-45.8)	.18
Younger than 60 y	44 (48)	31 (70)	4.5	49.2 (32.9-63.6)	24.0 (11.2-39.3)	
PS						
0	22 (24)	15 (68)	8.4	75.9 (51.4-89.1)	38.9 (16.8-60.7)	.006
1	49 (54)	33 (67)	3.4	41.5 (26.9-55.5)	22.5 (9.7-38.5)	
2 or 3	19 (22)	15 (79)	1.3	27.9 (10.2-49.0)	13.9 (1.3-41.1)	
Complications at diagnosis (n = 90)						
Absent	55 (61)	37 (67)	5.7	54.1 (39.4-66.7)	25.4 (12.9-40.1)	
Present	35 (39)	26 (74)	3.4	36.6 (20.7-52.8)	28.3 (13.5-45.1)	.06
Malignancies other than ATL	7 (8)	6 (86)	0.8	28.6 (4.1-61.2)	28.6 (4.1-61.2)	
Opportunistic infection	9 (10)	7 (78)	1.2	0	0	
Chronic pulmonary disease	13 (14)	10 (77)	4.1	38.5 (14.1-62.8)	25.6 (5.2-53.4)	
Autoimmune disease	6 (7)	3 (50)	11.4	62.5 (14.2-89.3)	62.5 (14.2-89.3)	
Hematologic factors						
WBC count (n = 90)						
At least 12.0 × 10^9/L	43 (48)	32 (74)	3.4	43.0 (27.6-57.5)	22.3 (9.9-37.8)	.24
Less than 12.0 × 10^9/L	47 (52)	31 (66)	5.3	51.0 (35.1-64.8)	28.5 (13.6-45.2)	
Total lymphocyte count (n = 90)						
At least 6.5 × 10^9/L	45 (50)	35 (78)	3.7	43.3 (28.2-57.5)	17.4 (6.8-32.0)	.34
Less than 6.5 × 10^9/L	45 (50)	28 (62)	5.3	51.4 (35.2-65.4)	36.8 (20.9-52.9)	
Neutrophil counts (n = 89)						
At least 7.5 × 10^9/L	17 (19)	14 (82)	2.3	29.4 (10.7-51.1)	14.7 (1.3-42.9)	.05
Less than 7.5 × 10^9/L	72 (81)	48 (67)	5.3	51.0 (38.3-62.4)	28.4 (16.6-41.3)	
Eosinophil count (n = 89)						
At least 0.4 × 10^9/L	17 (19)	11 (65)	4.0	34.9 (13.0-58.0)	23.2 (4.9-49.4)	.47
Less than 0.4 × 10^9/L	72 (81)	51 (71)	4.5	49.2 (36.8-60.5)	27.4 (16.0-40.1)	
Laboratory factors						
LDH (n = 90)						
Greater than NI	28 (31)	23 (82)	1.5	34.8 (17.3-53.0)	14.9 (3.9-32.7)	.004
Less than or equal to NI	62 (69)	40 (65)	5.4	52.9 (39.2-64.8)	31.8 (18.5-45.9)	
BUN (n = 87)						
Greater than NI	5 (6)	5 (100)	2.0	20.0 (0.8-58.2)	0	.18
Less than or equal to NI	82 (94)	56 (68)	4.5	48.9 (37.2-59.6)	28.4 (17.3-40.6)	
Albumin (n = 88)						
Less than 40.0 g/L	34 (39)	22 (65)	3.4	39.9 (22.4-56.8)	25.6 (8.9-46.4)	.22
At least 40.0 g/L	54 (61)	40 (74)	5.3	52.2 (37.9-64.7)	26.6 (14.3-40.6)	
Potential prognostic factors (n = 87)‡						
At least 1	47 (55)	34 (72)	2.9	38.7 (24.1-53.1)	18.1 (6.5-34.3)	.05
None	40 (45)	27 (68)	5.4	56.1 (39.2-70.0)	35.2 (19.3-51.6)	
Tumor lesions (n = 90)						
No. of lymph node lesions						
2 or more	24 (27)	16 (67)	2.1	37.5 (19.0-56.0)	30.0 (12.1-50.4)	.09
0 or 1	66 (73)	47 (71)	5.3	50.9 (37.5-62.8)	23.6 (12.2-37.2)	
No. of extranodal lesions						
3 or more	18 (20)	14 (78)	1.1	29.4 (10.7-51.1)	19.6 (4.2-43.3)	.005
1 or 2	72 (80)	49 (68)	5.3	51.6 (38.9-62.9)	26.8 (15.2-39.7)	
No. of total involved lesions						
4 or more	24 (27)	16 (67)	1.3	34.8 (16.6-53.7)	26.1 (8.8-47.6)	.03
2 or 3	42 (46)	30 (71)	4.5	49.5 (32.7-64.3)	13.1 (3.5-29.1)	
1	24 (27)	17 (71)	5.4	54.5 (32.1-72.4)	44.1 (22.8-63.5)	
Chemotherapy						
Received	12 (13)	12 (100)	1.4	25.0 (6.0-50.5)	0	.01
Not received	78 (87)	51 (65)	5.3	50.8 (38.6-61.8)	31.3 (19.3-44.0)	

WBC indocates white blood cell count; MST, median survival time (years); and NI, normal index.

*Rate of death in evaluated cases.

†Cumulative probability of survival rate was estimated with the Kaplan-Meier method, and the *P* value was calculated with the generalized Wilcoxon test.

‡PPFs indicate at least 1 of the following 3 factors: low serum albumin, high LDH, or high BUN.[13,14]

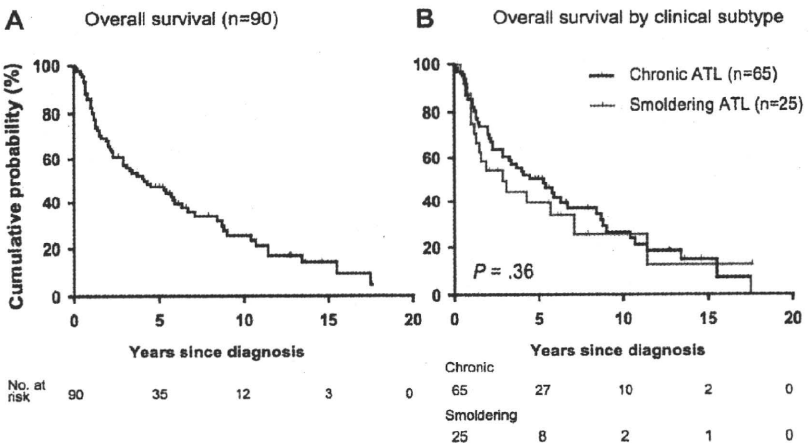

Figure 1. Survival of patients with indolent ATL. (A) For OS (n = 90), the median survival time was 4.1 years (95% CI, 2.9-6.3 years). No plateau was observed in the survival curves for OS. The estimated 5-, 10-, and 15-year survival rates were 47.2% (95% CI, 36.1%-57.5%), 25.4% (95% CI, 15.3%-36.8%), and 14.1% (95% CI, 6.2%-25.3%), respectively. (B) OS by clinical subtype (smoldering type vs chronic type). The estimated 15-year survival rate was 12.7% (95% CI, 1.1%-38.8%) with an MST of 2.9 years for smoldering type and 14.7% (95% CI, 5.7%-27.8%) with an MST of 5.3 years. There was no statistically significant difference ($P = .36$).

Among 25 patients with smoldering ATL, 17 patients (68%) died, and the estimated 15-year survival rate was 12.7% (95% CI, 1.1%-38.8%) with an MST of 2.9 years (95% CI, 1.3-7.1 years). Of the 17 patients who died, 15 died of acute ATL after transformation. Among 65 patients with chronic ATL, 46 (71%) died, and the estimated 15-year survival rate was 14.7% (95% CI, 5.7%-27.8%) with an MST of 5.3 years (95% CI, 2.9-6.7 years). Of the 46 patients who died, 29 died of acute ATL after transformation and 5 died of the disease severity. No statistically significant difference was observed in OS between subtypes ($P = .36$; Figure 1B). The overall estimated 5- and 10-year survival rates of both subtypes are shown in Table 2.

Effects of clinical factors on prognosis

Effects of clinical factors on prognosis were analyzed with the use of all the 90 patients together. Results of prognostic analyses (estimated 5- and 10-year OS rates and MST) with the use of Kaplan-Meier methods are summarized in Table 2. The survival rate was poor for patients with advanced PS ($P = .006$; Figure 2A), neutrophilia ($P = .05$; Figure 2B), and a higher LDH level ($P = .004$; Figure 2C). Patients with at least 1 of 3 PPFs for chronic ATL (a high level of LDH and BUN and a low level of albumin)[13,14] showed a poor survival rate compared with patients without ($P = .05$; Figure 2D). The difference in survival rates between patients with any complications and patients without was marginally significant ($P = .06$). Among patients with any complications, those with malignancies other than ATL or opportunistic infections at diagnosis showed a tendency of poor prognosis, although the number of patients in each category was too small (supplemental Figure 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). Although no difference was observed in survival rates between patients with involvement of more than 2 lymph node lesions and patients with less involvement ($P = .09$; Table 2), the survival rate of patients

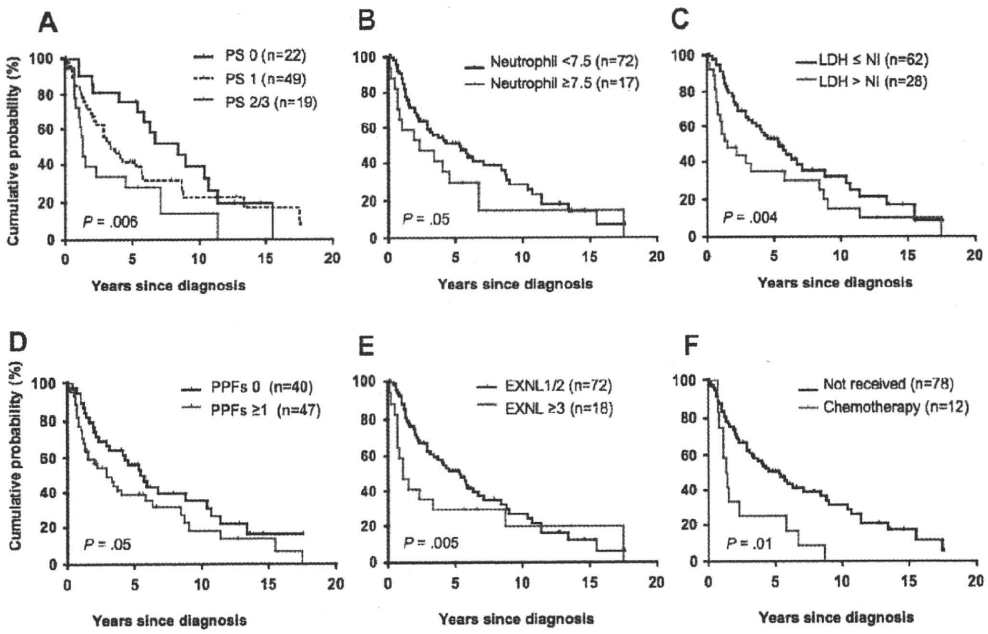

Figure 2. OS by clinical parameters. (A) OS by PS ($P = .006$). (B) OS by neutrophil count ($P = .05$). The unit is $\times 10^9$/L. (C) OS by LDH level ($P = .004$). NI indicates normal index. (D) OS by PPFs for chronic ATL that were defined based on low serum albumin, high LDH, or high BUN according to previous reports[13,14] ($P = .05$). (E) OS by the number of extranodal lesions (EXNL; $P = .005$). (F) OS by treatment states ($P = .01$).