VOLUME 28 · NUMBER 9 · MARCH 20 2010

JOURNAL OF CLINICAL ONCOLOGY

ORIGINAL REPORT

Phase I Study of KW-0761, a Defucosylated Humanized Anti-CCR4 Antibody, in Relapsed Patients With Adult T-Cell Leukemia-Lymphoma and Peripheral T-Cell Lymphoma

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Submitted July 27, 2009; accepted November 16, 2009; published online ahead of print at www.jco.org on February 22, 2010.

Presented in part at the 50th Annual Meeting of the American Society of Hematology, San Francisco, CA, December 6-9, 2008.

Authors' disclosures of potential conflicts of interest and author contributions are found at the end of this article.

Clinical Trials repository link available on JCO.org.

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0732-183X/10/2809-1591/$20.00

DOI: 10.1200/JCO.2009.25.3575

ABSTRACT

Purpose
KW-0761, a defucosylated humanized anti-CC chemokine receptor 4 (CCR4) antibody, exerts a strong antibody-dependent cellular cytotoxic effect. This phase I study assessed the safety, pharmacokinetics, recommended phase II dose and efficacy of KW-0761 in patients with relapsed CCR4-positive adult T-cell leukemia-lymphoma (ATL) or peripheral T-cell lymphoma (PTCL).

Patients and Methods
Sixteen patients received KW-0761 once a week for 4 weeks by intravenous infusion. Doses were escalated, starting at 0.01, 0.1, 0.5, and finally 1.0 mg/kg by a 3 + 3 design.

Results
Fifteen patients completed the protocol treatment. Only one patient, at the 1.0 mg/kg dose, developed grade 3 dose-limiting toxicities, skin rash, and febrile neutropenia, and grade 4 neutropenia. Other treatment-related grade 3 to 4 toxicities were lymphopenia (n = 10), neutropenia (n = 3), leukopenia (n = 2), herpes zoster (n = 1), and acute infusion reaction/cytokine release syndrome (n = 1). Neither the frequency nor severity of toxicities increased with dose escalation. The maximum tolerated dose was not reached. Therefore, the recommended phase II dose was determined to be 1.0 mg/kg. No patients had detectable levels of anti-KW-0761 antibody. The plasma maximum and trough, and the area under the curve of 0 to 7 days of KW-0761, tended to increase dose and frequency dependently. Five patients (31%; 95% CI, 11% to 59%) achieved objective responses: two complete (0.1; 1.0 mg/kg) and three partial (0.01; 2 at 1.0 mg/kg) responses.

Conclusion
KW-0761 was tolerated at all the dose levels tested, demonstrating potential efficacy against relapsed CCR4-positive ATL or PTCL. Subsequent phase II studies at the 1.0 mg/kg dose are thus warranted.

J Clin Oncol 28:1591-1598. © 2010 by American Society of Clinical Oncology

INTRODUCTION

The successful use of monoclonal antibodies (mAb) has evolved into a promising approach to treating cancer over the last decade. In the field of hematologic malignancies, development of the therapeutic mAb rituximab has changed the standard of therapy for patients with B-cell lymphomas and has markedly improved prognosis.[1-3] In contrast, the prognosis of patients with T-cell neoplasms remains very poor.[4] The 5-year overall survival (OS) for common subtype of peripheral T-cell lymphoma (PTCL), such as PTCL not otherwise specified (NOS) and angioimmunoblastic T-cell lymphoma, is 32% compared with only 14% for adult T-cell leukemia lymphoma (ATL).[4] A recent phase III trial for newly diagnosed aggressive ATL demonstrated that a dose-intensified multidrug chemotherapy with vincristine, cyclophosphamide, doxorubicin, and prednisone (VCAP), doxorubicin, ranimustine, and prednisone (AMP), and vindesine, etoposide, carboplatin, and prednisone (VECP) was more effective than biweekly cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP).[5] However, the median survival time and OS at 3 years were still unsatisfactory, at approximately 13 months and 24%, respectively.[5,6]

CC chemokine receptor 4 (CCR4) is a chemokine receptor expressed on T-helper type 2[7] and regulatory T cells (Treg).[8-10] Because numerous studies, including our own, have demonstrated CCR4 to be expressed on certain types of T-cell neoplasms,[11-17] we hypothesized that this molecule might represent a novel molecular target for immunotherapy against relapsed or refractory T-cell lymphomas.[16-21] Accordingly, we developed KW-0761, a next generation humanized anti-CCR4 mAb, with a defucosylated Fc region, which markedly enhanced antibody-dependent cellular cytotoxicity (ADCC) due to increased binding affinity to the Fcγ receptor on effector cells.[21,22]

Herein, we report the results of a phase I study designed to assess the safety, pharmacokinetics, recommended phase II dose, and efficacy of KW-0761 in patients with relapsed CCR4-positive ATL and other peripheral T-cell lymphomas (PTCL).

PATIENTS AND METHODS

Investigational Drug and Eligibility

KW-0761 is a defucosylated humanized immunoglobulin G1 (IgG1) 1 mAb generated from a mouse anti-CCR4 mAb[7] by Kyowa Hakko Kirin Co Ltd.[23,24]

Patients between 20 and 69 years of age with CCR4-positive aggressive ATL (acute type, lymphoma type, or unfavorable chronic type)[25,26] or PTCL with CCR4 expression were eligible. CCR4 expression was confirmed by immunohistochemistry or flow cytometry using an anti-CCR4 mAb (KM2160, Kyowa Hakko Kirin Co Ltd),[12,14,15] and confirmed by the review committee with a central evaluation. Patients with relapse after at least one prior course of chemotherapy were eligible. All patients were required to have an Eastern Cooperative Oncology Group performance status of 0 or 1. Eligibility criteria also included the following laboratory values: an absolute neutrophil count ≥ 1,500/uL, platelet count ≥ 75,000/uL, hemoglobin ≥ 8.0 g/dL, AST ≤ 2.5 × the upper limit of the normal range (UNL), ALT ≤ 2.5 × UNL, total bilirubin ≤ 1.5 × UNL, serum creatinine ≤ 1.5 × UNL, corrected serum calcium ≤ 11.0 mg/dL, negative for hepatitis B surface antigen and for hepatitis B virus DNA, and arterial partial oxygen pressure ≥ 65 mmHg or arterial blood oxygen saturation ≥ 90%. All subjects underwent electrocardiography to confirm the absence of abnormalities requiring treatment and that the left ventricular ejection fraction was at least 50%.

Patients were excluded if they had any severe complication, an infectious complication or active tuberculosis, a history of organ transplantation, active concurrent cancers, CNS involvement, a bulky mass requiring emergent radiotherapy, or tested positive for hepatitis C virus antibody and/or HIV antibody.

The institutional review boards of the participating institutions approved this study, and all patients gave written informed consent according to the Declaration of Helsinki.

Study Design

This was a multicenter dose-escalation study with three to six patients at each dose level to determine the maximum-tolerated dose (MTD) and estimate the recommended phase II dose. Cohorts of patients received KW-0761 at 0.01, 0.1, 0.5, and 1.0 mg/kg, weekly for 4 weeks by intravenous infusion. Premedications (antihistamine and antipyretic) were administered before each KW-0761 treatment.

If no dose-limiting toxicity (DLT) was observed in a cohort of three patients at a given dose level, the next cohort of three new patients would be treated with the next higher dose. If DLT was experienced by one or two of the three patients at any dose, three additional patients would be treated at the same dose level. If three or more patients at a given dose level exhibited DLT, this dose would be considered to exceed the MTD and the dose escalation would thus be halted. The recommended phase II dose was defined as one dose level below the MTD or the maximum dose level judged to be tolerable. An expanded cohort of three additional newly enrolled patients was also treated at the recommended phase II dose. Patients who relapsed after achieving responses to KW-0761 were allowed to be re-treated with this antibody.

Toxicity Evaluation and Definition of DLT

Patients treated at each dose level were evaluated weekly during therapy and until 4 weeks after the last infusion to assess toxicity. Toxicity was graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events version 3. Human anti-KW-0761 antibodies in the plasma of patients were detected by an enzyme-linked immunosorbent assay. The plates were coated with KW-0761 to capture any anti-KW-0761 antibodies, followed by addition of biotinylated KW-0761, and then horseradish peroxidase–labeled avidin. Detection sensitivity of this assay was 5 ng/mL as standard antibody equivalent in plasma.

DLT was defined as an adverse event or a laboratory abnormality that occurred within 28 days after the first infusion, judged to be related to KW-0761 and meeting any of the following criteria: ≥ grade 4 hematologic toxicity except lymphopenia, ≥ grade 4 symptoms judged to be consistent with an acute infusion reaction/cytokine release syndrome or with tumor lysis

Table 1. Patient Demographic and Clinical Characteristics by Cohort

Characteristic	Cohort and Dosage					Total
	1: 0.01 mg/kg	2: 0.1 mg/kg	3: 0.5 mg/kg	4: 1.0 mg/kg	Expanded: 1.0 mg/kg	
No. of patients	3	4*	3	3	3	16
Median age, years						62
Range	46-68	55-66	60-69	62-64	55-62	46-69
Sex						
Male	2	2	2	0	2	8
Female	1	2	1	3	1	8
Diagnosis						
ATL	2	4	3	2	2	13
PTCL	1 (MF)	0	0	1 (PTCL-NOS)	1 (PTCL-NOS)	3
No. of prior chemotherapy regimens						
1	2	2	2	1	2	9
2	0	0	0	2	0	2
≥ 3	1	2	1	0	1	5

Abbreviations: ATL, adult T-cell leukemia-lymphoma; PTCL, peripheral T-cell lymphoma; NOS, not otherwise specified; MF, mycosis fungoides.
*One patient enrolled at 0.1 mg/kg was withdrawn due to early progressive disease.

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syndrome, and ≥ grade 3 nonhematologic toxicities. The independent data monitoring committee evaluated the safety data at all dose levels.

Responses

Responses were evaluated within 2 weeks and again at 4 weeks after the last KW-0761 infusion. The antitumor effects were determined according to criteria described previously.[26,27-29] The overall response (OR) rate included patients with a complete response (CR), CR unconfirmed, or a partial response (PR). Progression-free survival (PFS) was defined from the day of the first KW-0761 infusion until the day of progressive disease (PD) detection or death due to any cause. The tumor response and PFS of each subject were confirmed by the efficacy assessment committee with a central evaluation based on computed tomography imaging.

Pharmacokinetics

Blood was drawn into a heparin-containing tube before and after the infusion in all patients and plasma concentrations of KW-0761 were assessed using an enzyme-linked immunosorbent assay. One blood sample was obtained before each infusion, six during the 0- to 72-hour period after the first or fourth infusion, one immediately after the second or third infusion, and four in the 7 to 28 days after the fourth infusion. The pharmacokinetic parameters of plasma KW-0761 concentrations were calculated by employing a noncompartment model using WINNonlin (Scientific Consulting, Apex, NC) software; plasma maximum (C_{max}) and trough (C_{trough}) drug concentrations after each administration of KW-0761, and the plasma half-life ($t_{1/2}$) and area under the blood concentration time curve ($AUC_{0\text{-}7days}$) after the first and the fourth infusions.

Table 2. Grade 2 or Higher Nonhematologic and Hematologic Adverse Events by Cohort

Adverse Event	Cohort 1 (n = 3)		Cohort 2 (n = 4)		Cohort 3 (n = 3)		Cohort 4 and Expanded (n = 6)	
	Grade 2	Grade 3	Grade 2	Grade 3	Grade 2	Grade 3	Grade 2	Grade 3
Nonhematologic*								
Cardiac arrhythmia and general								
Prolonged QTc	1	—	—	—	—	—	—	—
Vasovagal episode	—	—	—	—	—	—	1†	—
Hypertension	—	—	—	—	—	—	1	—
Hypotension	1†	—	—	—	—	—	—	—
Constitutional symptoms								
Fever	—	—	1†	—	—	—	2 (1†)	—
Dermatology/skin								
Pruritus	—	—	—	—	—	—	1	—
Rash	1	—	—	—	—	—	2	1
Gastrointestinal								
Constipation	1	—	—	—	—	—	—	—
Infection								
Febrile neutropenia	—	—	—	—	—	—	—	1
Herpes zoster‡	—	1	—	—	—	—	—	—
Metabolic								
Alkaline phosphatase	—	—	1†	—	—	—	—	—
ALT	—	—	1	1†	—	—	—	—
AST	—	—	—	1†	—	—	—	—
γ-GTP	—	—	—	1†	—	—	—	—
CRP increased	—	—	—	—	—	—	1†	—
Pain								
Lymph node	—	—	—	—	—	—	1	—
Pulmonary/upper respiratory								
Hypoxemia	—	—	2†	—	—	—	1	—
Syndrome								
Acute infusion reaction/cytokine release	1	—	2	1	1	—	2	—

	Cohort 1 (n = 3)			Cohort 2 (n = 4)			Cohort 3 (n = 3)			Cohort 4 (n = 6)		
	Grade 2	Grade 3	Grade 4	Grade 2	Grade 3	Grade 4	Grade 2	Grade 3	Grade 4	Grade 2	Grade 3	Grade 4
Hematologic*												
Leukopenia	1	—	—	1	—	—	2	1	—	1	1	—
Lymphopenia§	1	1	—	1	1	1	1	2	—	1	3	2
Neutropenia	1	—	—	1	1	—	—	1	—	1	—	1
Thrombocytopenia	1	—	—	—	—	—	—	—	—	—	—	—
Eosinophilia	1	—	—	—	—	—	—	—	—	—	—	—

Abbreviations: QTc, corrected QT interval; γ-GTP, γ-glutamyl transpeptidase; CRP, C-reactive protein.
*KW-0761–related adverse events.
†Adverse events observed as the acute infusion reaction/cytokine release syndrome.
‡Observed 2.5 months after the last administration.
§Includes abnormal cells and was excluded from the definition of dose-limiting toxicities.

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RESULTS

Patient Characteristics

Sixteen patients (13 ATL, two PTCL-NOS, one mycosis fungoides) were enrolled in this phase I study (Table 1). Patients characteristics both at first presentation and at study entry are listed in Appendix Table A1 (online only). Four patients were enrolled in cohort 2 because one participant (203) withdrew due to PD after receiving the first infusion. The other 15 patients completed the planned treatment. All 16 enrolled patients were evaluated for toxicity and response on an intent-to-treat basis.

Adverse Events and Nonhematologic Toxicities

All adverse events ≥ grade 2 are listed in Table 2.

The grade 3 nonhematologic toxicities were herpes zoster, skin rash, febrile neutropenia, elevations of ALT, AST, and γ-glutamyl transpeptidase (γ-GTP), and acute infusion/cytokine release syndrome (n = 1, each). All other toxicities observed were ≤ grade 2, and there were no grade 4 or grade 5 nonhematologic toxicities. Among the grade 3 toxicities, increases liver transaminases and γ-GTP were judged to be infusion-related toxicity. Neither the frequency nor the severity of toxicities increased with dose escalation. None of our patients had detectable human anti-KW-0761 antibody. Recovery from toxicities was observed in all cases.

Hematologic Toxicities

Lymphopenia occurred in 14 (88%) of the 16 patients: grade 2 or grade 3 in 11 and grade 4 in three. Grade 4 neutropenia, which developed in one patient, was associated with a febrile episode. Other hematologic toxicities were leukopenia, thrombocytopenia, and eosinophilia. These hematologic toxicities, which were ≤ grade 3, occurred at all the dose levels, but were transient. Recovery to normal or baseline levels was eventually seen in all cases.

Infusion-Related Toxicities

As presented in Table 2, seven (44%) of the 16 patients exhibited ≥ grade 2 acute infusion reaction or cytokine release syndrome. In six cases, the severity was grade 2, and in one grade 3. Overall, 14 patients (88%) had such events with a severity of at least grade 1. These adverse events occurred primarily at the first infusion, then became less frequent with subsequent treatments. The common infusion-related events were vasovagal episodes, hypotension, fever, hypoxemia, and elevations of alkaline phosphatase, C-reactive protein (CRP), liver transaminases, and γ-GTP. None of the patients required interruption of antibody infusion due to these toxicities.

Only one patient (201) who developed grade 2 infusion-related toxicities needed steroid administration for his infusion reactions. He was given one dose of 100 mg hydrocortisone with symptomatic improvement. The remaining patients did not need steroids.

Dose Escalation and DLT

In cohort 1, no DLT was observed during the DLT observation period, although one patient (102) developed grade 3 herpes zoster 2.5 months after the last infusion. This adverse event was treated with topical dressing by ointment and acyclovir and resolved in 1 week. Another patient (103) in cohort 1 showed a grade 3 increase in liver transaminase due to hepatitis B virus reactivation (grade 2) 6 months after the last infusion. At the onset, this patient was receiving the second course of KW-0761 because of PD after achieving PR with the first course, according to the protocol. This event resolved with the antiviral drug entecavir. This event was not judged to represent DLT by the independent data monitoring committee. In cohort 2, one patient (203) showed grade 3 liver function impairment. The event was not, however, considered to represent DLT, instead being judged to be an acute infusion reaction and cytokine syndrome toxicity. Patients in cohorts 3 and 4 developed neither grade 3 nonhematologic or grade 4 hematologic toxicities, nor acute infusion reaction and cytokine syndrome toxicities. Therefore, the MTD was not reached by cohort 4 and the maximum dose of 1.0 mg/kg was thus selected as the dose for the expanded cohort. In the expanded cohort, one patient (412) exhibited grade 4 neutropenia and grade 3 skin rash and febrile neutropenia (Appendix Fig A1, online only), possibly related to KW-0761 treatment. In total, one of the six patients at the 1.0 mg/kg dose level showed a DLT. Taking all data into account, the recommended phase II dose was determined to be 1.0 mg/kg.

Pharmacokinetics

KW-0761 exhibited dose-proportional pharmacokinetics. The plasma C_{max} and C_{trough} as well as the $AUC_{0\text{-}7days}$ increased dose and frequency dependently, as presented in Figure 1 and Table 3. At 1.0 mg/kg, the mean values (± standard deviation [SD]) of C_{max}, C_{trough}, and $AUC_{0\text{-}7days}$ after the first infusion were 21,758 ± 3,495 ng/mL, 7,544 ± 3,009 ng/mL, and 1,879,383 ± 464,447 ng × hours/mL, respectively, while the corresponding values after the fourth infusion were 41,374 ± 5,317 ng/mL, 19,637 ± 3,826 ng/mL, and 4,224,459 ± 533,158 ng × hours/mL. The $t_{1/2}$ was prolonged at the 0.5 and 1.0 mg/kg dose levels as compared with lower doses. The mean value ± SD of $t_{1/2}$ after the fourth infusion at 1.0 mg/kg was 438 ± 76 hours (18.3 ± 3.2 days). There were no significant correlations between any of the pharmacokinetic parameters and either the clinical response to treatment or adverse events.

Responses

Five (31%; 95% CI, 11% to 59%) of the 16 enrolled patients achieved objective responses, including two (13%) with CR and three (19%) with PR (Table 4). The two patients achieving CR had acute-type ATL and their CR status was maintained until the last follow-up (12 and 3 months) without subsequent therapy. Two other acute-type

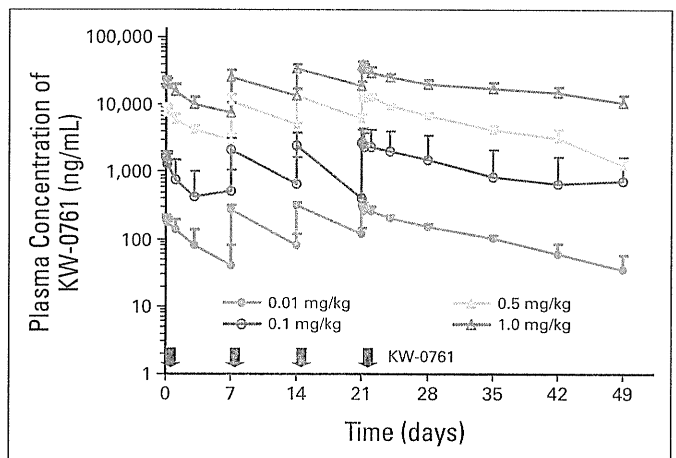

Fig 1. Mean KW-0761 plasma concentration profile by cohort: bar indicates upper limit of standard deviation.

Table 3. Mean Value of Pharmacokinetic Parameters of KW-0761 by Cohort

Dose (mg/kg) by Frequency	No.	C_{max} (ng/mL) Mean	C_{max} (ng/mL) SD	C_{trough} (ng/mL) Mean	C_{trough} (ng/mL) SD	$AUC_{0\text{-}7\ days}$ (ng × hours/mL) Mean	$AUC_{0\text{-}7\ days}$ (ng × hours/mL) SD	$t_{1/2}$ (hours) Mean	$t_{1/2}$ (hours) SD
0.01	3								
4th		323.7	56.7	151.6	12.4	34,301	4,455	244	117
0.1	3								
4th		2,806.7	1,664.5	1,515.2	1,873.4	327,212	322,031	201	196
0.5	3								
4th		15,181.2	872.0	6,824.7	872.9	1,615,135	143,225	332	122
1	6								
1st		21,758.0	3,495.4	7,544.2	3,008.8	1,879,383	464,447	133	111*
4th		41,373.7	5,316.6	19,636.7	3,825.7	4,224,459	533,158	438	76

Abbreviations: C_{max}, plasma maximum; C_{trough}, plasma trough; AUC, area under the curve; $t_{1/2}$, terminal half-life; SD, standard deviation.
*n = 2.

ATL and one PTCL-NOS patient showed PR, and one of these three patients maintained PR until the last follow-up (6 months). The median progression-free survival was 46 days although some patients remain progression free at last follow-up.

Clinical response was observed even at 0.01 mg/kg (Table 4). It is noteworthy that tumor cells disappeared rapidly from peripheral blood in most patients after KW-0761 infusion, as documented in patient 204 (Fig 2). Two other representative cases are also shown in Appendix Figures A1 and A2 (online only). These patients had ATL (102) and PTCL-NOS (401) and had previously been treated with VCAP plus AMP plus VECP and CHOP, respectively. The ATL patient (102) showed systemic skin involvement of ATL cells, and a lytic bone lesion. This patient received KW-0761 once a week for 4 weeks by intravenous infusion at 0.01 mg/kg, and 3 weeks later, his skin and bone lesions were assessed as stable disease according to the response criteria. Subsequently, both lesions gradually diminished in size, and by 1 year after treatment, the disease had completely disappeared, and this patient was categorized as showing CR. His CR status was maintained until the last follow-up (Appendix Fig A2, online only). The PTCL-NOS patient (401) had an enlarged inguinal lymph node and lymphoma cell involvement in peripheral blood and the skin. This patient received KW-0761 once a week for 4 weeks by intravenous

Table 4. Summary of Clinical Response of Each Patient

Patient No. by Cohort	Sex	Age (years)	Disease	No. of Infusions	Response: PB	Response: Skin	Response: LN*	Response: OR	PFS (days)
1									
101	M	46	MF tumor stage	4	—	PD	SD	PD	29
102	M	60	ATL acute	4	—	SD	—	SD→ CR†	617+
103	F	68	ATL acute	4	CR	—	CR	PR‡	85
2									
201	M	55	ATL acute	4	CR	PR	SD	SD	50
202	F	66	ATL acute	4	PR	—	SD	SD	36
203	M	66	ATL acute	1	—	—	SD	PD‡	8
204	F	57	ATL acute	4	CR	CR	—	CR	379+
3									
301	M	60	ATL acute	4	—	PD	—	PD	36
302	M	64	ATL acute	4	—	—	PD	PD	29
303	F	69	ATL lymphoma	4	—	—	SD	PD‡	29
4									
401	F	64	PTCL-NOS	4	CR	CR	PR	PR	198+
402	F	62	ATL acute	4	CR	CR	PR	PR	64
403	F	64	ATL lymphoma	4	—	—	SD	SD	43
Expanded									
411	M	55	ATL acute	4	—	PD	—	PD	28
412	M	62	ATL acute	4	CR	—	—	CR	107+
413	F	58	PTCL-NOS	4	—	—	SD	SD	110+

Abbreviations: PB, peripheral blood; LN, lymph node; PFS, progression-free survival; OR, overall response; M, male; MF, mycosis fungoides; PD, progressive disease; SD, stable disease; F, female; ATL, adult T-cell leukemia-lymphoma; CR, complete response; PR, partial response; PTCL-NOS, peripheral T-cell lymphoma, not otherwise specified.
*Target lesions among measurable enlarged lymph nodes and tumor nodules in extranodal organs.
†The diseases had disappeared by 1 year after treatment and 102 was categorized as showing CR.
‡Patients had nontarget lesions (nonincrease on 103, increase on 203) and new tumor legions (303).

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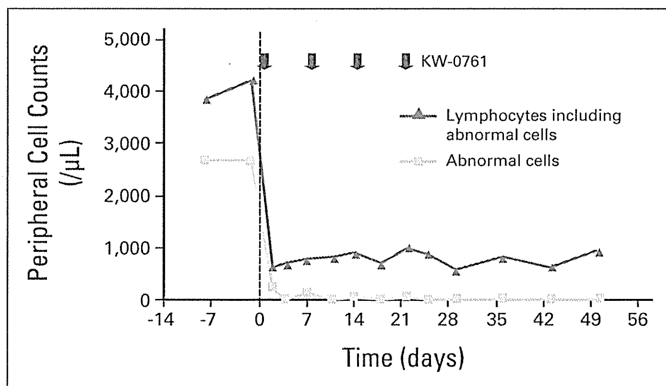

Fig 2. Response to KW-0761 in a representative patient (204). The time course of lymphocytes and adult T-cell leukemia-lymphoma (ATL) cells in peripheral blood of a patient with acute-type ATL treated with 0.1 mg/kg KW-0761 is shown.

infusion at 1.0 mg/kg. Lymphoma cells rapidly decreased after the first infusion and had completely disappeared before the second infusion. The skin lesions also resolved completely after the last infusion, while the lymph node remained somewhat enlarged, indicating PR in this case. The PR status was maintained for at least 6 months until the last follow-up (Appendix Fig A3, online only).

DISCUSSION

KW-0761 is a first-in-class therapeutic antibody targeting CCR4. In addition, this phase I study was the first clinical trial to examine the safety and efficacy of this next-generation defucosylated therapeutic antibody against hematologic malignancies. In humans, however, up to 15% of IgG does not contain fucose, and its physiological importance has yet to be fully elucidated,[30,31] although defucosylated antibodies markedly enhanced ADCC due to increased binding affinity to the Fcγ receptor on effector cells in vitro and in a mouse model.[21,22]

In this study, one patient showed DLT (grade 3 skin rash and febrile neutropenia; grade 4 neutropenia) at the 1.0 mg/kg dose in the expanded cohort. These toxicities were judged to possibly be related to KW-0761, although a causal association with trimethoprim/sulfamethoxazole could not be excluded. Further safety assessment is needed to determine whether KW-0761 itself might directly cause these toxicities. All other toxicities and symptoms including infusion reactions were mild to moderate and easily managed. The incidence and severity of infusion-related toxicity were the highest at the first infusion, diminishing with subsequent infusions, as has been observed with other antibody therapies.[32,33] The other important adverse event was viral reactivation. Hepatitis B virus reactivation and varicella-zoster virus infection were observed. These episodes might be related to a reduction in the number of CCR4-expressing cells caused by KW-0761 infusion, resulting in an alteration of the immune balance. Alterations in the proportions of each T-cell subset including Treg cells, due to this treatment, are currently being evaluated in detail in an ongoing phase II study.

Although the number of patients was small, it would be noteworthy that objective responses were achieved in 31% of patients, with 13% of CR. This is a particularly promising result since the response rate of relapsed patients with ATL to conventional chemotherapy with a single agent is reportedly extremely low.[6,34-36] Clinical responses were observed even at 0.01 mg/kg, which is approximately 1/1,000 of the rituximab dose. The clinical effect observed at the 0.01 mg/kg dose of KW-0761 would be consistent with this defucosylated mAb markedly enhancing ADCC.[22-24]

Pharmacokinetic analyses of KW-0761 revealed plasma C_{max}, C_{trough}, and $AUC_{0\text{-}7days}$ for both the first and the fourth infusion increased as the dose was increased. The $t_{1/2}$ after the fourth administration at 1.0 mg/kg was almost 18 days, which is nearly equal to the $t_{1/2}$ of circulating endogeneous human IgG,[37] indicating good stability of KW-0761 in the human body. In addition, in this study, no anti-KW-0761 antibody was detected, suggesting that the antigenicity of this novel defucosylated mAb agent was not therapeutically problematic. The C_{trough} level of 10 μg/mL was achieved after the fourth infusion of KW-0761 at 1.0 mg/kg. The in vitro study using primary ATL cells from patients demonstrated profound autologous ADCC mediated by 10 μg/mL KW-0761,[17] suggesting that an antibody concentration sufficient to exert ADCC against primary leukemia/lymphoma cells can be achieved clinically at this dose.

Increased Treg cells in the tumor microenvironment are thought to play an important role in tumor escape from host immunity in several different types of cancer.[38] Emerging recent evidence has demonstrated that the presence of Treg cells among tumor infiltrating lymphocytes is the main obstacle to successful tumor immunotherapy. Therefore, depletion of Treg cells around tumors is a potentially promising strategy for boosting tumor-associated antigen-specific immunity.[19,38-41] We previously reported that chimeric anti-CCR4 mAb actually depleted CD4-positive, CCR4-positive, and forkhead box protein P3–positive Treg cells both in vitro[17,41] and in vivo in a murine model.[21] The unexpected long-term CR in one patient (102) after stable disease at the 0.01 mg/kg dose of KW-0761 might be related to such a KW-0761-induced Treg reduction, resulting in enhancing the tumor immunity against ATL cells. However, there is no direct evidence for this and further studies are needed to assess the validity of this concept.

In summary, the results of this phase I trial show that KW-0761 infusion is tolerated at all dose levels tested in patients with relapsed CCR4-positive PTCL, including ATL and PTCL-NOS. This preliminary evidence of antitumor activity, in addition to the good tolerability and reasonable pharmacokinetics of KW-0761, warrants further investigation including a single-agent phase II study at the 1.0 mg/kg dose level and combination studies with conventional chemotherapeutic agents in patients with ATL and PTCL.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following author(s) indicated a financial or other interest that is relevant to the subject matter under consideration in this article. Certain relationships marked with a "U" are those for which no compensation was received; those relationships marked with a "C" were compensated. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

Employment or Leadership Position: Shiro Akinaga, Kyowa Hakko Kirin Co Ltd (C) **Consultant or Advisory Role:** Michinori Ogura, Kyowa Hakko Kirin Co Ltd (C) **Stock Ownership:** None **Honoraria:** None **Research Funding:** None **Expert Testimony:** None **Other Remuneration:** None

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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

Submitted December 9, 2009; accepted April 20, 2010. Prepublished online as *Blood* First Edition paper, May 6, 2010; DOI 10.1182/blood-2009-12-257410.

The online version of this article contains a data supplement.

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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.

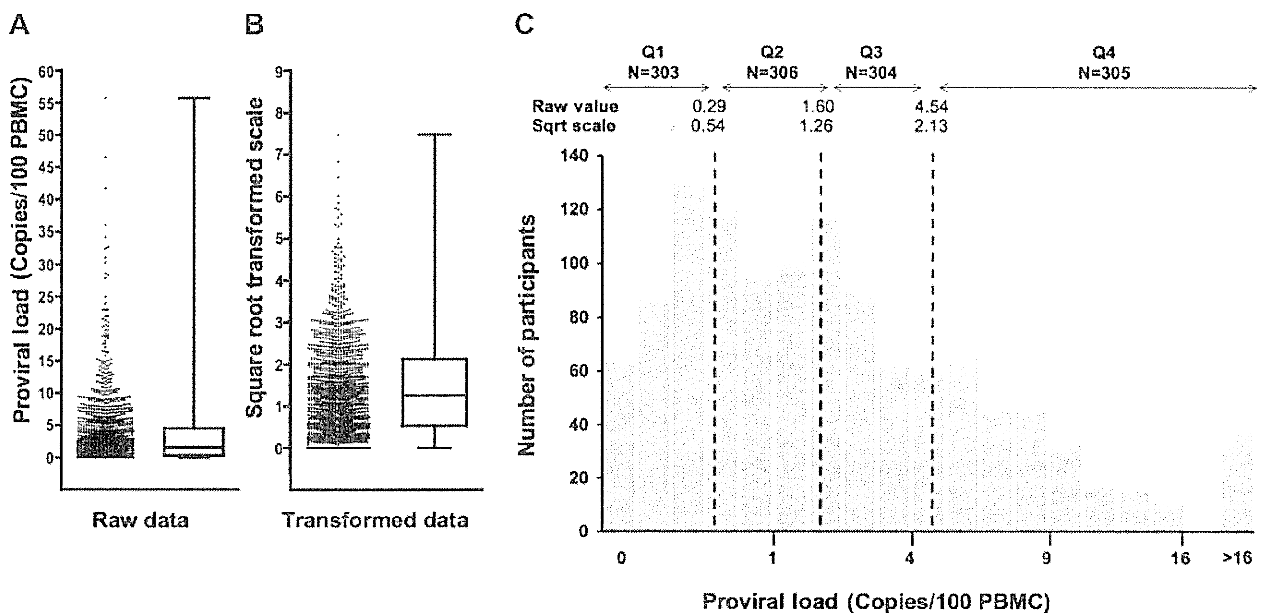

Figure 1. Distribution of baseline HTLV-1 proviral load levels among 1218 asymptomatic HTLV-1 carriers. (A) Scatter plot of raw data of proviral load (left) and the vertical box and whiskers plot (right): the box delineates 25th percentile (0.29 copies/100 periopheral blood mononuclear cells [PBMCs]), 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

			Frequency of subjects by VL level, n (% of row)			
Demographic characteristics	**No.**	**Median VL (range) (copies/100 PBMCs)**	**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, ×10³/mm³	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

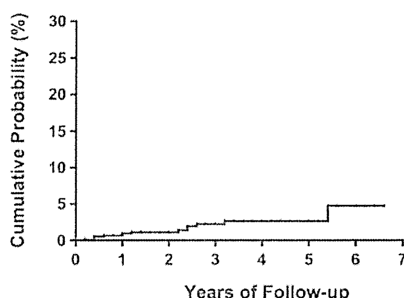

Figure 2. Probability of progression to ATL among 1218 asymptomatic HTLV-1 carriers.

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]

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

			Frequency of ATL by VL level, n (% of subjects in each quartile in Table 2)		
Demographic characteristics	No. of subjects	No. of ATLs (% of subjects)	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 ($\sim > 4$ copies/100 PBMCs) are in a high-risk group for developing ATL (this group accounted for $\sim$ 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.

Acknowledgments

The authors thank staff members in all collaborating institutions and Mr Makoto Nakashima, Ms Takako Akashi, and other technical members in the central office of the JSPFAD for efforts in sample processing and biologic assays.

This work was supported by the Ministry of Education, Culture, Sports, Science and Technology of Japan (Grant-in-Aid for Scientific Research on Priority Areas 17015047).

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.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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

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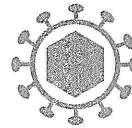

RESEARCH Open Access

High Human T Cell Leukemia Virus Type-1(HTLV-1) Provirus Load in Patients with HTLV-1 Carriers Complicated with HTLV-1-unrelated disorders

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Abstract

Background: To address the clinical and virological significance of a high HTLV-1 proviral load (VL) in practical blood samples from asymptomatic and symptomatic carriers, we simultaneously examined VL and clonal expansion status using polymerase chain reaction (PCR) quantification (infected cell % of peripheral mononuclear cells) and Southern blotting hybridization (SBH) methods.

Results: The present study disclosed extremely high VL with highly dense smears with or without oligoclonal bands in SBH. A high VL of 10% or more was observed in 16 (43.2%) of a total of 33 samples (one of 13 asymptomatic carriers, 8 of 12 symptomatic carriers, and 7 of 8 patients with lymphoma-type ATL without circulating ATL cells). In particular, an extremely high VL of 50% or more was limited to symptomatic carriers whose band findings always contained at least dense smears derived from polyclonally expanded cells infected with HTLV-1. Sequential samples revealed that the VL value was synchronized with the presence or absence of dense smears, and declined at the same time as disappearing dense smears. Dense smears transiently emerged at the active stage of the underlying disease. After disappearance of the smears, several clonal bands became visible and were persistently retained, explaining the process by which the clonality of HTLV-1-infected cells is established. The cases with only oligoclonal bands tended to maintain a stable VL of around 20% for a long time. Two of such cases developed ATL 4 and 3.5 years later, suggesting that a high VL with oligoclonal bands may be a predisposing risk to ATL.

Conclusion: The main contributor to extremely high VL seems to be transient emergence of dense smears detected by the sensitivity level of SBH, corresponding to polyclonal expansion of HTLV-1-infected cells including abundant small clones. Major clones retained after disappearance of dense smears stably persist and acquire various malignant characteristics step by step.

Background

Human T-cell leukemia virus type-1 (HTLV-1) is thought to infect mainly CD4 T-cells, and to cause T-cell malignancy adult T-cell leukemia (ATL) after a long latency, a degenerative nervous disorder of HTLV-1-associated myelopathy (HAM), and so on [1,2]. During the clinically asymptomatic period preceding the diseases, the HTLV-1-infected cell number is low, at about less than 2 - 3% per 100 blood mononuclear cells (MNC). Therefore, infected cells in asymptomatic (healthy) carriers are considered to proliferate polyclonally because the provirus integrates at a random site [3]. Recent work using real-time polymerase chain reaction (PCR) quantification for HTLV-1 provirus (proviral load: VL) and inverse PCR indicates that clonal expansion of HTLV-1-infected cells is important for the maintenance of infection [4-6]. Interestingly, the proviral integration sites in genomic DNA in asymptomatic and symptomatic carriers without ATL is either random or constant, implying the difference in clonality detected by Southern blotting hybridization (SBH) [7,8]. Thus, high VL corresponding to an increased number of polyclonal or monoclonal infected cells is one of the key

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events in HTLV-1-associated pathology. Therefore, a high VL with clonal expansion has potential as a biomarker to predict patients predisposed to ATL or HAM [9,10]. On the other hand, HTLV-1-infected individuals who are complicated by opportunistic infections, such as parasites, mycosis, viruses and some bacteria, and abnormal immunity due to aging are known to show an increased proviral load with polyclonal expansion [11-13]. This condition associated with polyclonal expansion of the infected cells was considered to be the intermediate state prior to progression to ATL [14], but the pathological and clinical correlation between clonality and level of VL is not fully understood. Recently, we have had frequent opportunities to see unusual or indeterminate ATL patients or carriers with high VL with discrete band(s) in SBH, but no circulating ATL cells, especially among the elderly.

Accordingly, to address what kind of clonal infected cells contribute to high VL, and clarify unusual ATL or carrier states, we simultaneously analyzed HTLV-1 proviral load and SBH status using the same blood samples. In contrast to the maintenance of stable VL in asymptomatic carriers with no-bands or only faint discrete bands, the VL in symptomatic carriers with complications unrelated HTLV-1 tended to have high VL with dense smears with/without discrete band(s), consisting of mainly polyclonal expansion and partial oligoclonal expansion of the infected cells.

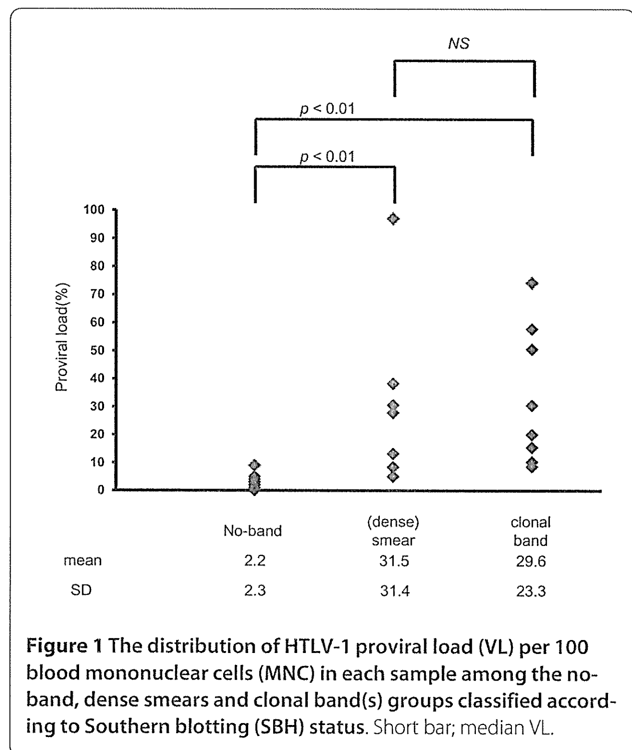

Figure 1 The distribution of HTLV-1 proviral load (VL) per 100 blood mononuclear cells (MNC) in each sample among the no-band, dense smears and clonal band(s) groups classified according to Southern blotting (SBH) status. Short bar; median VL.

Results

Sample features and SBH band status

The median age of the 29 subjects who donated peripheral blood was 66 years old (range, 49-81). No circulating ATL cells were found morphologically or immunophenotyically in any samples, including 8 samples of lymphoma-type ATL employed as a control. Subsequently, all 33 samples were divided into 3 groups; 13 asymptomatic healthy carriers (median age, 60), 12 symptomatic carriers (median age, 68) with complications unrelated to HTLV-1 such as infectious diseases (Strongyloides, hepatic disorders due to HBV and HCV, chronic pneumonitis or bronchitis) and immune-disorders (Crow-Fukase syndrome, RA, and chronic eczema, and reactive unknown adenitis) and 8 patients with lymphoma-type ATL. The distribution of SBH band patterns in each group is summarized in Table 1 and the median proviral loads of the no-band, dense smears and clonal band groups were 2.0% (range, 0.1 - 9.0), 27.9% (5.0-97.4), and 20.1% (8.3-74.3), respectively, as shown in Table 1 and Figure 1.

Characteristic band patterns in samples with high VL

Although SBH in asymptomatic carriers gave no clonal band with or without very faint Smears, SBH in some symptomatic carriers gave characteristic band patterns, as shown in Figure 2. Those were mainly a mixture type of dense smears and discrete oligoclonal bands in symptomatic carriers with high VL, such as cases 1 (VL, 97%), 3 (74%), 4 (57%), and 5 (21%). On the other hand, in samples from lymphoma-type ATL, the mixture type was detected in only case 10 and the clonal band type was detected in case 10 to 15. For all sample tested, the relationship between VL and band status is depicted in Figure 3, showing no-band or vague smears in all but one of the healthy carriers, either dense smears or a mixture of dense smears and oligoclonal bands (open circle+S:O+S) in symptomatic carriers and mainly discrete clonal band in patients with lymphoma-type ATL. In particular, an extremely high VL of 50% or more was limited to symptomatic carriers whose band findings always contained at least dense smears. Moreover, as shown in Figure 4, sequential samples disclosed that a higher VL value was synchronized with the transient emergence of dense smears, and declined at the same time as disappearing dense smears. After that, several discrete bands became visible and were persistently retained.

Clinical features in symptomatic carriers and patients with lymphoma-type ATL

Clinico-hematological features in 15 cases with a high VL of 10% or more and distinctive band patterns are summarized in Additional file 1. Of 8 symptomatic carriers, complicated disorders were mainly associated with abnormal immunity or non-bacterial pathogens. Two of 8

Table 1: The distribution of HTLV-1 SBH band status in samples without circulating ATL cells among three HTLV-1-seropsitive groups, asymptomatic (healthy), symptomatic carriers with HTLV-1-unrelated disorders and patients with lymphoma type ATL.

HTLV-1 seropositive persons	No.	SBH			
		no-band*	dense smears	clonal band(s)	total
asymptomatic (healthy)	13	11 (84.6%)	2 (15.4%)	0 (0%)	100%
symptomatic, (complication unrelated to HTLV-1)	12	4 (33.3%)	2 (16.7%)	6(4)**(50.0%)	100%
patients with lym. type ATL***	8	1 (12.5%)	3 (37.5%)	4 (1)**(50.0%)	100%
total	33	16 (48.5%)	7(21.2%)	10 (5)**(30.3%)	100%

*: no-band with or without vague smears
**: aberrant bands showing broad bands
***: patients with lym. Type ATL was defined as cases with no morphological and immunophenotypical abnormal lymphocytes
(): the number of cases with co-existence of clonal band(s) and dense smear bands

symptomatic carriers developed ATL, case 3 in 4 years later and case 5 in 3.5 years later, respectively. For lymphoma-type ATL, SBH for Lymph node (LN) suspension cells gave positive results in 5 of 6 samples tested. The band size was different in case 11 and was accordant in case 14 between PB and LN, while the other band profiles were very similar to those of symptomatic carriers; they were like a relic of the symptomatic carriers' past.

Discussion

Recent studies including our previous studies [13,15] suggest that VL in asymptomatic carriers may be approximately one copy per 25 to 1000 MNC. Even in patients with HAM whose VL are known to be high, the VL may be as high as one copy per 10 to 100 MNC. Therefore, we defined a VL of 10% or more per 100 MNC as unusually high.

In the present study, we found that the results of VL and SBH status in healthy carriers were the same as those of the past reports, while there was an extremely high VL with a characteristic band status of high dense smears with or without clonal bands in elderly symptomatic carriers. A VL of 10% or more (range, 10 to 97.4%) was detected in 16 (43.5%) of all samples, 1 (6.3%)/16 asymptomatic healthy carriers, 8 (61.5%)/13 symptomatic carriers unrelated to HTLV-1 and 7(87.5%)/8 patients with lymphoma-type ATL without circulating ATL cells. On the other hand, in SBH analysis, no visible aberrant bands were detectable in low VL samples with less than 10%. All but one of the asymptomatic carriers (mean age; 60) were of this pattern. In contrast, the high VL samples with 10% or more displayed distinctive band patterns accompanied by dense smears with or without discrete clonal band(s), indicating that an increase in polyclonally infected-cells corresponding to dense smears contributed to a high VL. As triggering factors for HTLV-1-infected cells, various microbes and abnormal immunity due to aging in symptomatic carriers were suspected. Furthermore, the observations from sequential samples also support the contribution of dense smears to the elevation of VL. This helps explain the process by which the clonality of HTLV-1-infected cells is established after the disappearance of dense smears.

It is now recognized that clonal expansion of HTLV-1-infected cells is the norm in nonmalignant disease [11]. In the present study, of 13 asymptomatic and 12 symptomatic carriers, the incidence of clonality was 24.0% (6/25 cases), of which 4 cases were accompanied by dense smears and maintained a higher VL. In other word, this suggests that polyclonal expansion, rather than oligoclonal expansion, contributes to a high VL. The contribution of clonal expansion to the elevation of VL in carriers is thought to be small because VL in HAM patients is generally reported to be around 10% on average. In fact, Furukawa et al. [8] reported a high frequency of clonality of 20% in HAM patients and 16% in carriers in families of HAM patients, while the VL was at most 10 to 20% in general. On the other hand, patients co-infected with