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

	Cohort 1 (n = 3)		Cohort 2 (n = 4)		Cohort 3 (n = 3)		Cohort 4 and Expanded (n = 6)	
Adverse Event	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

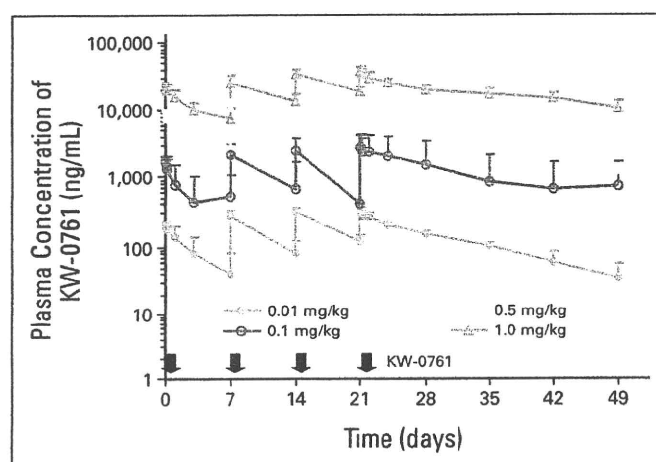

Fig 1. Mean KW-0761 plasma concentration profile by cohort: bar indicates upper limit of standard deviation.

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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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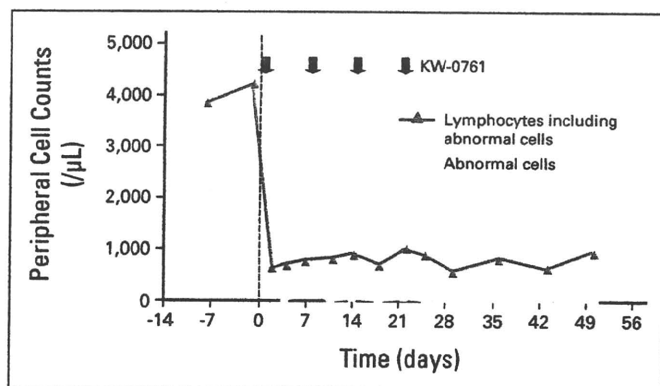

Fig 2. Response to KW-0761 in a representative patient (204). The time course of lymphocytes and adult T-cell leukemia-lymphoma (ATL) cells in peripheral blood of a patient with acute-type ATL treated with 0.1 mg/kg KW-0761 is shown.

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.

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Trends in HTLV-1 Prevalence and Incidence of Adult T-Cell Leukemia/Lymphoma in Nagasaki, Japan

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Most previous studies aimed at estimating the number of human T-cell leukemia virus type-1 (HTLV-1) carriers in endemic areas have been based on seroprevalence rates in blood donors; however, this may result in underestimation because of the healthy donor effect. People who have health problem do not donate blood. In the present study, the number of HTLV-1 carriers in Nagasaki City was estimated based on the seroprevalence rates in a hospital-based population from Nagasaki University Hospital. In accordance with previous reports, seroprevalence of HTLV-1 was higher in females, and year of birth-specific seroprevalence showed a significant annual decline in both genders (P for trend: <0.0001). The estimated number of HTLV-1 carriers in Nagasaki City was 36,983. The incidence of adult T-cell leukemia/lymphoma (ATLL) among HTLV-1 carriers was estimated using data from the Nagasaki Prefectural Cancer Registry. The estimated annual incidence of ATLL was 61 per 100,000 HTLV-1 carriers, and the crude lifetime risk of the development was 7.29% for males and 3.78% for females. There is a large pool of HTLV-1 carriers aged over 70 years, and a continuing development of cases of ATLL among the elderly is therefore expected. ***J. Med. Virol. 82:668–674, 2010.*** © 2010 Wiley-Liss, Inc.

KEY WORDS: HTLV-1; ATLL; seroprevalence; retrovirus; epidemiology

INTRODUCTION

Human T-cell leukemia virus type-1 (HTLV-1) is the causative agent of adult T-cell leukemia/lymphoma (ATLL), HTLV-1-associated myelopathy/tropical spastic paraparesis, and HTLV-1-associated uveitis [Uchiyama et al., 1977; Poiesz et al., 1980; Yoshida et al., 1984; Gessain et al., 1985; Osame et al., 1987; Mochizuki et al., 1992]. HTLV-1 carriers are clustered in the southwestern districts of Japan, including Nagasaki, as well as among native Andeans, North Iranians, Central Africans, and African descendants in the Caribbean and South America [Tajima and Takezaki, 2003]. Such regional clustering is, at least in part, explained by vertical transmission of the virus by breast-feeding in closed communities [Kinoshita et al., 1984; Hino et al., 1985]. To prevent vertical transmission, the Adult T-Cell Leukemia/Lymphoma Prevention Program, which is a prefecture-wide breast-feeding intervention study for HTLV-1 carrier mothers, was initiated in Nagasaki in the middle of 1987 [Hino et al., 1996], and HTLV-1-positive pregnant women were advised to refrain from breast-feeding. It has also been demonstrated that HTLV-1 can be transmitted by blood transfusion, and routine screening of blood donors for anti-HTLV-1 antibodies has been conducted at all blood centers in Japan since 1986 [Inaba et al., 1999].

Previous studies found that age-specific rates of HTLV-1 seropositivity among residents and blood donors in endemic areas of Japan have declined annually [Tokudome et al., 1989; Yamaguchi et al.,

Dr. Koga and Dr. Iwanaga contributed equally to this work.

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1992]. However, the results for HTLV-1 seropositivity in blood donors might have been underestimated, because blood is solely procured by voluntary donation in Japan and is limited to those aged 16–69 years old; in addition, people who have been notified that they are HTLV-1 carriers will not revisit a blood center. Furthermore, any age-specific analysis usually involves a birth cohort effect. Because the majority of Japanese HTLV-1 carriers were infected through mother-to-infant transmission, a birth cohort-specific analysis, rather than an age-specific analysis provides a more appropriate means of understanding trends in HTLV-1 seroprevalence.

Nearly 20 years have passed since the commencement of the Adult T-Cell Leukemia/Lymphoma Prevention Program and screening for anti-HTLV-1 antibodies in blood centers. The first aim of this study was to determine a more reliable estimate of the longitudinal trend in seroprevalence of HTLV-1 in Nagasaki City, using data from a hospital-based population from Nagasaki University Hospital. The second aim was to estimate the incidence of ATLL among HTLV-1 carriers by applying the hospital-based seroprevalence rate to ATLL records held in a population-based local cancer registry, namely, the Nagasaki Prefectural Cancer Registry [Soda and Ikeda, 1997].

MATERIALS AND METHODS

Patients

The results of anti-HTLV-1 antibody tests performed on 12,848 blood samples from 10,261 patients (males: 5,523; females: 4,738) who visited Nagasaki University Hospital between April 2000 and March 2007 were assessed retrospectively. Only the result of the first examination for each patient was considered, to avoid duplication of patients. Some patients were examined repeatedly at different times, but no patients produced conflicting results. The median age of the patients was 61 years (range: 0–99). Patients with HTLV-1-associated diseases were more likely to visit certain departments, such as the hematology, dermatology, neurology, and ophthalmology departments, and the data were therefore also analyzed by the department. The cardiovascular surgery department tested routinely for anti-HTLV-1 antibodies in all patients before surgery.

Anti-HTLV-1 Antibody Assay

The presence of anti-HTLV-1 antibodies in serum samples was assayed using a chemiluminescent enzyme immunoassay (CLEIA) (Lumipulse HTLV-1, Fujirebio, Inc., Tokyo, Japan) [Nishizono et al., 1991; Yoshiki et al., 1993], using an index cut-off value of 1.0. This assay is known to be less affected by non-specific reactions than the particle agglutination test [Fujiyama et al., 1995a,b; manufacturer's data sheet]. The validity of this assay was also verified in the Central Laboratory of Nagasaki University Hospital by comparison with the particle agglutination test (Serodia HTLV-1, Fujirebio, Inc.) and Western blotting (Genelabs Diagnostics Pte., Tsukuba, Japan). The sensitivity of CLEIA is adequate to detect almost all the HTLV-1 carriers, but there may be a small number of false-positive results (<0.5%) in which the HTLV-1 infection is not confirmed by the WHO criteria for Western blotting [unpublished observation; Fujiyama et al., 1995b]. However, the impact of this on the results of the current study was supposed to be marginal.

Statistical Analysis

The patients were categorized by year of birth into nine groups: before 1926, 1927–1936, 1937–1946, 1947–1956, 1957–1966, 1967–1976, 1977–1986, 1987–1996, and 1997–2007. The seroprevalence rates of HTLV-1 were calculated by dividing the number of seropositive subjects by the number of subjects tested in each group. The exact binomial 95% confidence intervals (CI) for seroprevalence were calculated as required. Comparisons of seroprevalence among categories were performed by calculating seroprevalence ratios and the 95% CI via a log-binomial regression model using PROC GENMOD. Trend tests were performed using the Cochran–Armitage test. Continuous data were compared using the Mann–Whitney test or the Kruskal–Wallis test. Post hoc analyses were performed using Dunn's multiple comparison test. Categorical data were compared using the χ^2 test or Fisher's exact test.

To estimate the year of birth-specific incidence of ATLL in HTLV-1 carriers, a two-step estimation procedure was performed. In the first step, the number of HTLV-1 carriers in the population of Nagasaki City was estimated by applying the sex- and year of birth-specific HTLV-1 seroprevalences of patients in Nagasaki University Hospital (excluding those in the departments usually involved in treating HTLV-1-related diseases, such as hematology, dermatology, neurology, and ophthalmology), to the 2006 census data for Nagasaki City (population size 454,203 in 2006). In the second step, the year of birth-specific incidence of ATLL in HTLV-1 carriers was estimated, based on the cases registered with the Nagasaki Prefectural Cancer Registry between 1990 and 2005 as the numerator, and the number of HTLV-1 carriers estimated in the first step as the denominator. The diagnosis of ATLL in the Nagasaki Prefectural Cancer Registry data was reevaluated by one of the authors (Y.Y.). The annual incidence of ATLL was estimated as the number of cases per 100,000 HTLV-1 carriers. The rates were compared using Poisson regression analysis, and relative risk (RR) and the 95% CI were calculated.

All statistical analyses were performed using the SAS version 9.1 (SAS Institute Japan, Tokyo, Japan) with a two-tailed significance level of 0.05. This study was approved by the institution's ethical committee and by the Nagasaki Prefectural Cancer Registry committee.

RESULTS

Seroprevalence of HTLV-1 in Patients

The presence of anti-HTLV-1 antibodies was confirmed in 1,392 (males: 653; females: 739) out of 10,261

TABLE I. HTLV-1 Seroprevalence and Age at Examination in Patients by Medical Department

Medical department	No. examined	Median age at examination (range)	No. positive for HTLV-1	Seroprevalence (%)
Hematology	1,052	55 (12–95)	223	21.20
Neurology	436	62 (12–99)	75	17.20
Dermatology	934	59 (0–92)	159	17.02
Ophthalmology	339	54 (5–86)	43	12.68
Cardiovascular surgery	1,417	69 (10–94)	180	12.70
Others	6,083	60 (0–97)	712	11.70
Total	10,261	61 (0–99)	1,392	13.57

patients. The overall seroprevalence was 13.57% (95% CI: 12.90–14.23%). Table I shows seroprevalence of HTLV-1 analyzed by department. As expected, the seroprevalence was higher among patients in the departments usually involved in treating HTLV-1-related diseases, such as hematology (21.2%), neurology (17.2%), and dermatology (17%). The seroprevalence among patients in the ophthalmology department was relatively low (12.68%) compared with those of the other three departments. The seroprevalence among patients attending the cardiovascular surgery department (12.7%) was comparable to that of the ophthalmology department and was slightly higher than that of the other departments not usually involved in treating HTLV-1-related diseases (11.7%); however, the difference was not significant ($P = 0.33$).

The overall seroprevalence was significantly higher in females than in males (15.6% vs. 11.82%, $P < 0.0001$) (Table II). This gender difference in seroprevalence existed in every year of examination, with a female/male ratio of around 1.3 (95% CI: 1.2–1.5). There was a trend toward a decline in annual seroprevalence during the study period from 14.53% in 2000 to 12.78% in 2007 (Fig. 1), but it was not statistically significant (*P* for trend: 0.22).

Seroprevalence of HTLV-1 in Patients by Year of Birth

Year of birth-specific seroprevalence rates showed a significant annual decline from 15.85% in those born before 1926 to 0% in those born during 1987–2007 in males (*P* for trend: <0.0001) and from 22.32% to 0% in females (*P* for trend: <0.0001) (Table III and Fig. 2). The annual decline in seroprevalence was especially rapid for those born between 1947 and 1976. The seroprevalence was consistently higher in females than males in each cohort born before 1966, but the predominance in females became weaker in those born after 1967.

Estimation of the Number of HTLV-1 Carriers in the Population

The sex- and year of birth-specific numbers of individuals corresponding to each birth cohort in the hospital data were extracted from the 2006 census data for Nagasaki City. Table IV shows the breakdown and the estimated number of HTLV-1 carriers in Nagasaki City based on the HTLV-1 seroprevalence of patients at Nagasaki University Hospital by year of birth. The estimated number of HTLV-1 carriers in Nagasaki City was 36,983 (12,755 males and 24,228 females). More than 90% of the carriers were born before 1966 and were thus more than 43 years old in 2009.

Estimation of the Incidence of Adult T-Cell Leukemia/Lymphoma Among HTLV-1 Carriers in Nagasaki City

On the basis of the Nagasaki Prefectural Cancer Registry data, a total of 360 ATLL cases (males: 188;

TABLE II. HTLV-1 Seroprevalence in Patients by Year of Examination and Gender

Year of examination	Total: No. positive/no. examined	Total: Seroprevalence (%)	Male: No. positive/no. examined	Male: Seroprevalence (%)	Female: No. positive/no. examined	Female: Seroprevalence (%)	F/M ratio[a] (95% CI)
2000	195/1,342	14.53	88/717	12.27	107/625	17.12	1.4 (1.1–1.8)
2001	171/1,288	13.28	85/711	11.95	86/577	14.90	1.2 (0.9–1.6)
2002	169/1,172	14.42	87/649	13.41	82/523	15.68	1.2 (0.9–1.5)
2003	171/1,243	13.76	91/690	13.19	80/553	14.47	1.1 (0.8–1.4)
2004	163/1,289	12.65	76/699	10.87	87/590	14.75	1.4 (1.0–1.8)
2005	176/1,261	13.96	75/664	11.30	101/597	16.92	1.5 (1.1–2.0)
2006	182/1,375	13.24	79/721	10.96	103/654	15.75	1.4 (1.1–1.9)
2007	165/1,291	12.78	72/672	10.71	93/619	15.02	1.4 (1.1–1.9)
Total	1,392/10,261	13.57	653/5,523	11.82	739/4,738	15.60	1.3 (1.2–1.5)

95% CI, 95% confidence intervals.
[a]F/M ratio indicates the female-to-male ratio for seroprevalence.

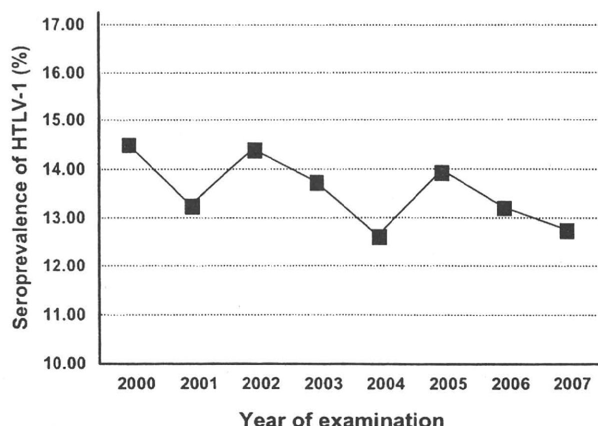

Fig. 1. Changes in annual seroprevalence of HTLV-1 in patients visiting Nagasaki University Hospital. There was an annual decline in seroprevalence, though this was not statistically significant (P for trend: 0.22).

females: 172) diagnosed between 1990 and 2005 (16 years) in Nagasaki City (Table V) were confirmed. The median age of the patients was 67 years (range: 33–99 years). No patient was born after 1967. The estimated annual incidence of ATLL was 61 per 100,000 HTLV-1 carriers, with a significantly higher rate in males (92) than females (44) (RR: 2.75, 95% CI: 2.37–3.19). There was a clear annual decline in prevalence by year of birth until 1966 (P for trend: <0.0001), with the highest rate in carriers born before 1926 (375 for males, 112 for females, and 171 in total) (Table V). The mean life span of Japanese individuals in 2008 was 79.19 years for males and 85.99 years for females, and the crude lifetime risk of developing ATLL in HTLV-1 carriers was thus 7.29% for males and 3.78% for females.

DISCUSSION

The results of this study indicate a clear annual decline in the year of birth-specific seroprevalence of HTLV-1, similar to that reported previously among blood donors in another area of Japan where the virus is endemic, namely, Kumamoto prefecture [Oguma et al., 1992; Yamaguchi et al., 1992]. The trend toward a rapid annual decline in HTLV-1 seroprevalence seen in the cohorts born from 1947 to 1976 is explained by improved sanitation, increased popularity of bottle-feeding, and the reduced duration of breast-feeding. The Adult T-Cell Leukemia/Lymphoma Prevention Program may have had a considerable impact on the decline in Nagasaki, because the seroprevalence in patients born after 1987, when this program was started, was zero. A decrease in seroprevalence of HTLV-1 has also been reported outside of Japan. In French Guiana, the prevalence in pregnant women older than 25 years was 8.3%, while that in those aged 25 years or younger was 2.8% [Tortevoye et al., 2000]. A recent article confirmed this trend [Tortevoye et al., 2005]. In a community-based follow-up study in Guinea-Bissau, the prevalence decreased from 3.5% in 1996 to 2.3% in 2006 [da Silva et al., 2009]. In the present study, a higher level of prevalence was found in females, which has been documented previously and was interpreted as showing that HTLV-1 is transmitted more easily from husband to wife than from wife to husband [Tajima et al., 1987; Kondo et al., 1989; Tokudome et al., 1989; Yamaguchi et al., 1992]. The recent reduction in this difference may be due to a decrease in the number of children and the frequent use of condoms as contraception in Japan.

Seroprevalence was higher in patients in the hematology, dermatology, and neurology departments than in other departments, because patients with HTLV-1-associated diseases visit these departments. In contrast, the prevalence in patients in the ophthalmology department was almost the same as that in patients in departments not usually involved in treating HTLV-1-related diseases. This is probably because of a low incidence of HTLV-1-associated uveitis. The prevalence in patients attending departments not usually involved in treating HTLV-1-related diseases was expected to indicate the background prevalence in Nagasaki City. The cardiovascular surgery department routinely tests

TABLE III. HTLV-1 Seroprevalence in Patients by Birth Year and Gender

	Total		Male		Female		
Year of birth	No. positive/no. examined	Seroprevalence (%)	No. positive/no. examined	Seroprevalence (%)	No. positive/no. examined	Seroprevalence (%)	F/M ratio[a] (95% CI)
Before 1926	246/1,316	18.69	117/738	15.85	129/578	22.32	1.4 (1.1–1.8)
1927–1936	458/2,568	17.83	224/1,465	15.29	234/1,103	21.21	1.4 (1.2–1.6)
1937–1946	312/1,961	15.91	139/1,079	12.88	173/882	19.61	1.5 (1.2–1.9)
1947–1956	236/1,710	13.80	115/915	12.57	121/795	15.22	1.2 (1.0–1.5)
1957–1966	91/990	9.19	36/481	7.48	55/509	10.81	1.4 (1.0–2.2)
1967–1976	37/910	4.07	18/456	3.95	19/454	4.19	1.1 (0.6–2.0)
1977–1986	12/588	2.04	4/281	1.42	8/307	2.61	1.8 (0.6–6.0)
1987–1996	0/155	0	0/78	0	0/77	0	—
1997–2007	0/63	0	0/30	0	0/33	0	—
Total	1,392/10,261	13.57	653/5,523	11.82	739/4,738	15.60	1.4 (1.2–1.5)[b]

95% CI, 95% confidence intervals.
[a]F/M ratio indicates the female-to-male ratio for seroprevalence.
[b]The F/M ratio was adjusted for the year of birth.

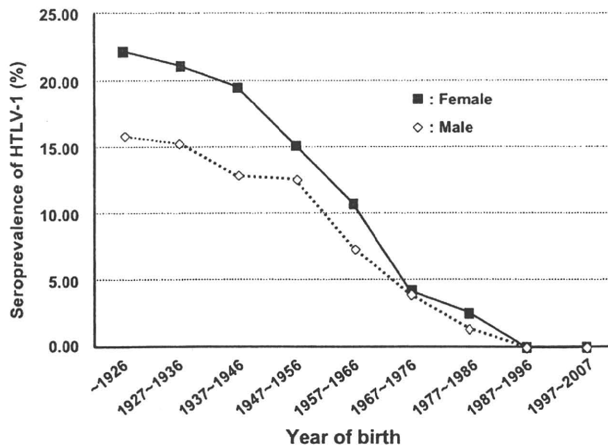

Fig. 2. Seroprevalence of HTLV-1 in patients by year of birth. The HTLV-1 seroprevalence rate showed a significant annual decline by birth year in both genders (*P* for trend: <0.0001).

for anti-HTLV-1 antibodies in all patients before surgery. The prevalence among patients in this department was 12.7%, which was slightly higher than that in other departments not usually involved in treating HTLV-1-related diseases (11.76%). This could be due to the fact that the median age of the patients in the cardiovascular surgery department (69 years) was greater than that in other departments (60 years).

Most previous studies that have examined seroprevalence of HTLV-1 among blood donors in endemic areas used age-specific but not year of birth-specific seroprevalence. The blood center of Nagasaki prefecture reported year of birth-specific seroprevalence based on data from 253,824 blood donors and found a 3.11% (7,889) seropositivity [Chiyoda et al., 2001]. The seroprevalence was 8.95% in donors born before 1936, 7.3% in those born between 1937 and 1946, 5.11% in those born between 1947 and 1956, 2.9% in those born between 1957 and 1966, 1.51% in those born between 1967 and 1976, and 1.29% in those born between 1977 and 1983. A recent report confirmed this trend [Iwanaga et al., 2009]. These prevalence rates are, however, approximately 50% lower than those estimated in the present study. The results based on blood donors do not reflect the real prevalence and are underestimates. However, there it is also possible that hospital-based prevalence results are overestimated. The seroprevalence of HTLV-1 was 0.3% in patients at a genitourinary medicine department at a hospital in southeast London, while those in blood donors in the north of England and north London were very low, in the range of 0.0014–0.005% [Turner et al., 2008]. The seroprevalence of HTLV-1/2 in pregnant women in Argentina (0.191%) was 10 times higher than that in blood donors (0.019%) [Trenchi et al., 2007].

The year of birth-specific annual incidence of ATLL among HTLV-1 carriers indicated a clear trend of higher rates in earlier year of birth, due to the tendency of this disease to develop in the elderly. The crude lifetime risk of developing ATLL in HTLV-1 carriers was 7.29% for males and 3.78% for females. A study in Saga prefecture, another area of Japan in which HTLV-1 is endemic, estimated age- and sex-specific prevalence of HTLV-1 among blood donors [Tokudome et al., 1989]. The sex- and age-specific numbers of HTLV-1 carriers were also estimated by applying the results to the entire population of Saga prefecture. The crude annual incidence rates among 100,000 HTLV-1 carriers of 40–79 years old were 115.9 for males and 66.4 for females, and the cumulative risks were 4.5% for males and 2.6% for females. Two other studies found similar results. The Ehime ATLL study group reported that the crude annual incidence rate among 100,000 male carriers aged over 30 years was 145.3 while that for females was 55.2 [Kondo et al., 1989]. In a recent cohort study of the populations of the small islands in Nagasaki prefecture, the crude annual incidence rate among 100,000 carriers of 30 years old or older was estimated at 137.7 for males and 57.4 for females, and the cumulative risk for individuals of 30–79 years of age was estimated at approximately 6.6% for males and 2.1% for females [Arisawa et al., 2000]. It is interesting that the lifetime

TABLE IV. Estimation of the Number of HTLV-1 Carriers in the Population

	Size of population[a]		Seroprevalence of HTLV-1 (%)[b]		Estimated no. HTLV-1 carriers[c]		
Year of birth	Male	Female	Male	Female	Male	Female	Total
Before 1926	9,107	19,993	12.98	20.38	1,182	4,075	5,257
1927–1936	19,916	28,268	13.41	19.18	2,671	5,422	8,093
1937–1946	24,519	29,823	10.67	18.56	2,616	5,535	8,151
1947–1956	36,358	38,642	9.16	12.30	3,330	4,753	8,083
1957–1966	26,808	29,887	6.34	9.15	1,700	2,735	4,434
1967–1976	27,368	29,991	3.69	3.90	1,010	1,170	2,180
1977–1986	23,628	26,050	1.04	2.07	246	539	785
1987–1996	23,341	22,809	0	0	0	0	0
1997–2007	19,175	18,520	0	0	0	0	0
Total	210,220	243,983	9.99	14.28	12,755	24,228	36,983

[a]Data were obtained from the 2006 census data for Nagasaki City.
[b]Data were based on the results of patients at Nagasaki University Hospital, with the exception of the departments of hematology, dermatology, neurology, and ophthalmology, during the years of examination (2000–2007).
[c]The rate was calculated by multiplying sex- and year of birth-specific numbers by sex- and year of birth-specific HTLV-1 seroprevalence.

TABLE V. Estimation of the Incidence of Adult T-Cell Leukemia/Lymphoma Among HTLV-1 Carriers in Nagasaki City

Year of birth	Estimated no. HTLV-1 carriers[a]			No. adult T-cell leukemia/lymphoma in NPCR[b]			Estimated annual incidence of adult T-cell leukemia/lymphoma among HTLV-1 carriers (per 100,000)[c]		
	Male	Female	Total	Male	Female	Total	Male	Female	Total
Before 1926	1,182	4,075	5,257	71	73	144	375	112	171
1927–1936	2,671	5,422	8,093	60	52	112	140	60	86
1937–1946	2,616	5,535	8,151	30	24	54	72	27	41
1947–1956	3,330	4,753	8,083	24	18	42	45	24	32
1957–1966	1,700	2,735	4,434	3	5	8	11	11	11
1967–1976	1,010	1,170	2,180	0	0	0	0	0	0
1977–1986	246	539	785	0	0	0	0	0	0
1987–1996	0	0	0	0	0	0	0	0	0
1997–2007	0	0	0	0	0	0	0	0	0
Total	12,755	24,228	36,983	188	172	360	92	44	61

[a]Data are cited from Table IV.
[b]We used cases registered with the Nagasaki Prefecture Cancer Registry (NPCR) during 1990–2005 (a total of 16 years).
[c]The rate was calculated using number of adult T-cell leukemia/lymphoma in the NPCR as the numerator and the estimated number of HTLV-1 carriers as the denominator.

risk of developing ATLL in HTLV-1 carriers in the present study was higher than those reported in other studies, although it is possible that the number of HTLV-1 carriers was overestimated because of the hospital-based nature of the current study. In this context, it is necessary to consider the shift in age distribution of patients: the median age of patients before 1990 in Nagasaki prefecture was younger than 65, but that in recent years was over 70 (data from Nagasaki Prefectural Cancer Registry). If we therefore evaluate the lifetime risk for a group in which the patient age is limited to 79, the results will exclude patients older than this age. The number of HTLV-1 carriers is apparently decreasing in most areas of the world in which HTLV-1 is endemic, including Nagasaki. However, it is also true that the incidence of ATLL, at least in Nagasaki City or the Nagasaki prefecture, has not changed in the past 20 years (data from Nagasaki Prefectural Cancer Registry). There is still a large pool of elderly HTLV-1 carriers over 70 years old in Japan, and the continuous development of ATLL among individuals in this pool is expected. Further studies in HTLV-1 endemic areas outside Japan are needed to confirm these trends.

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Downregulation of CDKN1A in Adult T-Cell Leukemia/Lymphoma despite Overexpression of CDKN1A in Human T-Lymphotropic Virus 1-Infected Cell Lines▽†

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Human T-lymphotropic virus 1 (HTLV-1) causes an aggressive malignancy of T lymphocytes called adult T-cell leukemia/lymphoma (ATLL), and expression of HTLV-1 Tax influences cell survival, proliferation, and genomic stability in the infected T lymphocytes. Cyclin-dependent kinase inhibitor 1A (CDKN1A/p21$^{waf1/Cip1}$) is upregulated by Tax, without perturbation of cell cycle control. During an analysis of the gene expression profiles of ATLL cells, we found very low expression of *CDKN1A* in ATLL-derived cell lines and ATLL cells from patient samples, and epigenetic abnormalities including promoter methylation are one of the mechanisms for the low *CDKN1A* expression in ATLL cells. Three HTLV-1-infected cell lines showed high levels of expression of both *CDKN1A* and *Tax*, but expression of *CDKN1A* was detected in only two of six ATLL-derived cell lines. In both the HTLV-1-infected and ATLL cell lines, we found that activated Akt phosphorylates CDKN1A at threonine 145 (T145), leading to cytoplasmic localization of CDKNIA. In HTLV-1-infected cell lines, cytoplasmic CDKN1A did not inhibit the cell cycle after UV irradiation; however, following treatment with LY294002, a PI3K inhibitor, CDKN1A was dephosphorylated and relocalized to the nucleus, resulting in suppression of the cell cycle. In the ATLL cell lines, treatment with LY294002 did not inhibit the cell cycle but induced apoptosis with the cytoplasmic localization. Therefore, the low *CDKN1A* expression in ATLL cells may be a key player in ATLL leukemogenesis, and the abnormal genomic methylation may influence the expression of not only HTLV-1 *Tax* but also *CDKN1A* during long-term development of ATLL from the HTLV-1-infected T lymphocytes.

Human T-cell lymphotropic virus type 1 (HTLV-1) is the etiologic agent of adult T-cell leukemia/lymphoma (ATLL), a fatal CD4$^+$ leukemia (20, 21, 38). At present, an estimated 10 to 20 million people worldwide are infected with HTLV-1. The HTLV-1 infection is endemic in southwestern Japan, Africa, the Caribbean Islands, and South America. The prognosis of patients with aggressive ATLL remains poor, with a median survival time of less than 1 year despite advances in both chemotherapy and supportive care (28, 29, 37). The viral determinant critical for the progression to T-cell malignancy in HTLV-1 carriers is thought to be the HTLV-1 transactivator/oncoprotein Tax (1). Tax is a 40-kDa protein that functions as a transactivator of viral gene expression and is considered a key component of the leukemogenic process that results from HTLV-1 infection (12). Tax interacts with multiple transcription factors, such as cyclic AMP-responsive element binding protein (CREB), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) family members, TATA-binding protein (TBP), and transcription factor IIA (TFIIA). Tax also stimulates the transcription of many genes, including interleukin-2 (*IL-2*), *IL-2Rα*, *PCNA*, and *PTHrP*, as well as the protooncogenes c-*fos* and c-*sis* (17). Intriguingly, Tax increases the levels of cyclin-dependent kinase 1A (*CDKN1A/p21^{waf1}*) expression in HTLV-1-infected cell lines (5, 13). The *CDKN1A* gene product was originally thought to be purely a cell cycle inhibitor; however, HTLV-1-transformed T cells grow and proliferate normally, despite abundant *CDKN1A* expression. On the other hand, the majority of ATLL cells do not produce a large amount of Tax protein *in vivo* since methylation and deletion of HTLV-1 genomic DNA are frequently found in ATLL cells (14, 30, 32). Therefore, many important differences may exist between the intracellular environments of ATLL cells and HTLV-1-infected cells because several types of transformation events must be accumulated in order for ATLL to develop.

Recently, we reported that tumor suppressor in lung cancer 1 (TSLC1/IgSF4/CADM1) is overexpressed in acute-type ATLL cells in a DNA microarray-based survey of gene expression (24). Expression of a cell adhesion molecule, TSLC1, plays an important role in the organ infiltration of ATLL cells (6). In this report,

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we examined the expression profile of ATLL cells, focusing on genes regulated by HTLV-1 infection. Within the Tax-regulated genes, we found that *CDKN1A* was specifically downregulated in ATLL cells compared with $CD4^+$ T lymphocytes, while *CDKN1A* was upregulated in the HTLV-1-infected cell lines. Compared with HTLV-1-infected cell lines, a majority of ATLL-derived cell lines and primary ATLL cells showed DNA methylation of the *CDKN1A* promoter region, with low or no expression of *CDKN1A* and *Tax*; however, no DNA methylation in *CDKN1A* was found in the three HTLV-1-infected cell lines that showed high levels of *CDKN1A* and *Tax*. Interestingly, CDKN1A was mainly localized in the cytoplasm in HTLV-1-infected cell lines and ATLL cell lines and was also phosphorylated at threonine 145 (T145) by activated Akt in both cell lines. After treatment with the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002, Akt was dephosphorylated, and CDKN1A was dephosphorylated at T145 and relocalized to the nucleus in HTLV-1-infected cell lines, which increased the percentage of G_1-arrested cells. However, dephosphorylated CDKN1A remained in the cytoplasm in ATLL cells to induce apoptosis after LY294002 treatment; the reason for this is unknown. Therefore, we found that CDKN1A showed different expression patterns and different functions in HTLV-1-infected and ATLL-derived cell lines. Since many kinds of genetic and epigenetic abnormalities are accumulated in the genomes of HTLV-1-infected cells during the course of development to ATLL cells, different expression patterns and functions of CDKN1A in both cell lines might reflect the developmental changes from HTLV-1-infected T cells to ATLL cells.

MATERIALS AND METHODS

Patient samples. All samples were collected at the time of admission to the hospital before the patients started chemotherapy. The diagnosis of ATLL was based on clinical features, hematologic characteristics, and the presence of anti-HTLV-1 antibodies in patient sera. The integration of monoclonal HTLV-1 provirus into the DNA of leukemic cells was confirmed by Southern blot hybridization. Peripheral blood mononuclear cells (PBMCs) from patients with ATLL were isolated using Histopaque (Sigma, Saint Louis, MO) by density gradient centrifugation. Each patient had more than 90% leukemic cells in the blood at the time of analysis. The study was approved by the Institutional Review Board (IRB) of the Faculty of Medicine, University of Miyazaki. Informed consent was obtained from all blood and tissue donors according to the Helsinki Declaration.

Cell lines. Human T-cell acute lymphoblastic leukemia (T-ALL) cell lines (Jurkat, MOLT4, and MKB-1), HTLV-1-infected T-cell lines (MT-2, MT-4 and HUT102), and ATLL-derived cell lines (ED, Su9T01, and S1T) were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. The interleukin-2 (IL-2)-dependent ATLL-derived cell lines KOB, SO4, and KK1 were maintained in complete RPMI 1640 medium supplemented with 100 Japan reference units (JRU)/ml of recombinant human IL-2 (Takeda Chemical Industries, Osaka, Japan).

Oligonucleotide microarray and data analyses. Five micrograms of total RNA was hybridized to the HU95A microarray (Affymetrix, Santa Clara, CA). The standard protocol used for sample preparation and microarray processing is available from Affymetrix and has been described previously (24). Expression data were analyzed using Microarray Suite, version 5.0 (Affymetrix).

RT-PCR. Total RNA was extracted using Trizol (Life Technologies, Carlsbad, CA) and converted to cDNA using an RNA-PCR kit (Takara Shuzo, Kyoto, Japan) according to the manufacturer's protocol. The following primers (forward and reverse, respectively) were used: for *CDKN1A*, 5′-ATGTCAGAACCGGC TGGGGAT-3′ and 5′-TAGGGCTTCCTCTTGGAGAAG-3′ (annealing temperature of 55°C); for HTLV-1 *Tax*, 5′-CCGGCGCTGCTCTCATCCCGAT-3′ and 5′-GGCCGAACATAGTCCCCCAGAG-3′ (60°C); for p53, 5′-CCAAGCT TATGGAGGAGCCGCAGTCAGATCCTAGCG-3′ and 5′-TCACAACCTCC GTCATGTGC-3′ (55°C); for β-actin, 5′-TCCTTCTGCATCCTGTCGGCT-3′ and 5′-CCAGAGATGGCCACGGCTGCT-3′ (55°C). The reverse transcription-PCR (RT-PCR) products were run on agarose gels and visualized by ethidium bromide staining.

Western blot analysis. Cells were lysed with sodium dodecyl sulfate (SDS) sample buffer (0.05 M Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0.01% bromophenol blue, 0.6% 2-mercaptoethanol), subjected to SDS polyacrylamide gel electrophoresis, and then transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% nonfat dried milk in Tris-HCl-buffered saline plus 0.1% Tween 20 and incubated with anti-p21 (C-19; Santa Cruz Biotechnology, Santa Cruz, CA), anti-p27 (C-19; Santa Cruz), anti-Tax (MI73: a kind gift of M. Matsuoka, Institute for Virus Research, Kyoto University), anti-p53 (Ab-6; EMD Biosciences, San Diego, CA), anti-Akt (9272; Cell signaling), anti-phospho-Akt (Ser473; 9271, Cell signaling Technology, Beverly, MA), anti-phospho-CDKN1A (Thr145; sc-20220-R, Santa Cruz), or anti-β-actin (AC-15; Sigma) antibodies overnight at 4°C and then incubated with horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG for 2 h at room temperature. Immunoblots were then reacted with Lumi-Light Plus reagent (Roche Diagnostics, Indianapolis, IN). Can Get Signal (Toyobo, Osaka, Japan) was used to enhance antigenic signals.

Assay for HTLV-1 proviral copy numbers. The primers and the probe for the *pX* gene region of HTLV-1 provirus were as follows: the forward primer (pX2-S, 5′-CGGATACCCAGTCTACGTGTT-3′: positions 7359 to 7379), the reverse primer (pX2-AS, 5′-CAGTAGGGCGTGACGATGTA-3′; positions 7458 to 7439), and the 6-carboxyfluorescein (FAM)-labeled probe (5′-FAM-CTGTGT ACAAGGCGACTGGTGCC-TAMRA-3′, where TAMRA is 6-carboxytetramethylrhodamine) (31). The nucleotide position numbers of HTLV-1 provirus are according to the published reports (25). RNase P control reagent (Applied Biosystems, Foster City, CA) was used for the primers and the probe for the human RNase P DNA gene as an internal control.

Cell growth analysis. Cells were seeded in six-well plates at 1×10^6 cells/ml and treated with UV radiation (20 J/m^2) and/or LY294002 (20 μM). Rates of proliferation were determined by counting the number of cells every 24 h using the trypan blue exclusion method.

Real-time quantitative RT-PCR. Real-time RT-PCR was performed on an ABI Prism 7700 SDS using a predeveloped TaqMan RT-PCR kit (Applied Biosystems). The expression levels of *CDKN1A* mRNA and the internal reference β-actin were measured following the manufacturer's instructions. The primers and probes were purchased from Applied Biosystems as TaqMan Gene Expression Assays.

MSP with bisulfite treatment. One microgram of genomic DNA was treated with sodium bisulfite as described previously (22). Methylation-specific PCR (MSP) primers for *CDKN1A* were designed according to published literature (40). The following primer sets were used: 5′-GTTGTTTGTTGGAATTCGGT TAG-3′ and 5′-CGACGAATCCGCGCC-3′ for the methylated *CDKN1A* sequence, located at −182 to +48 upstream of the transcription start site (230 bp), and 5′-GTTTGTTGGAATTTGGTTAG-3′ and 5′-CAACAAATCCACACCC AA-3′ for the unmethylated *CDKN1A* sequence (230 bp). Each MSP reaction mixture incorporated approximately 10 ng of bisulfite-treated DNA, 20 pmol of each primer, 100 pmol of the deoxynucleoside triphosphates (dNTPs), 2 μl of 10× PCR buffer, 0.2 μl of ExTaq Polymerase (Takara, Tokyo, Japan), and 0.2 μl of Anti-ExTaq monoclonal antibody (Takara), in a final reaction volume of 20 μl. Cycle conditions were the following: 1 cycle of 5 min at 95°C; 35 cycles of 30 s at 95°C, 30 s at 50°C, and 30 s at 72°C; and a final cycle of 5 min at 72°C. PCR products were run on 2% agarose gels and visualized by ethidium bromide staining.

Immunofluorescence staining. Cells were fixed with 1% formaldehyde, permeabilized with 0.5% Triton X-100, stained with antibodies against CDKN1A or CDKN1B followed by Alexa Fluor 488 green-conjugated second antibody (Life-Technologies), and then observed under a confocal microscope (Olympus IX81 fluorescence microscope; Olympus, Tokyo, Japan). Cells were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI) to visualize nuclei.

Cell cycle and apoptosis analyses. Serum-starved cells (2×10^6) were treated with UV (20 J/m^2) and/or LY294002 (20 μM) for the time periods indicated in the figure legends and stained with propidium iodide (PI). The cells were then subjected to fluorescence-activated cell sorter (FACS) analysis. The percentage of cells in each phase of the cell cycle was computed by using the ModFit LT software program (Becton Dickinson, San Jose, CA). For apoptosis analysis, cells were harvested and stained using an annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (MBL Nagoya, Japan) according to the manufacturer's protocol.

Statistical analysis. For the cell growth shown in Fig. 3A and 4C, statistical analyses of the associations between treated and control cells were performed by a Student's t test with significance set at P values of <0.05 or <0.001, and all data

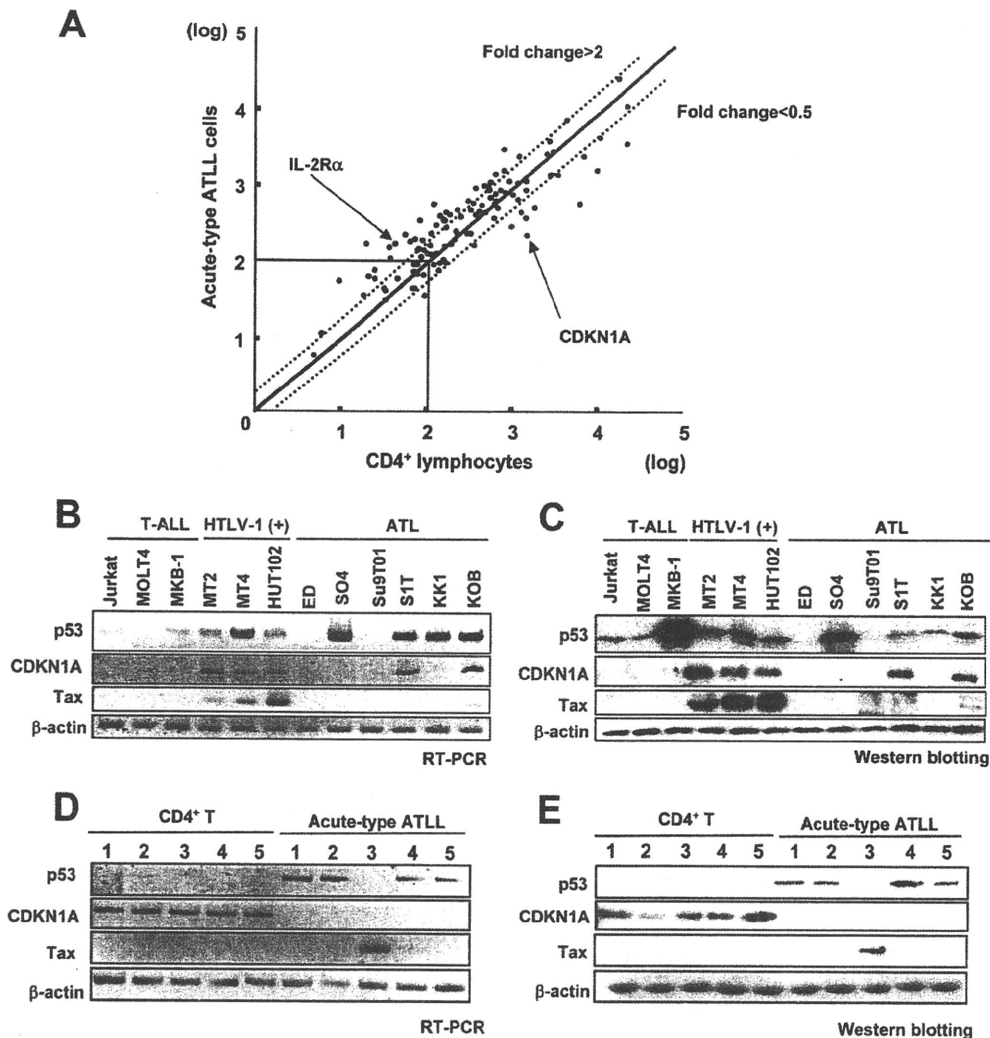

FIG. 1. Downregulation of *CDKN1A* in ATLL cells. (A) cDNA microarray analysis of gene expression changes in acute-type ATLL. The scatter plot of DNA microarray signal intensities of ATLL cells from acute-type ATLL patients plotted against the signal intensities of $CD4^+$ T lymphocytes of healthy volunteers is shown. Each dot shows a series of genes which were regulated by HTLV-1 infection (19). The flanking diagonal lines indicate a 2-fold change in expression. Data points (marked with arrows) corresponding to differentially expressed genes *IL-2Rα* and *CDKN1A* are also shown. Names of the genes that were regulated by HTLV-1 infection are listed in Table S1 in the supplemental material. (B and C) Expression of mRNA (B) and protein (C) for *p53*, *CDKN1A*, and *Tax* in three HTLV-1-uninfected T-ALL cell lines, three HTLV-1-infected cell lines [HTLV-1 (+)], and six ATLL-derived cell lines was examined by semiquantitative RT-PCR and Western blotting, respectively. The expression of β-actin is shown at the bottom as a control. (D and E) Expression of mRNA (D) and protein (E) for *p53*, *CDKN1A*, and *Tax* in five patients with acute-type ATLL and in five healthy controls ($CD4^+$ T).

are expressed as the means ± standard deviations of the values as analyzed by the *t* test.

RESULTS

Low expression of CDKN1A in ATLL cells. In a previous study, we reported that 192 genes were specifically upregulated in acute-type adult T-cell leukemia/lymphoma (ATLL) cells compared with $CD4^+$ T lymphocytes using gene expression profiles of a DNA microarray system (24). On the other hand, many genes were induced by HTLV-1 Tax, including *IL-2Rα*, *CDKN1A*, and others (4, 9, 18). Previously, the Tax-induced genes were reported by comparing the expression profiles of HTLV-1-infected cell lines with those of normal activated peripheral blood lymphocytes (PBLs) using Affymetrix Hu6800 arrays (19). In the present study we determined the expression patterns of a series of Tax-induced and Tax-suppressed genes in comparison with the expression profiles of acute-type ATLL cells and $CD4^+$ T lymphocytes using Affymetrix HU95 arrays. We detected 108 genes that were upregulated at least 2-fold (77 genes) or suppressed more than 0.5-fold (31 genes) in the HTLV-1-infected cells (see Table S1 in the supplemental material). Of those 108 genes, 22 (including *IL-2Rα*) were expressed at least 2-fold higher, and 28 genes were suppressed by at least 0.5-fold in ATLL cells compared with $CD4^+$ lymphocytes (Fig. 1A). Interestingly, 18 out of 28 genes upregulated by HTLV-1 infection were downregulated, with under 0.5-fold

expression, in ATLL cells (see Table S1-d in the supplemental material). Cyclin-dependent kinase inhibitor $p21^{waf1}$ (*CDKN1A*), an important gene induced by HTLV-1 infection, was one of the 18 oppositely regulated genes.

To confirm the downregulation of CDKN1A expression in ATLL cells, we initially determined expression of *p53*, *CDKN1A*, and *Tax* in three T-ALL cell lines (Jurkat, MOLT4, and MKB-1), the three HTLV-1-infected cell lines (MT-2, MT-4, and HUT102), and the six ATLL-derived cell lines (ED, SO4, Su9T01, S1T, KK1, and KOB) by semiquantitative RT-PCR and Western blotting (Fig. 1B and C). Three HTLV-1-infected cell lines highly expressed *CDKN1A* and *Tax* while only two of six ATLL cell lines expressed *CDKN1A* with low levels of Tax (S1T and KOB), but every ATLL cell line had minimal expression of *Tax*. Therefore, the CDKN1A expression in S1T and KOB is probably derived from the Tax expression. For reference, proviral DNA copy numbers in each cell line were calculated by the *pX* gene region of HTLV-1 DNA per RNase P DNA copy as an internal control (Fig. 2B). Three HTLV-1-infected cell lines and four out of six ATLL cell lines expressed various levels of *p53* mRNA and protein. Among them, MKB-1 showed a high level of p53 protein expression with a low level of *p53* mRNA expression, suggesting that the p53 protein degradation system might be inhibited in MKB-1. We next examined the expression of *p53*, *CDKN1A*, and *Tax* in $CD4^+$ T lymphocytes and leukemia cells from the patients with acute-type ATLL (Fig. 1D and E). In $CD4^+$ single-positive (SP) T lymphocytes, mRNA and protein of *CDKN1A* were clearly expressed, but *p53* and *Tax* were not expressed in any of the five samples. In ATLL cells, mRNA and protein of *CDKN1A* were not detected in any of the five samples. However, four out of five ATLL samples showed expression of *p53* mRNA and protein but not *Tax*. Tax was detected in one sample, but the same sample lacked p53. Compared with the *CDKN1A* expression levels in $CD4^+$ SP T lymphocytes and HTLV-1-infected cell lines (Fig. 2B), *CDKN1A* was highly expressed in HTLV-1-infected cell lines, but there was minimal expression of *CDKN1A* in ATLL-derived cell lines and primary ATLL cells and no expression of *CDKN1A* in primary $CD4^+$ lymphocytes from acute-type ATLL compared to $CD4^+$ lymphocytes from healthy volunteers. We next determined the mechanism for the low *CDKN1A* expression in ATLL cells because downregulation of *CDKN1A* expression in leukemia is considered one of the major events required for leukemogenesis.

Downregulation of *CDKN1A* due to methylation of its promoter region. Genomic mutations and allelic loss of the *CDKN1A* gene are rarely detected in many kinds of cancer cells, but epigenetic alteration of the *CDKN1A* locus is reported in select cases (3, 7, 11, 27). We initially determined the genetic alterations at the *CDKN1A* locus on chromosome 6p21.2 by spectral karyotyping of acute-type ATLL cells from 61 patients (10) and by a comparative genomic hybridization method using a high-density DNA microarray of 10 patient samples and eight ATLL cell lines. We found that no translocations or genetic deletions at the *CDKN1A* locus were found in any of the ATLL samples (data not shown). Since hypermethylation at the promoter region of *CDKN1A* was reported in acute lymphocytic leukemia in children (23), we further investigated epigenetic modifications in the promoter region of *CDKN1A* in leukemia cells from acute-type ATLL patients.

Leukemic cells from eight patients with acute-type ATLL and PBLs from two healthy volunteers were cultured with various concentrations of the DNA-demethylating agent 5-aza-2-deoxycytidine (5-aza-dC) or the histone deacetylase inhibitor trichostatin A (TSA) for 2 to 4 days. After the treatments, we found that levels of *CDKN1A* mRNA were increased in five of eight ATLL samples by 5-aza-dC but not by TSA although expression levels of *CDKN1A* did not change in PBLs from two healthy volunteers (Fig. 2A and Table 1). Along with the patient samples, various kinds of T-cell leukemia cell lines (three T-ALL, three HTLV-1-infected, and six ATLL-derived) were treated with 5-aza-dC and/or TSA, and the expression of *CDKN1A* was determined by quantitative RT-PCR (Fig. 2B). *CDKN1A* was highly expressed in all three HTLV-1-infected cell lines and in two of six ATLL cell lines (S1T and KOB), but it was expressed at lower levels in all three T-ALL and four of six ATLL cell lines (ED, SO4, Su9T01, and KK1). After the 5-aza-dC treatment, upregulation of *CDKN1A* expression was observed in two of three T-ALL cell lines (Jurkat and MOLT4) and three of six ATLL cell lines (ED, Su9T01, and KK1). After the TSA treatment, upregulation of *CDKN1A* expression was observed in one T-ALL (MKB-1) and one ATLL (KK1) cell line, suggesting that downregulation of *CDKN1A* in T-ALL and ATLL cell lines was frequently due to DNA methylation of the *CDKN1A* promoter region.

We next examined the methylation status of the promoter region of *CDKN1A* in various types of T-cell leukemia cell lines and primary ATLL cells by methylation-specific PCR (MSP). Since the basal expression of *CDKN1A* is reportedly due to six tandem Sp1 binding sites in the *CDKN1A* promoter region adjacent to exon 1 (Materials and Methods), a 230-bp fragment of the *CDKN1A* promoter region containing Sp1 binding sites in CpG islands was treated with bisulfite and amplified by a pair of methylation- or nonmethylation-specific primers. As shown in Fig. 2C, the promoter region in all three T-ALL cell lines (Jurkat, MOLT4, and MKB-1) was methylated, but it was unmethylated in all three HTLV-1-infected cell lines (MT-2, MT-4, and HUT102). In ATLL cell lines, the promoter region was methylated in two cell lines (ED and Su9T01), partially methylated in three cell lines with lower expression of *CDKN1A* (SO4, S1T, and KK1), and not methylated in one cell line with high expression of *CDKN1A* (KOB) (Fig. 2B and C). On the other hand, all five DNA samples from lymphocytes in peripheral blood from healthy volunteers were not methylated and showed high expression levels of *CDKN1A*; three samples from ATLL leukemia cells were partially methylated, and two samples were methylated, with minimal expression of *CDKN1A* (Fig. 1D and 2D). After 23 DNA samples from primary acute-type ATLL cells were examined for promoter methylation by MSP analysis, we found that the promoter region of *CDKN1A* was methylated in 22 of 23 samples, with partial methylation of 17 samples (Table 2). Therefore, the promoter region of *CDKN1A* was methylated in most of the ATLL cells with lower *CDKN1A* expression levels.

CDKN1A does not contribute to G_1 arrest in response to UV irradiation in HTLV-1-infected and ATLL-derived cell lines. To examine the functional differences between *CDKN1A* expression in HTLV-1-infected and ATLL cell lines, we investigated the p53-mediated DNA repair responses to UV irradiation (Fig. 3A), with the MOLT4 T-ALL cell line as a control.

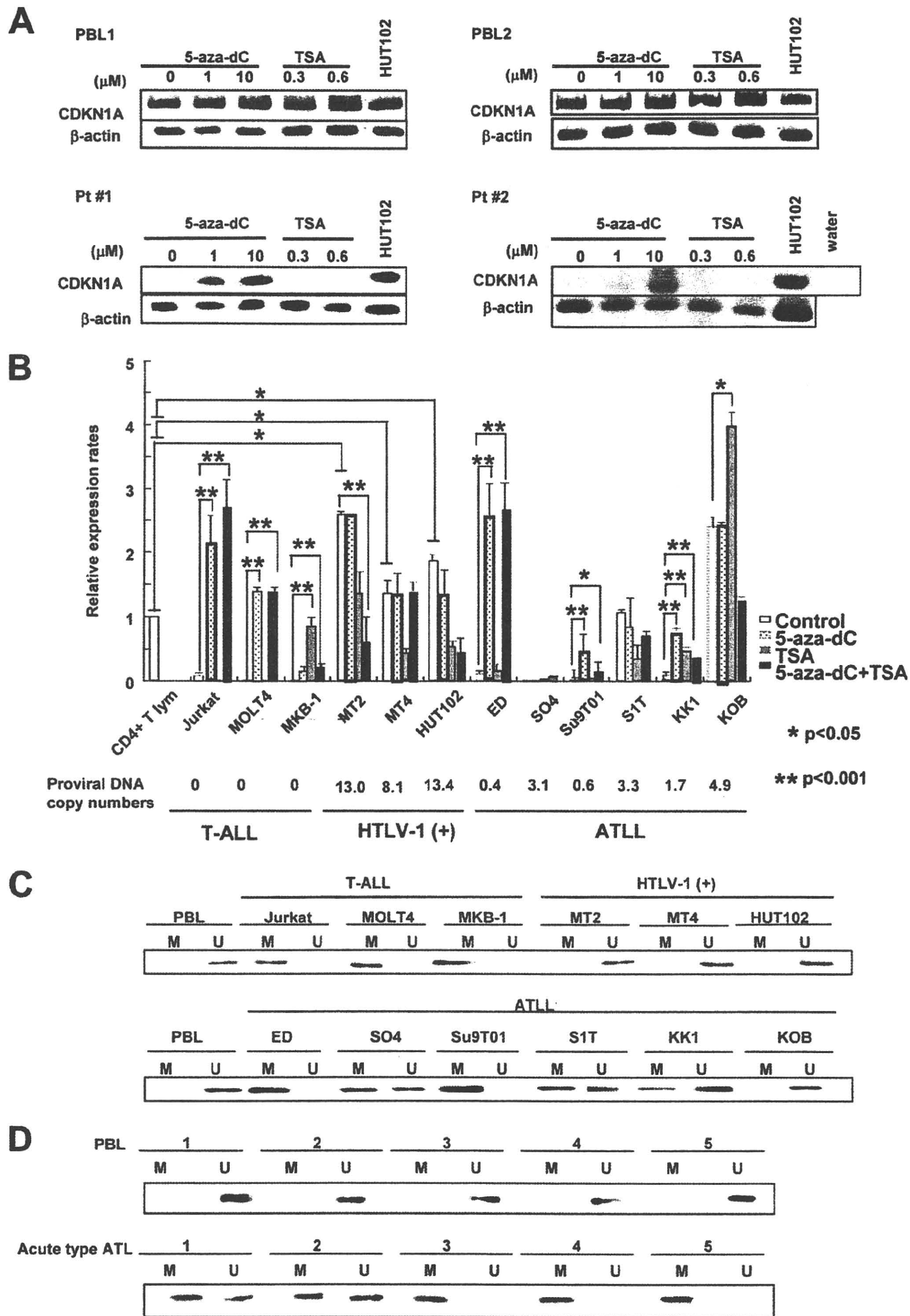

FIG. 2. Promoter methylation of *CDKN1A* is involved in suppression of *CDKN1A* expression in ATLL cells. (A) Semiquantitative RT-PCR of *CDKN1A* expression was performed with mRNA isolated from PBLs from two healthy volunteers and leukemia cells from two acute-type ATLL patients (Pt 1 and 2) after treatment with 10 μM 5-aza-dC for 72 h or with 1.2 μM TSA for 24 h. The HUT102 cell line was used as a control. (B) Quantitative RT-PCR of *CDKN1A* expression was performed with mRNA isolated from various T-leukemia cell lines treated with 10 μM 5-aza-dC for 72 h, with 1.2 μM TSA for 24 h, or with 1.2 μM TSA for 24 h followed by 10 μM 5-aza-dC for 48 h. *CDKN1A* mRNA expression levels were normalized to β-actin mRNA expression and are expressed relative to the mRNA level in the normal CD4$^+$ T lymphocytes. Proviral DNA copy numbers for HTLV-1 were measured in each cell line. Statistical analysis was done by a Student's *t* test. (C and D) MSP analysis of the *CDKN1A* gene promoter. DNAs from various T-leukemia cell lines (C) and primary ATLL cells (D) were treated with bisulfite and subjected to MSP. A product in the U gel indicates that the *CDKN1A* gene is unmethylated; a product in the M gel indicates that the gene is methylated. CD4$^+$ T lymphocytes of healthy volunteers were used as a control.

TABLE 1. Induction of the *CDKN1A* gene transcription in acute-type ATLL cells or PBLs after treatment with 5-aza-dC or TSA[a]

Treatment	*CDKN1A* transcription (no. of samples)			
	Unchanged		Upregulated	
	ATLL cells	PBLs	ATLL cells	PBLs
Control	8	2	0	0
5-Aza-dC	3	2	5	0
TSA	8	2	0	0

[a] Leukemic cells from eight patients with acute-type ATLL and PBLs from two healthy volunteers were cultured with or without 5-aza-dC or TSA for 2 to 4 days. After the treatment, expression of CDKN1A was determined by RT-PCR (Fig. 2A).

TABLE 2. Methylation status of the promoter region of *CDKN1A* as determined by MSP

Methylation status of *CDKN1A* promoter region	No. of samples by cell line[a]			No. of primary samples by type[b]	
	T-ALL	HTLV-1-infected	ATLL	PBL	Acute-type ATLL
Methylated	3	0	2	0	6
Partially methylated	0	0	3	0	17
Unmethylated	0	3	1	5	1
Total (% of methylation rates)	3 (100)	3 (0)	6 (82)	5 (0)	24 (96)

[a] Cell lines used were three T-ALL cell lines (Jurkat, MOLT4, and MKB-1), three HTLV-1-infected T-cell lines (MT-2, MT-4, and HUT102), and six ATLL-derived cell lines (ED, SO4, Su9T01, S1T, KK1, and KOB).

[b] For primary samples, PBLs were obtained from five healthy volunteers, and ATLL cells were from 24 patients with acute-type ATLL.

Three HTLV-1-infected cell lines with high expression levels of *CDKN1A* (MT-2, MT-4, and HUT102) did not change their rates of growth after high-dose UV irradiation (20 J/m^2); however, MOLT4 and ATLL-derived cell lines either without (ED and Su9T01) or with low (S1T and KOB) *CDKN1A* expression significantly decreased their rates of growth after UV irradiation. To further investigate the differences between the responses of HTLV-1-infected and ATLL-derived cell lines to UV irradiation, protein expression levels of p53, CDKN1A, and CDKN1B (p27/KIP1) were examined before and after UV irradiation since both CDKN1A and CDKN1B induce cell cycle arrest following DNA damage. After UV irradiation, the expression level of CDKN1A in KOB cells increased 24 h after UV irradiation and was mostly sustained until 72 h (data not shown). In MOLT4 cells, the levels of p53, CDKN1A, and CDKN1B markedly increased up to 72 h postirradiation (data not shown). On the other hand, there was no significant change in any of these proteins at any of the time points after the UV irradiation in the MT-2 cells (data not shown). In two of the HTLV-1-infected cell lines (MT-2 and HUT102), the expression levels of p53, CDKN1A, and CDKN1B were similar before and 72 h after UV irradiation (Fig. 3B). On the other hand, ATLL-derived ED and Su9T01 cells did not express either p53, CDKN1A, or CDKN1B while in the S1T and KOB cell lines the levels of p53, CDKN1A, and CDKN1B were slightly increased after UV irradiation. The HTLV-1-infected cell lines did not respond to UV irradiation with the p53-mediated DNA repair pathway, suggesting that the overexpression of CDKN1A in HTLV-1-infected cells did not contribute to the cell cycle arrest. Therefore, we next determined the subcellular localization of CDKN1A and CDKN1B in these leukemia cell lines, with MOLT4 cells used as a control, by immunofluorescence staining (Fig. 3C). In MOLT4 cells, both CDKN1A and CDKN1B were weakly expressed and localized in the nucleus and cytoplasm. After UV irradiation, both CDKN1A and CDKN1B were significantly induced and mostly localized in the nucleus of MOLT4 cells. Interestingly, both CDKN1A and CDKN1B proteins mainly localized in the cytoplasm in both HTLV-1-infected and ATLL-derived cell lines before and after UV irradiation. Therefore, cytoplasmic CDKN1A and CDKN1B may contribute to cellular responses other than cell cycle arrest in response to UV irradiation in HTLV-1-infected and ATLL-derived cell lines.

Cytoplasmic CDKN1A induced by phosphorylated Akt in HTLV-1-infected and ATLL-derived cells. Since recent reports show that cytoplasmic CDKN1A is phosphorylated at threonine 145 (T145) of CDKN1A by phosphorylated Akt (p-Akt) (39), we next examined the phosphorylation status of CDKN1A and the activation of the PI3K/Akt signaling pathway in HTLV-1-infected and ATLL cell lines. As shown in Fig. 4A, a high degree of p-Akt was detected in three HTLV-1-infected cell lines (MT-2, MT-4, and HUT102) and four ATLL cell lines (SO4, S1T, KK1, and KOB), compared with low expression of p-Akt in three peripheral lymphocytes from healthy volunteers. The expression levels of CDKN1A and CDKN1B were higher in three HTLV-1 cell lines than in lymphocytes and two ATLL cell lines (S1T and KOB) (data not shown). CDKN1A was highly phosphorylated at T145 in three HTLV-1-infected cell lines and two ATLL cell lines (S1T and KOB), and these cell lines also exhibited activated PI3K/Akt signaling, as evidenced with p-Akt. These data suggest that the phosphorylation of CDKN1A was probably derived from activation of PI3K/Akt signaling.

To investigate the relationship between activation of PI3K/Akt signaling and CDKN1A phosphorylation, the expression level of p-Akt was determined in these cell lines after treatment with the PI3K inhibitor LY294002. After the treatment with LY294002, the expression levels of p-Akt and p-CDKN1A were significantly decreased in two HTLV-1-infected cell lines (MT-2 and HUT102) and two ATLL cell lines (S1T and KOB) in parallel (Fig. 4B). The LY294002 treatment did not inhibit the growth rate of the control MOLT4 T-ALL cell line, with low Akt phosphorylation (data not shown), but significantly inhibited the growth rates of HTLV-1-infected cell lines and ATLL cell lines (Fig. 4C). The treatment with LY294002 also induced cytoplasmic CDKN1A and CDKN1B to relocalize to the nucleus in both HTLV-1-infected cell lines, but CDKN1A and CDKN1B did not relocalize to the nucleus in ATLL cell lines (Fig. 4D).

To determine the difference of cellular responsiveness after LY294002 treatment between HTLV-1-infected and ATLL cell lines, the cell cycle progression of MT-2 HTLV-1-infected cells and KOB ATLL cells was examined before and after UV irradiation and/or LY294002 treatment. In MT-2 HTLV-1-infected cells, approximately 60% of the cells were arrested in the G_1 phase after LY294002 with or without UV irradiation (Fig. 4E). However, ~13% of the KOB ATLL cells were in apoptotic cell death after LY294002 and/or UV irradiation

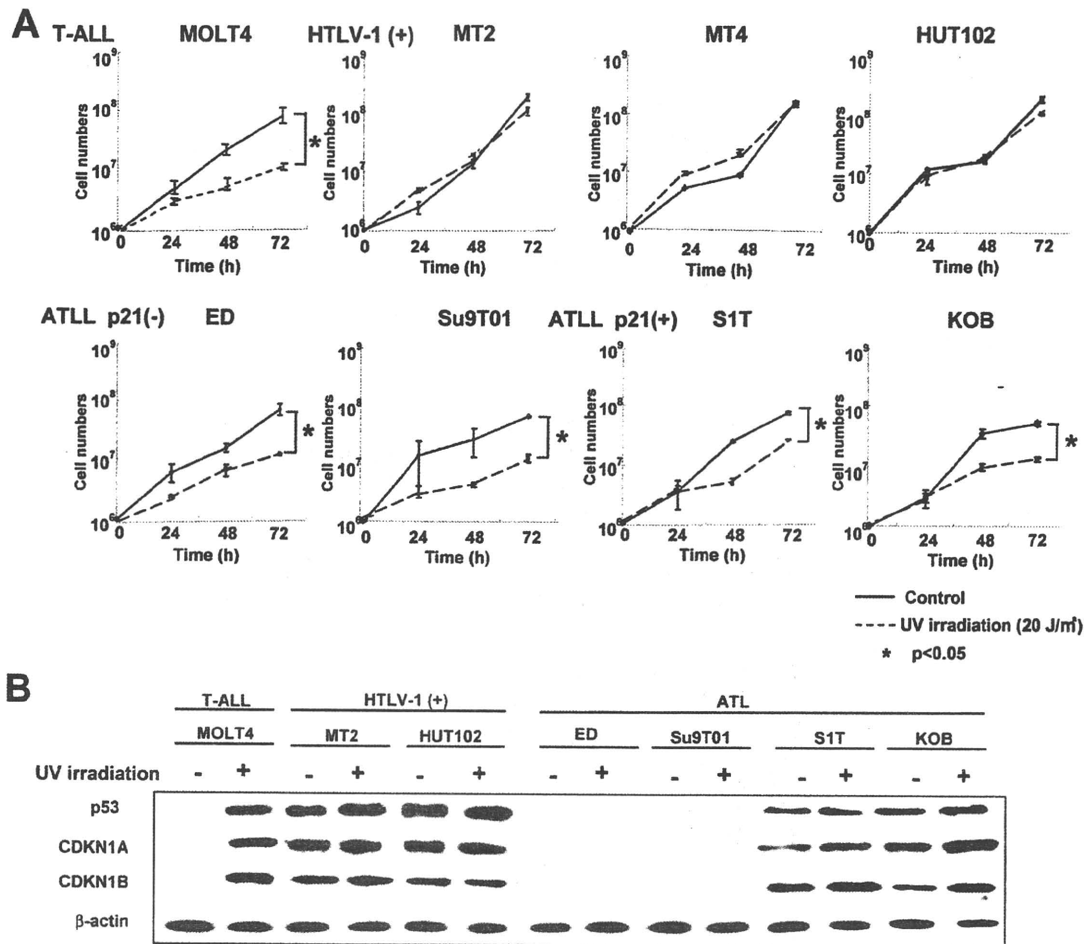

FIG. 3. Loss of functional CDKN1A response to UV irradiation in HTLV-1-infected and ATLL cell lines. (A) Cell growth curves of an HTLV-1-negative T-ALL cell line (MOLT4), three HTLV-1-infected cell lines (MT-2, MT-4, and HUT102), and four ATLL-derived cell lines (ED, Su9T01, S1T, and KOB) before and after UV irradiation. Viable cells were counted using the trypan blue exclusion method at the indicated times after 20 J/m^2 UV irradiation. Samples at 0 h were nonirradiated controls. A Student's t test was used for statistical analysis. (B) Western blot analyses of p53, CDKN1A, and CDKN1B following UV irradiation. Each cell line, either untreated (−) or UV irradiated (+; 20 J/m^2),was cultured for 72 h to examine protein expression. (C) Subcellular localization of CDKN1A and CDKN1B with or without (control) UV irradiation. Localization of CDKN1A and CDKN1B was detected by confocal immunofluorescence analysis. DAPI stain was used to visualize the nuclei. Magnification, ×400; bar, 25 μm.

(Fig. 4E and F). In the HTLV-1-infected cell lines, the relocalization of dephosphorylated CDKN1A to the nucleus was correlated with G_1 cell cycle arrest after PI3K/Akt inactivation. On the other hand, T145-dephosphorylated CDKN1A remained in the cytoplasm but could not prevent apoptotic cell death after treatment with LY294002 and/or UV irradiation in ATLL cells. Therefore, along with the phosphorylation of CDKN1A at T145, the lower expression of *CDKN1A* in ATLL cells may contribute to the dysregulation of the p53-dependent cell cycle arrest.

DISCUSSION

In this study, we showed that *CDKN1A* was frequently downregulated in ATLL cells and that the mechanism of the transcriptional repression primarily involved promoter methylation, in spite of the upregulation of *CDKN1A* in HTLV-1-infected cell lines by Tax. Because severe methylation of the *CDKN1A* promoter region has been reported in acute lymphoblastic leukemia and was associated with an unfavorable clinical outcome (23), the downregulation of *CDKN1A* in ATLL cells by promoter methylation is probably one of the important events for ATLL leukemogenesis. Low expression of *CDKN1A* was detected in primary acute-type ATLL cells and a few ATLL cell lines with higher promoter methylation, but *CDKN1A* was expressed in some ATLL cell lines with partial promoter methylation. Since CDKN1A in HTLV-1-infected and ATLL cell lines was mostly localized to the cytoplasm when CDKN1A was phosphorylated at T145 by p-Akt, CDKN1A did not contribute to p53-dependent G_1 arrest after UV irradiation in either cell line. Therefore, downregulation of *CDKN1A* transcription in ATLL cells may contribute to ATLL leukemogenesis by permitting progression of the cell cycle.

Earlier results have indicated that the loss of CDKN1B ($p27^{KIP1}$) plays a critical role in T-cell transformation following HTLV-1 infection (2). However, the expression levels of