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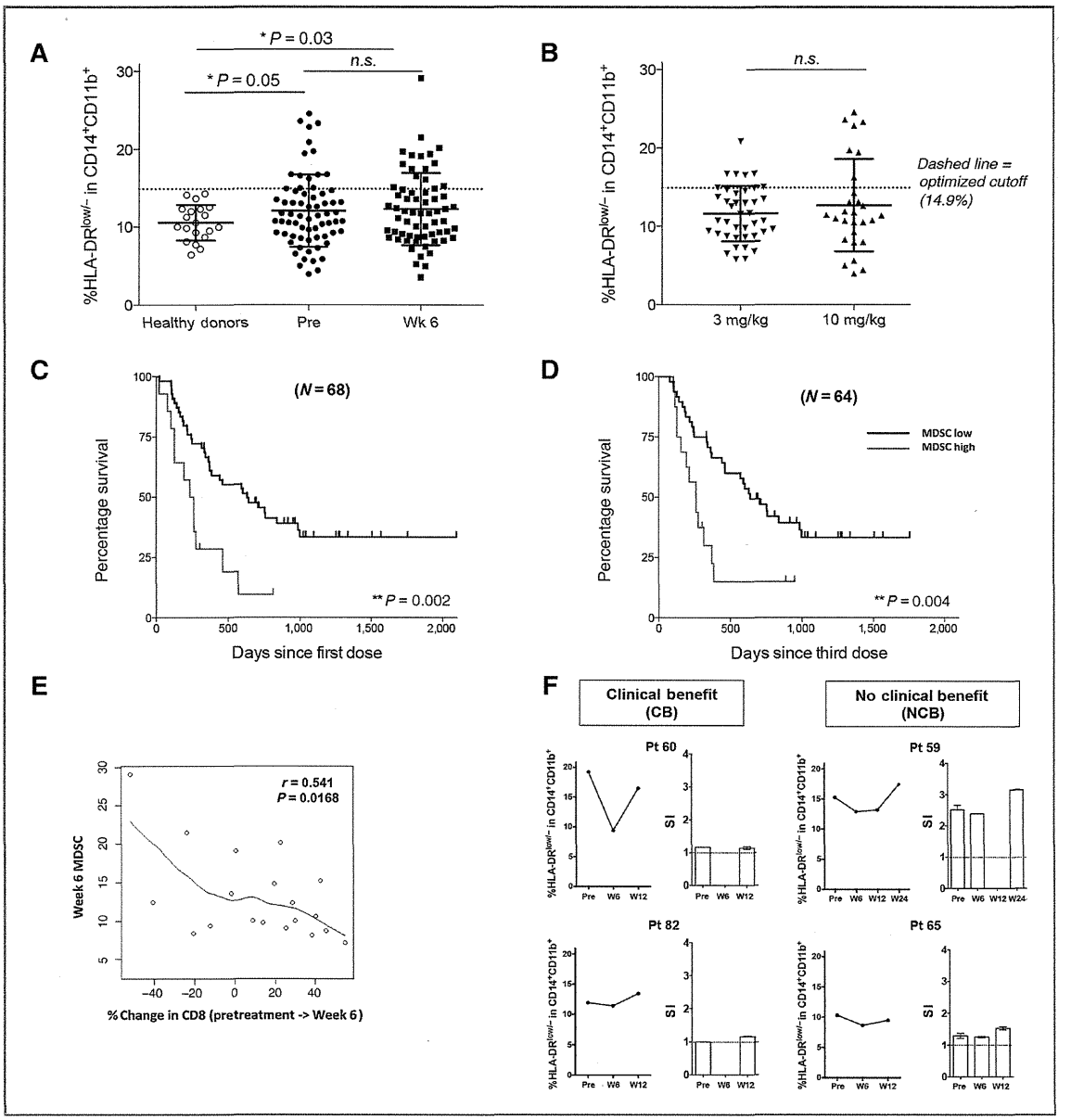

Figure 2. Functionally suppressive m-MDSCs are increased in patients with metastatic melanoma who are less likely to achieve prolonged OS following ipilimumab. A, PBMCs from patients with advanced melanoma and from healthy donors analyzed for %m-MDSC based on CV_{HLA-DR}. The frequency of m-MDSC in healthy donors ($n = 20$) and patients with melanoma analyzed at pretreatment baseline and week 6 (healthy donors vs. pretreatment, $P = 0.05$; healthy donors vs. week 6, $P = 0.03$). B, pretreatment values for subsets of patients treated with ipilimumab 10 mg/kg ($n = 28$) or 3 mg/kg ($n = 40$). C, OS based on m-MDSC quantity at pretreatment baseline. D, OS from 6 weeks after start of ipilimumab treatment. E, correlation between percentage change in CD8 T cells and week 6 m-MDSC frequency ($r = -0.541$; $P = 0.02$). Percentage change in CD8 T cells = [(wk 6 absolute CD8 − baseline absolute CD8)/(baseline absolute CD8)]. F, average SI graphed for 2 patients with melanoma with clinical benefit and 2 patients with melanoma with nonclinical benefit assessed at week 24. SI = (% proliferated $CD3^+$ T cells in CD14-depleted PBMCs)/(% proliferated $CD3^+$ T cells in CD14-PBMCs with $CD14^-$ cells added back).

the percentage change in total ALC [(week 6 − pretreatment)/(pretreatment)] and pretreatment or week 6 m-MDSC frequency. Data on $CD4^+$ and $CD8^+$ T-cell subsets were available for 19 of the 40 patients treated with ipilimumab at 3 mg/kg. We observed a statistically significant inverse correlation only between percentage change in absolute $CD8^+$ T-cell number

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Table 2. Univariate analysis of relationship between m-MDSC and OS at pretreatment baseline and week 6 after ipilimumab

	Ipilimumab treated					
	Pretreatment			Week 6		
	n	HR (95% CI)	*P*	*n*	HR (95% CI)	*P*
MDSC < 14.9%	68	0.35 (0.18–0.70)	0.002	64	0.38 (0.19–0.75)	0.004
ALC ≥ 1,000 cells/μL	68	0.73 (0.41–1.33)	0.303	64	0.22 (0.11–0.45)	<0.001
LDH < 250	68	0.33 (0.18–0.59)	<0.001	65	0.37 (0.20–0.68)	0.001
Monocytes < 300 cells/μL	68	0.70 (0.25–1.95)	0.495	64	1.77 (0.69–4.51)	0.233

and m-MDSC frequency at week 6 ($r = -0.54$; $P = 0.0168$; Fig. 2E), and no correlation was observed between that and percentage change in $CD4^+$ T-cell numbers on therapy.

We next assessed for suppressive function by measuring T-cell proliferation in PBMCs in the presence or absence of $CD14^+$ cells. We inferred that suppressive function was present if enhanced proliferation was observed among PBMCs stimulated with anti-CD3 and IL2 in the absence of CD14-expressing cells (Supplementary Fig. S2). Proliferation of $CD3^+$ T cells was increased to a greater extent in the absence of CD14-expressing cells only in PBMC samples taken from patients who did not achieve clinical benefit as measured at week 24 imaging (Fig. 2F). These data suggest that higher frequency of m-MDSCs in patients with inferior outcomes is correlated with diminished T-cell proliferation *in vitro*.

Discussion

We developed an objective methodology to evaluate m-MDSC frequency in the peripheral blood of patients with metastatic melanoma receiving immunotherapy with ipilimumab at our center. In our single institution cohort, we found that patients with metastatic melanoma have a greater frequency of m-MDSC than a group of healthy donors. An m-MDSC quantity before treatment and at week 6 that was outside the healthy donor range that we defined was significantly associated with inferior OS, independent of LDH (at baseline and week 6) and ALC (at week 6) in a multivariate model. Our observations suggest that m-MDSC frequency is a novel prognostic indicator of OS in patients with metastatic melanoma treated with ipilimumab.

The CV-based cutoff presented here represents an objective methodology for determining m-MDSC composition independent of fluorescence variability in FACS analysis. The cutoff level derived here was consistent with a level greater than the 99th percentile of a preliminary cohort of healthy donor m-MDSCs, suggesting that our method enables distinction of normal versus abnormal $CV_{HLA\text{-}DR}$ and m-MDSC evaluation in a prospective fashion. Thus, we suggest that using healthy donors as a calibration can lead to an easily implementable, automated, and objective tool for monitoring the frequency of m-MDSCs within patients' blood samples, and distinguish between "normal" and "high" ranges. However, it is important to note the preliminary nature of our healthy donor range and that further study of the effects of age, gender, body mass index, and nonmalignant comorbid conditions on $CV_{HLA\text{-}DR}$ are necessary to propose a "cutoff" value capable of prospectively segregating patients with melanoma more or less likely to benefit from ipilimumab.

As the CV is defined as the ratio of the SD to the mean, we obtained a metric independent of nonbiologically meaningful fluctuations in sample handling, FACS protocol, and fluorescence intensity. Although using "non-normalized" metrics for $HLA\text{-}DR^{low/-}$ populations (GMFI and SD; Supplementary Fig. S3) replicates the reported observations, the survival-based cutoffs determined here by GMFI or SD do not represent universal standards, and would be expected to be unstable differentiating factors with subsequent validation. Using CV effectively captures either the decreasing GMFI and/or increasing SD of the HLA-DR fluorescence intensity characteristic of cellular populations with higher numbers of m-MDSCs and eliminates replicate variability. By establishing a protocol in

Table 3. Multivariate analysis of relationship between m-MDSC and OS at pretreatment baseline and week 6 after ipilimumab treatment

	Ipilimumab					
	Pretreatment			Week 6		
	n	HR (95% CI)	*P*	*n*	HR (95% CI)	*P*
MDSC ≤ 14.9%	68	0.47 (0.23–0.94)	0.033	63	0.38 (0.18–0.81)	0.012
ALC ≥ 1,000 cells/μL	—	—	—	63	0.21 (0.10–0.46)	<0.001
LDH < 250	68	0.38 (0.21–0.69)	0.002	63	0.29 (0.15–0.56)	<0.001

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which healthy donor CV provides the necessary threshold for a "normal" CV range, we can achieve a robust identification of patients with high m-MDSC composition.

ALC rise on therapy has been associated with improved OS following ipilimumab therapy. In a small cohort of patients at our center treated with ipilimumab at 10 mg/kg, we found that $CD4^+$ lymphocytes, $CD4^+CD25^+$ regulatory T cells, and $CD8^+$ lymphocytes increased with therapy. However, increases in the absolute number of $CD8^+$ lymphocytes were significantly greater among patients who achieved clinical benefit from ipilimumab when compared with patients who did not benefit (34). In the current analysis, we observed inverse correlations between percentage change in $CD8^+$ T cells with m-MDSC frequency *in vivo*. These findings are consistent with an *in vitro* suppression assay in which higher frequencies of m-MDSCs were associated with greater suppressive activity. We propose that in patients with melanoma receiving ipilimumab, the pharmacodynamic effects on lymphocyte subset increase and the quantity of m-MDSCs are interrelated. Evaluations of m-MDSC quantity and changes in T-cell subsets are worthy of further study as pharmacodynamic markers of therapeutic efficacy and are perhaps sufficient to guide risk-adapted clinical trials. Furthermore, taken together, the observations reported here suggest the hypothesis that m-MDSC suppression of lymphocytes may be limiting the therapeutic benefit of ipilimumab. A larger cohort of patients will need to be studied to confirm our findings and to assess whether escalating ipilimumab dose or combination therapies, including m-MDSC–directed therapies, can modulate $CD8^+$ and m-MDSC interactions.

In our study, we developed an objective method to evaluate pretreatment $Lin^-CD14^+HLA\text{-}DR^{low/-}$ m-MDSC frequency building on the phenotype reported in the literature by other research groups (5, 6, 21). Similar to results from Gros and colleagues (19) and Meyer and colleagues (21), we found a greater frequency of these cells in the peripheral blood of patients with melanoma in comparison with healthy donors. These cells coexpress CD11b (Supplementary Fig. S1) and in most cases also coexpress CD33 (data not shown). Similar to findings by other authors, we also found that disease course paralleled m-MDSC frequency, that is, patients without radiographic benefit following ipilimumab tended to have increasing frequencies of m-MDSCs over time (data not shown). Nevertheless, the prognostic significance of m-MDSC frequency in our analysis was independent of LDH, a known prognostic marker associated with disease burden in patients with melanoma (35).

Clinically significant m-MDSC accumulation characterized with diverse myeloid phenotypic markers has been observed in a number of malignancies in humans (4–10). Young and colleagues measured $CD34^+$ natural suppressor cells and found that excess of $CD34^+$ cells at the tumor site was associated with relapse of head and neck cancer (4). Solito and colleagues (36) and Gabitass and colleagues (37) have reported that the quantity of m-MDSCs with an immature myeloid phenotype (Lin^-, $HLA\text{-}DR^-$, $CD11b^+$, and $CD33^+$) is a prognostic marker in breast and colorectal cancer or gastric, esophageal, and pancreatic cancer, respectively. In renal cell carinoma, Zea and colleagues have described $CD33^+$, $CD15^+$ granulocytic MDSCs (10), whereas in melanoma, both Filipazzi and colleagues and Poschke and colleagues have found that m-MDSC function is within the monocytic $CD14^+$, $HLA\text{-}DR^{low/-}$ cell population (5,6). Our report adds to this emerging literature with the first description of statistically significant associations between m-MDSC accumulation, survival outcomes, and specific lymphocyte parameters following an immunomodulatory antibody therapy.

In summary, we have developed a method that enables accurate measurement of $Lin\text{-}CD14^+HLA\text{-}DR^{low/-}$ m-MDSCs independent of technical variables related to sample processing time and flow cytometry. Using this method, we demonstrate for the first time that higher pretreatment quantities of $Lin\text{-}CD14^+HLA\text{-}DR^{low/-}$ m-MDSCs are associated with inferior OS in patients with metastatic melanoma treated with ipilimumab. Inverse correlations between $CD8^+$ T-cell increases and m-MDSC frequency in patients treated with ipilimumab suggest a role for m-MDSC–mediated lymphocyte suppression in OS following ipilimumab therapy. Further prospective studies are needed to validate m-MDSC measurement as a prognostic biomarker for melanoma and other disease states. Uniform methods of analysis, along with the use of Cyto-Chex tubes, make it possible for similar studies to proceed across multiple laboratories.

Disclosure of Potential Conflicts of Interest

M.A. Postow reports receiving a commercial research grant from Bristol-Myers Squibb and is a consultant/advisory board member for the same. J.D. Wolchok reports receiving a commercial research grant and other commercial research support from Bristol-Myers Squibb and is a consultant/advisory board member for the same. A.M. Lesokhin reports receiving a commercial research grant from Bristol Myers-Squibb and is a consultant/advisory board member for Bristol-Myers Squibb and Efranat, Inc. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: S. Kitano, M.A. Postow, J. Yuan, J.D. Wolchok, A.M. Lesokhin

Development of methodology: S. Kitano, K.S. Panageas, M. Adamow, J. Yuan, P. Wong, J.D. Wolchok, A.M. Lesokhin

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Kitano, M.A. Postow, T. Rasalan, M. Adamow, P. Wong, J.D. Wolchok, A.M. Lesokhin

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Kitano, M.A. Postow, C.G.K. Ziegler, D. Kuk, K.S. Panageas, M. Adamow, J. Yuan, G. Altan-Bonnet, J.D. Wolchok, A.M. Lesokhin

Writing, review, and/or revision of the manuscript: S. Kitano, M.A. Postow, D. Kuk, K.S. Panageas, J. Yuan, G. Altan-Bonnet, J.D. Wolchok, A.M. Lesokhin

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M.A. Postow, C. Cortez, A.M. Lesokhin

Study supervision: J.D. Wolchok, A.M. Lesokhin

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Human T-cell Lymphotropic Virus Type I–Associated Adult T-cell Leukemia–Lymphoma: New Directions in Clinical Research

Kunihiro Tsukasaki[1] and Kensei Tobinai[2]

Abstract

Adult T-cell leukemia–lymphoma (ATL) is a distinct malignancy of regulatory T cell (Treg)/TH2 cells caused by human T-cell lymphotropic virus type I (HTLV-1), with a high frequency of expression of CD3/CD4/CD25/CCR4 and FoxP3 in about half of the cells. However, in primary ATL cells, although expression of the virus, including the Tax oncoprotein, appears just after an *in vitro* culture, integration sites of the provirus into the host genome are random, and chromosomal/genetic abnormalities are complex. ATL is thus a single disease entity that is caused by HTLV-1 and possesses diverse molecular features. The clinical features and prognosis of ATL vary, and this has led to subtypes classified into four categories: acute, lymphomatous, chronic, and smoldering types, based on lactate dehydrogenase and calcium values and organ involvement. Approximately 15 to 20 million individuals are infected with HTLV-1 worldwide, 1.1 million of whom reside in Japan, and the annual incidence of ATL has been estimated to be approximately 1,000. HTLV-1 infection early in life, mainly from breast feeding, is crucial for the development of ATL. The age-specific occurrence of ATL and complex genome abnormalities that accumulate with disease progression suggest a multistep carcinogenesis model following HTLV-1 infection. Various treatment options are available for ATL and consist of watchful waiting for indolent ATL, intensive chemotherapy followed by allogeneic hematopoietic stem cell transplantation for aggressive ATL, and a combination of IFNα and zidovudine for ATL with leukemic manifestation. Several promising new agents, including an anti-CCR4 antibody, are currently undergoing clinical trials associated with translational research.

See all articles in this *CCR Focus* section, "Paradigm Shifts in Lymphoma."

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Introduction

Adult T-cell leukemia–lymphoma (ATL) is a rare T-cell malignancy associated with human T-cell lymphotropic virus type I (HTLV-1; refs. 1–5). Several inflammatory diseases have also been associated with HTLV-1, including tropical spastic paraparesis (TSP)/HTLV-1–associated myelopathy (HAM), infective dermatitis, and HTLV-associated uveitis (6–9). Endemic areas have been identified for the virus and these diseases, including southwestern Japan, the Caribbean islands, tropical Africa, South America, the Middle East, and northern Oceania. Only a small percentage of HTLV-1 carriers infected through breast feeding develop the disease, which suggests multistep carcinogenesis (10–12). The diversity of the clinical features and prognosis of patients with this disease has led to its classification into four categories: acute, lymphomatous, chronic, and smoldering types, based on lactate dehydrogenase (LDH) and calcium values and organ involvement (13, 14). Various treatment options are available for ATL and consist of watchful waiting for indolent (smoldering and unfavorable chronic) ATL, intensive chemotherapy followed by allogeneic hematopoietic stem cell transplantation (allo-HSCT) for aggressive (unfavorable chronic, lymphomatous, and acute) ATL, and a combination of IFNα and zidovudine (IFN/AZT) for ATL with leukemic manifestation. ATL is more refractory to chemotherapy than other peripheral T-cell lymphomas (PTCL), but is relatively sensitive to potential HTLV-1–targeting therapies such as allo-HSCT and IFN/AZT (12). A recent phase II trial revealed that an anti-CC chemokine receptor (CCR4) antibody was effective against relapsed ATL (15). Furthermore, other promising new agents for PTCL, including ATL, are being developed. Recent advances in clinical and translational research on this disease, including molecular, epidemiologic, biologic, and therapeutic aspects, are summarized below.

Molecular Epidemiology of ATL

The seroprevalence of HTLV-1 was examined in 1,196,321 Japanese first-time blood donors between 2006 and 2007 (16). A total of 3,787 of them were confirmed to be positive

[1]Department of Hematology, National Cancer Center Hospital East, Kashiwanoha, Kashiwa, Chiba, Japan. [2]Department of Hematology, National Cancer Center Hospital, Tsukiji, Chuo-ku, Tokyo, Japan.

Corresponding Author: Kensei Tobinai, Department of Hematology, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan. Phone: 81-3-3542-2511; Fax: 81-3-3542-3815; E-mail: ktobinai@ncc.go.jp

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for the anti-HTLV-1 antibody. By applying a fitness curve to age ranges outside the blood donor age range, the present number of HTLV-1 carriers from age 0 to 99 years was estimated to be at least 1.08 million in Japan, approximately 10% lower than that reported in 1988. The adjusted overall prevalence rates of HTLV-1 were estimated to be 0.66% and 1.02% in men and women, respectively. Carrier numbers peaked among individuals in their 70s, markedly different from the previous peak observed among individuals in their 50s in the 1988 database, probably reflecting a birth cohort effect. Compared with the survey conducted in the 1980s, carriers were distributed not only in endemic regions in Japan, but throughout the country, particularly in the greater Tokyo metropolitan area (16). A high prevalence of HTLV-1 is also found in the Caribbean islands (African), tropical Africa (African), South America (Mongoloid), and northern Oceania (Melanesian; refs. 10, 11).

The three major routes of HTLV-1 transmission are mother-to-child infections (via breast milk), sexual intercourse, and blood transfusions (10, 11). The overall infection rate of HTLV-1 in children by seropositive mothers was previously estimated to be between 10% and 30% mainly through breast feeding (17). The reported risk factors for the development of ATL among HTLV-1 carriers include HTLV-1 infection early in life, an increase in age, male sex, family history of ATL, past history of infectious dermatitis, smoking, serum titers of the antibody against HTLV-1, HTLV-1 proviral load, and several HLA subtypes (11, 18). However, these were the findings of relatively small and not-comprehensive studies. A total of 1,218 asymptomatic HTLV-1 carriers (426 males and 792 females) were examined between 2002 and 2008 for a prospective cohort-study on the development of ATL in Japan (19). The proviral load at enrollment was significantly higher in males than in females [median, 2.1 vs. 1.4 copies/100 peripheral blood mononuclear cells (PBMC)], in those ages 40 or older, and in those with a family history of ATL. During the follow-up period, 14 participants developed acute ATL. Their baseline proviral loads were high (range, 4.2–28.6 copies/100 PBMC). Not only a higher proviral load, but also advanced age, family history of ATL, and the first opportunity for HTLV-1 testing during the treatment of other diseases were independent risk factors for the progression of ATL.

Although the incidence of ATL in HTLV-1–endemic areas is known to be high, population-based evidence concerning the incidence of ATL in nonendemic areas is scarce. Chihara and colleagues recently estimated the age-standardized incidence of ATL between 1993 and 2006 in Japan and between 1993 and 2008 in the United States, and assessed trends using a population-based cancer registry in Japan and Surveillance Epidemiology and End Results in the United States (20). A total of 2,055 patients in three prefectures in Kyushu and 1,380 patients in 12 prefectures in Honshu were diagnosed with ATL during the study period. In the United States, a total of 140 patients were diagnosed with ATL. This study showed that the age-standardized incidence in nonendemic areas in Japan and the United States significantly increased during this period [annual percentage change (95% confidence interval; CI); Japan-Honshu: +4.6% (1.1–8.2); U.S.: +6.2% (1.5–11.1)], whereas no change was observed in endemic areas in Japan (Japan-Kyushu: 0.0%; 1.6–1.7).

Biology of HTLV-1–Associated ATL

The HTLV-I gene encodes three structural proteins: Gag, Pol, and Env, and complex regulatory proteins such as Tax, which not only activate viral replication, but also induce the expression of several cellular genes important in the proliferation and antiapoptosis of ATL cells, including NF-κB (Fig. 1; refs. 5, 21, 22). The expression of these cellular proteins may enhance the multistep carcinogenesis of ATL, whereas expression of the viral proteins *in vivo* is suppressed by cytotoxic T cells. A new viral factor, HTLV-1 basic Zip factor (HBZ), which was encoded from the minus strand of mRNA, was recently discovered and may play a role in viral replication and T-cell proliferation because it is steadily expressed in most HTLV-1–infected cells and primary ATL cells whereas Tax is not (23). The polycomb-mediated epigenetic silencing of miR31 was more recently reported to be implicated in the aberrant and constitutive activation of NF-κB signaling in ATL cells (24). HBZ and miR31 may be good targets for the prevention as well as treatment of ATL.

ATL is a distinct malignancy of regulatory T cell (Treg)/TH2 cells caused by HTLV-1 with high frequency of expression of CD3/CD4/CD25/CCR4 and FoxP3 in about half of the cells (25, 26).

Figure 2 summarizes the multistep leukemogenesis of ATL, which consists of viral, epigenetic, and genetic factors. Regarding the viral factors, Tax, which is a strong transactivating factor of host genes and important in cell transformation, is considered to be crucial for the oligoclonal maintenance and expansion of HTLV-1–infected cells in the early phase of HTLV-1–infected individuals, the so-called healthy HTLV-1 carriers (10, 11, 22). However, the expression of Tax, which is very immunogenic, should be transient on each HTLV-1–infected cell escaping the immune surveillance of the host. Thereafter HTLV-1–infected cells can transform with a combination of the continued expression of HBZ, acquired epigenetic regulation of cell-transforming factors, full-blown development of ATL with the genetic/epigenetic loss of function of tumor suppressor genes and microRNAs (miRNA), and activation of oncogenes (12, 23, 24, 27–34). These abnormalities are acquired during the progression of ATL from the indolent to the aggressive subtypes. These abnormalities, excluding Tax, HBZ, and miR31, are very diverse, as revealed by the aneuploidy profile obtained using comparative genomic hybridization and microarray expression profile (35, 36). These findings indicated that ATL is a single disease entity associated with HTLV-1 that acquires diverse molecular abnormalities resembling the acute-crisis phase of chronic myeloid leukemia with similar diverse abnormalities caused by bcr/abl. Clonal selection during the progression of ATL is typically the consequence of clonal evolution.

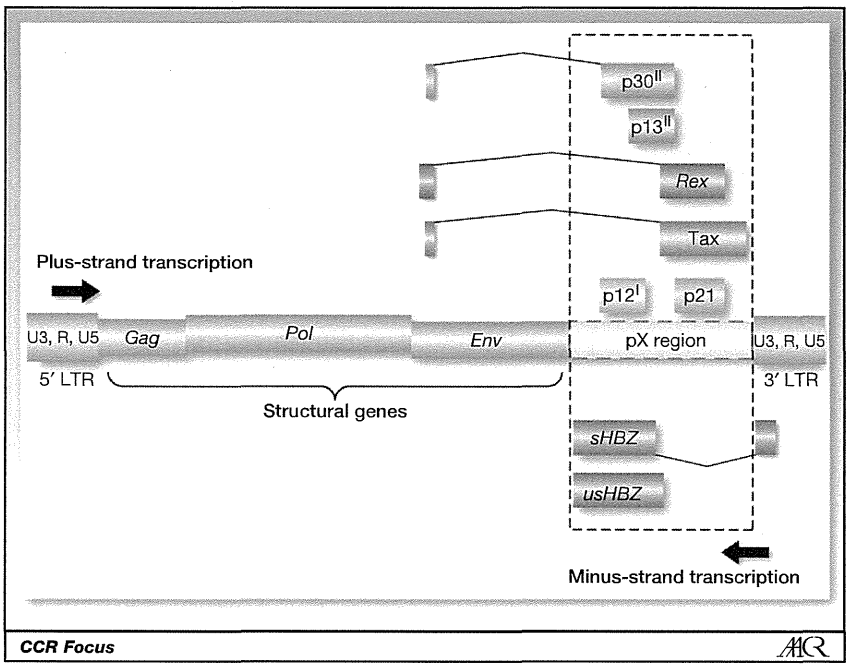

Figure 1. The structure of HTLV-1. The HTLV-I gene encodes three structural proteins, Gag, Pol, and Env, and complex regulatory proteins such as Tax, which not only activate viral replication, but also induce the expression of several cellular genes (5, 21). The expression of these viral proteins *in vivo* is suppressed by CTLs. HTLV-1 basic Zip factor (HBZ), encoded from the minus-strand mRNA, may play a role in viral replication and T-cell proliferation because it is steadily expressed in most HTLV-1–infected cells and primary ATL cells, whereas Tax is not. Reprinted with permission from Satou and Matsuoka (21).

Multiple subclones in lymph nodes originate from a common clone in many ATL cases, and a selected subclone among the lymph node subclones appears in the peripheral blood (37). Clonal changes, but not clonal evolution, have been reported in approximately 10% of cases progressing from indolent to acute ATL, and may reflect the emergence of multiple premalignant oligoclones in viral leukemogenesis, as suggested in Epstein–Barr virus-associated lymphomagenesis in immunocompromised hosts (38, 39). The genomic characteristics of proviral integration sites in malignant and nonmalignant clones, as well as the proviral features (genomic structure and 5′LTR methylation) that determine its capacity to express Tax, were recently identified using a sensitive high-throughput method for primary ATL cells (40).

ATL lesions in the peripheral blood are morphologically diagnosed in the same manner as other lesions involving the lymph nodes (13, 14). However, ATL cell atypia vary from the so-called flower cells with multilobulated nuclei to chronic lymphocytic leukemia (CLL)–like cells resembling normal lymphocytes (41). The monoclonal integration of HTLV-1 detected by Southern blotting hybridization (SBH) is used as a supportive method for the diagnosis of ATL with a threshold sensitivity of approximately 5%. However, SBH can also detect monoclonal integration in a small percentage of HTLV-1 carriers and approximately 10% of HAM/TSP patients (42, 43). Flow-cytometric analysis of T cells recently revealed that the expression of CADM1 and stepwise downregulation of CD7 were closely associated with the clonal expansion of HTLV-1–infected cells in ATL, and $CADM1^+$ cells with the downregulated expression of CD7 in asymptomatic HTLV-1 carriers exhibited common properties to those in indolent ATL carriers (44).

Treatment and Prognosis of ATL

The prognosis of ATL is worse than that of other PTCLs (45). The clinical subtype classification of ATL is very useful for decision making about the treatment of each patient (13). However, there is no plateau—rather an initial steep slope and subsequent gentle slope in the survival curves of aggressive and indolent ATL treated with chemotherapy and watchful waiting, respectively, although the prognosis of the latter is markedly better [median survival time (MST), 1 year vs. 5 years; refs. 13, 46]. Improved prognostic systems have been sought. From North America, a new prognostic score for ATL was reported, based on performance status (PS), stage, age, and calcium level at diagnosis (47). A recent retrospective survey in Japan on 807 patients with acute or lymphomatous ATL treated with chemotherapy, but not with allo-HSCT, developed a prognostic index based on five prognostic factors: stage, PS, age, serum albumin, and soluble IL2 receptor (48). In the validation sample, the index was reproducible with MSTs of 3.6, 7.3, and 16.2 months for patients at high, intermediate, and low risk, respectively. The Japan Clinical Oncology Group (JCOG)-Lymphoma Study Group (LSG) conducted a meta-analysis of three consecutive trials exclusively for aggressive ATL (see below; ref. 49). An overall survival (OS) analysis of 276 patients with aggressive ATL identified two significant prognostic factors, PS and hypercalcemia. In the validation sample, a proposed prognostic index using these two factors

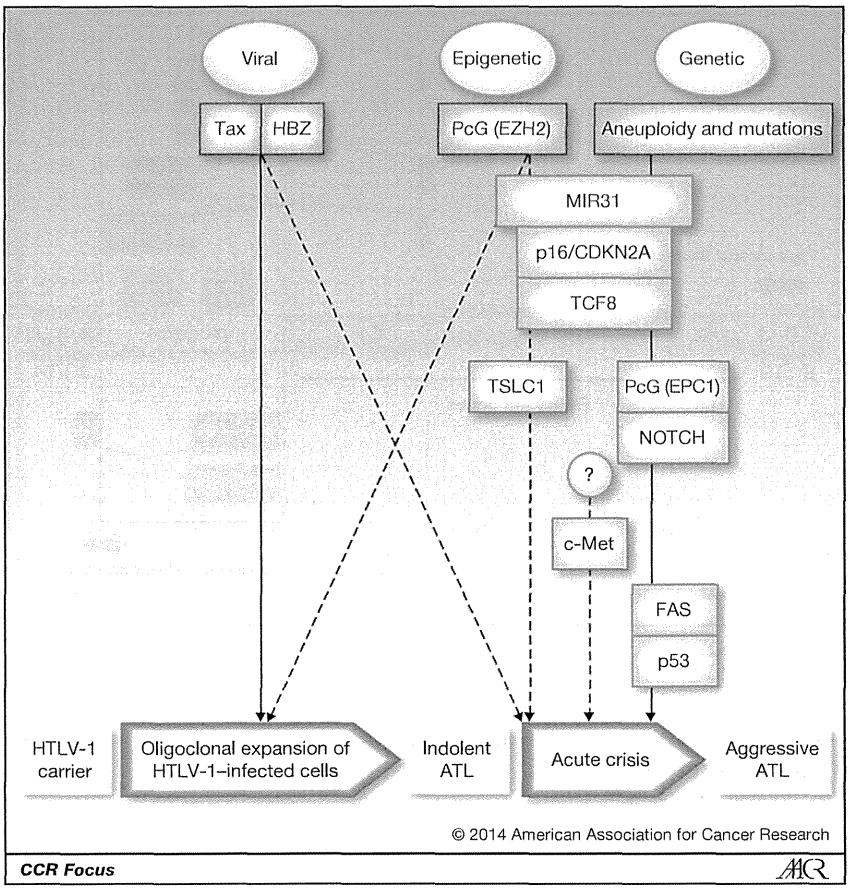

Figure 2. The role of viral, epigenetic, and genetic factors in the multistep leukemogenesis of ATL. Tax is considered crucial for the oligoclonal maintenance and expansion of HTLV-1–infected cells in early phase with the expression transient on each HTLV-1–infected cells escaping from the immune surveillance of hosts. Thereafter, continuing expression of HBZ, acquired epigenetic regulation of cell-transforming factors is followed by genetic/epigenetic loss of function of tumor suppressor genes and miRNAs, and activation of oncogenes (12, 23, 24, 27–34). Diverse abnormalities are acquired during the progression of ATL from indolent to aggressive disease.

in two strata revealed MSTs of 6.3 and 17.8 months for patients at high and low risk, respectively. However, the 5-year OS rates in both studies were less than 15%, even in the low-risk group; therefore, the subgroup with relatively favorable prognoses could not be identified. However, approximately 10% of patients with lymphoma-type ATL survived more than 10 years without allo-HSCT, which suggests that they may have been cured (49).

JCOG-LSG has consecutively conducted clinical trials on aggressive non-Hodgkin lymphoma (NHL), including ATL (50). Aggressive ATL has been exclusively studied from other NHLs after far worse response and survival rates were reported in earlier studies. A phase II trial (JCOG9303) for aggressive ATL using the LSG15 regimen, which consisted of six cycles of vincristine, cyclophosphamide, doxorubicin, and prednisone (VCAP); doxorubicin, ranimustine, and prednisone (AMP); and vindesine, etoposide, carboplatin, and prednisone (VECP) with the prophylactic use of G-CSF and intrathecal prophylaxis, revealed a promising response rate and MST. After JCOG9303, we conducted a phase III trial to compare modified (m)-LSG15 (VCAP-AMP-VECP) with CHOP-14, both supported by G-CSF and intrathecal prophylaxis. A higher 3-year survival rate (24% vs. 13%) and complete response rate (40% vs. 25%) with mLSG15 than with CHOP-14 suggested that the former was a more effective regimen at the expense of greater toxicities, which provided the basis for future investigations on the treatment of aggressive ATL (51). However, the MST of 13 months is still unsatisfactory.

A treatment strategy for ATL based on clinical subtypes, prognostic factors, and response to the initial therapy was suggested in an international consensus report (52). Patients with aggressive ATL generally have a very poor prognosis due to the multidrug resistance of ATL cells, large tumor burden with multiorgan failure, hypercalcemia, and/or opportunistic infections (10–13). Intensive chemotherapy such as mLSG15 is recommended for aggressive ATL (51, 52). Watchful waiting until disease progression has been recommended for indolent ATL, although the long-term prognosis of this disease was inferior to that of, for example, CLL (46, 52). Treatment decisions should be based on the ATL subclassification and the prognostic factors at onset and response to initial therapy (Table 1). The prognostic factors include clinical factors, such as PS, LDH, age, stage, number of involved lesions, and hypercalcemia, and molecular factors, such as Ki-67 expression, solubile IL2 receptor,

Table 1. Strategy for the treatment of adult T-cell leukemia–lymphoma proposed from an international consensus meeting

Smoldering- or favorable chronic-type ATL.
- Consider inclusion in prospective clinical trials.
- Symptomatic patients (skin lesions, opportunistic infections, and so on): consider AZT/IFNα or watch and wait.
- Asymptomatic patients: consider watch and wait.

Unfavorable chronic- or acute-type ATL.
- Recommend: inclusion in prospective clinical trials.
- If outside clinical trials, check prognostic factors (including clinical and molecular factors if possible):
  - Good prognostic factors: consider chemotherapy (VCAP-AMP-VECP evaluated by a randomized phase III trial against biweekly CHOP) or AZT/IFNα (evaluated by a retrospective worldwide meta-analysis).
  - Poor prognostic factors: consider chemotherapy followed by conventional or reduced-intensity allogeneic HSCT (evaluated by retrospective or prospective Japanese analyses, respectively).
  - Poor response to initial therapy with chemotherapy or AZT/IFNα: consider conventional or reduced-intensity allogeneic HSCT.

Lymphoma-type ATL.
- Recommend: inclusion in prospective clinical trials.
- If outside clinical trials, consider chemotherapy (VCAP-AMP-VECP).
- Check prognostic factors and response to chemotherapy (including clinical and molecular factors if possible):
  - Favorable prognostic profiles and good response to initial therapy: consider chemotherapy.
  - Unfavorable prognostic profiles or poor response to initial therapy with chemotherapy: consider conventional or reduced-intensity allogeneic HSCT.

Options for clinical trials (first line).
- Test the effect of up-front allogeneic HSCT.
- Test promising targeted therapies such as arsenic trioxide + IFNα, bortezomib + chemotherapy, or antiangiogenic therapy.
- Consider a phase II global study testing pegylated IFN and AZT.

Options for clinical trials (relapse or progressive disease).
- Test the effect of promising targeted therapies such as arsenic trioxide and IFNα, bortezomib, a purine nucleotide phosphorylase inhibitor, histone deacetylase inhibitors, monoclonal antibodies, antiangiogenic therapy, and survivin, β-catenin, syk, and lyn inhibitors, etc.
- Consider conventional or reduced-intensity allogeneic HSCT when possible.

Abbreviation: CHOP, cyclophosphamide, doxorubicin, vincristine, and prednisone.

 Tsukasaki K, Hermine O, Bazarbachi A, Ratner L, Ramos JC, Harrington W Jr, et al. Definition, prognostic factors, treatment, and response criteria of adult T-cell leukemia-lymphoma: a proposal from an international consensus meeting. J Clin Oncol 2009;27:453–9.

alteration of p53 or p15INK4B/p16INK4A, and overexpression of IRF-4 (47–49, 52). Initial relatively small phase II studies and recent retrospective meta-analyses suggested that IFN/AZT therapy may be promising, especially for types with leukemic manifestation (53–55). The therapeutic effects of IFN/AZT are not considered to be attributable to direct cytotoxic effects on leukemic cells (56). A possible mechanism of the combination for ATL includes AZT treatment of ATL cell lines resulting in telomere attrition, which reprograms cells to undergo p53-dependent senescence, and IFN alone suppressing the expression of HTLV-1 and cell cycling, whereas IFN/AZT induces p53 signaling and apoptosis in HTLV-1–infected cells (57, 58).

Allo-HSCT is promising for the treatment of aggressive ATL, possibly reflecting graft-versus-ATL effect, including the nonmyeloablative conditioning regimen (59–61). Minimal residual disease following allo-HSCT, which is detected as the HTLV-1 proviral load, was markedly less than that after chemotherapy or AZT/IFN therapy, which suggested the presence of a graft-versus-ATL effect as well as graft-versus-HTLV-1 activity (62). ATL with abnormalities in tumor suppressor genes such as p53 was reportedly resistant to IFN/AZT therapy as well as chemotherapy. Allo-HSCT may overcome this resistance (52). It remains unclear which type of allo-HSCT (myeloablative or reduced intensity conditioning) is more suitable for the treatment of ATL. Furthermore, selection criteria with respect to responses to previous treatments, the sources of stem cells, and the HTLV-1 viral status of the donor have yet to be determined.

Translational Research and Clinical Trials of New Agents for ATL

Translational research is mandatory for the development of new agents against specific disease subtypes such as PTCLs, including ATL. Research on the biology of HTLV-1–infected cells and ATL cells revealed that the IL2, IL9, IL15

pathways in conjunction with the JAK–STAT or Wnt pathways are crucial in this disease (63). Several mouse models of ATL development exist, including NOG mice in which the growth and proliferation of the primary cells of aggressive and indolent ATL are marked and resemble the site of organ involvement and hypercalcemia, thymus-derived leukemia–lymphoma in mice transgenic for the Tax gene, and an HTLV-1–infected intra-bone marrow injection of human $CD133^+$ stem cells into a NOG mouse model to recapitulate distinct ATL-like symptoms as well as HTLV-1–specific immune responses (64–66). These mouse models are useful for translational research on ATL; for example, the synergistic effects of the combination of IFN/AZT with As(2)O(3) were reported in a Tax transgenic mouse model (67).

Several promising new agents for ATL are currently undergoing translational research and some are now in clinical trials. Among them are an anti-CCR4 antibody, mogamulzumab, which has been approved for relapsed/refractory ATL in Japan; a CD30-directed antibody–drug conjugate, brentuximab vedotin, which is being assessed in a global phase III trial with chemotherapy for untreated PTCLs, including ATL; and an immunomodulatory agent, lenalidomide, which is in phase II testing for relapsed aggressive ATL.

Histone deacetylase inhibitors

Gene expression governed by epigenetic changes is crucial to the pathogenesis of cancer. Histone deacetylases are enzymes that are involved in the remodeling of chromatin, and play a key role in the epigenetic regulation of gene expression. The histone deacetylase inhibitor (HDACi) LBH589 exhibits significant anti-ATL effects by activating a novel RAIDD–caspase-2 pathway *in vitro* and in mice with the expression modulation of ATL-related proteins, including Tax and CCR4 (68). However, a phase II study of LBH589 for CTCL and indolent ATL was terminated because of severe infections associated with the shrinkage of skin tumors and formation of ulcers in patients with ATL. Romidepsin, another HDACi, was recently approved for the treatment of relapsed/refractory PTCL by the FDA. Further studies are needed to evaluate the efficacy of HDACis for PTCL/cutaneous T-cell lymphoma (CTCL), including ATL.

Proteasome inhibitors

The proteasome inhibitor bortezomib suppresses the activation of NF-κB, which is constitutively expressed in all subtypes of ATL cells and HTLV-1–infected cells, and has been implicated in oncogenesis as well as resistance to anticancer agents and apoptosis. This agent effectively inhibits the growth of ATL cells both *in vivo* and *in vitro* (69). A phase II study of bortezomib is now ongoing for ATL in Japan.

CD30-directed antibody–drug conjugates

The TNF receptor family member CD30 is an activation marker of lymphocytes, and signaling through CD30 is associated with cell proliferation. Some PTCLs, including ATL, as well as Hodgkin lymphoma and anaplastic large-cell lymphoma (ALCL), express CD30. Most ATL cells in less than 10% of ATL cases express CD30, similar to ALCL, whereas several to 10% of ATL cells express CD30 in the remaining ATL cases (14). To enhance the antitumor activity of CD30-directed therapy, the antitubulin agent monomethyl auristatin E was attached to a CD30-specific mAb by an enzyme-cleavable linker to produce the antibody–drug conjugate brentuximab vedotin (SGN-35). Brentuximab vedotin induced durable objective responses with acceptable toxicities in most patients with relapsed or refractory CD30-positive Hodgkin lymphoma/ALCL in several phase I and II studies (70). Regarding newly diagnosed CD30-positive PTCLs, including ATL, a phase I study of brentuximab vedotin + CHP, in which VCR was omitted to avoid its additive neurotoxicity, revealed promising results (71).

Anti-CCR4 antibody

CCR4 is expressed on the neoplastic cells of most patients with ATL, and this expression has been associated with the cutaneous manifestation and poor prognosis. The aberrant expression of Fra-2 promotes that of CCR4 and cell proliferation in ATL cells (72). The defucosylated humanized anti-CCR4 mAb (mogamulizumab), the ADCC activity of which was stronger than that of the usual antibody in preclinical analysis using primary ATL and effector cells, was approved for the treatment of relapsed/refractory ATL in Japan based on the results of phase I and II studies, with a response rate of approximately 50% and manageable toxicities, including moderate to severe skin reactions (15, 73, 74). The findings of a subsequent randomized phase II study on intensive chemotherapy (mLSG15) ± mogamulizumab for untreated aggressive ATL have recently been reported (75). This combination was anticipated because the former was more effective for ATL cells in lymph nodes than those in the peripheral blood, whereas the opposite was true for the latter (15, 51). The combination was well tolerated and produced a higher complete response rate [52% (95% CI, 33–71) vs. 33% (CI, 16–55)], respectively. Clinical trials of mAbs for ATL and other PTCLs include a humanized anti-CD52 mAb (alemtuzumab) and a humanized anti-CD2 mAb (siplizumab).

Other new agents

Other new agent trials for ATL and/or PTCL that are ongoing or in preparation in Japan include studies of IL2 fused with the diphtheria toxin targeting CD25; a novel purine nucleoside phosphorylase inhibitor, forodesine; an anti-folate, pralatrexate, an FDA-approved agent with clinical activity in T-cell malignancies, including ATL; an organic arsenic; and the immunomodulatory agent lenalidomide (76).

Conclusions

ATL cases are separately treated on the basis of the aggressive-versus-indolent subtypes, with prompt treatment using combination chemotherapy, followed by

allo-HSCT versus watchful waiting until disease progression, respectively. Therefore, future issues to be resolved in the treatment of this intractable disease with diverse clinical features include new standard treatments between watchful waiting and intensive chemotherapy ± allo-HSCT. IFN/AZT and mogamulizumab are promising treatment options, especially for aged patients. Another aspect is multimodality treatments for ATL with an extremely poor prognosis.

Two prospective studies are ongoing for ATL by JCOG-LSG. One is a phase II study of mLSG15 and mogamulizumab followed by allo-HSCT with myeloablative or non-myeloablative conditioning for aggressive ATL (JCOG0907). The other is a phase III trial for indolent ATL to compare IFN/AZT with watchful waiting (JCOG1111).

Furthermore, as described in more detail in the *CCR Focus* article by O'Connor and colleagues (77), more than 10 promising new agents for PTCL/CTCL, including ATL, are undergoing clinical trials or are in preparation with translational research. Future clinical trials on ATL should be carefully and appropriately conducted to ensure that the international consensus on ATL management is continually updated to establish evidence-based practical guidelines.

Disclosure of Potential Conflicts of Interest

K. Tsukasaki reports receiving commercial research grants from Celgene, Kyowa-Kirin, and Takeda. K. Tobinai reports receiving commercial research grants from Celgene, Kyowa-Kirin, Mundipharma, and Takeda. No other potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: K. Tsukasaki, K. Tobinai
Development of methodology: K. Tobinai
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K. Tsukasaki, K. Tobinai
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K. Tsukasaki, K. Tobinai
Writing, review, and/or revision of the manuscript: K. Tsukasaki, K. Tobinai
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K. Tsukasaki, K. Tobinai
Study supervision: K. Tobinai

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Clinical Cancer Research

Human T-cell Lymphotropic Virus Type I–Associated Adult T-cell Leukemia–Lymphoma: New Directions in Clinical Research

Kunihiro Tsukasaki and Kensei Tobinai

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Molecular and Cellular Pathobiology

Cancer Research

Molecular Characterization of Chronic-type Adult T-cell Leukemia/Lymphoma

Noriaki Yoshida[1,2,3], Kennosuke Karube[1], Atae Utsunomiya[4], Kunihiro Tsukasaki[5], Yoshitaka Imaizumi[5], Naoya Taira[6], Naokuni Uike[7], Akira Umino[1,8], Kotaro Arita[1,9], Miyuki Suguro[1], Shinobu Tsuzuki[1], Tomohiro Kinoshita[10], Koichi Ohshima[3], and Masao Seto[1,2,3]

Abstract

Adult T-cell leukemia/lymphoma (ATL) is a human T-cell leukemia virus type-1–induced neoplasm with four clinical subtypes: acute, lymphoma, chronic, and smoldering. Although the chronic type is regarded as indolent ATL, about half of the cases progress to acute-type ATL. The molecular pathogenesis of acute transformation in chronic-type ATL is only partially understood. In an effort to determine the molecular pathogeneses of ATL, and especially the molecular mechanism of acute transformation, oligo-array comparative genomic hybridization and comprehensive gene expression profiling were applied to 27 and 35 cases of chronic and acute type ATL, respectively. The genomic profile of the chronic type was nearly identical to that of acute-type ATL, although more genomic alterations characteristic of acute-type ATL were observed. Among the genomic alterations frequently observed in acute-type ATL, the loss of *CDKN2A*, which is involved in cell-cycle deregulation, was especially characteristic of acute-type ATL compared with chronic-type ATL. Furthermore, we found that genomic alteration of *CD58*, which is implicated in escape from the immunosurveillance mechanism, is more frequently observed in acute-type ATL than in the chronic-type. Interestingly, the chronic-type cases with cell-cycle deregulation and disruption of immunosurveillance mechanism were associated with earlier progression to acute-type ATL. These findings suggested that cell-cycle deregulation and the immune escape mechanism play important roles in acute transformation of the chronic type and indicated that these alterations are good predictive markers for chronic-type ATL. *Cancer Res; 74(21); 6129–38. ©2014 AACR.*

[1]Division of Molecular Medicine, Aichi Cancer Center Research Institute, Nagoya, Japan. [2]Department of Cancer Genetics, Nagoya University Graduate School of Medicine at Aichi Cancer Center Research Institute, Nagoya, Japan. [3]Department of Pathology, Kurume University School of Medicine, Kurume, Japan. [4]Department of Hematology, Imamura Bun-in Hospital, Kagoshima, Japan. [5]Department of Hematology, Atomic Bomb Disease and Hibakusha Medicine Unit, Atomic Bomb Disease Institute, Nagasaki University, Nagasaki, Japan. [6]Department of Internal Medicine, Heart Life Hospital, Nakagusukuson, Japan. [7]Department of Hematology, National Hospital Organization Kyushu Cancer Center, Fukuoka, Japan. [8]Hematology and Oncology, Mie University Graduate School of Medicine, Tsu, Japan. [9]Third Department of Internal Medicine, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama, Japan. [10]Department of Hematology and Cell Therapy, Aichi Cancer Center, Nagoya, Japan.

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Current address for N. Yoshida: Department of Pathology and Laboratory Medicine/Diagnostic Pathology, Nagoya University Graduate School of Medicine, Nagoya, Japan; current address for K. Karube: Hematopathology Section, Laboratory of Pathology, Hospital Clínic, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), University of Barcelona, Barcelona, Spain; and current address for K. Tsukasaki: Department of Hematology, National Cancer Center Hospital East, Kashiwa, Japan.

Corresponding Author: Masao Seto, Department of Pathology, Kurume University School of Medicine, 67 Asahimachi, Kurume, Fukuoka 830-0011, Japan. Phone: 81-942-35-3311, ext. 3181; 81-942-31-7547 (direct); Fax: 81-942-31-0342; E-mail: seto_masao@kurume-u.ac.jp and mseto@aichi-cc.jp

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Introduction

Adult T-cell leukemia/lymphoma (ATL) is a human T-cell leukemia virus type-1 (HTLV-1)–induced neoplasm (1, 2). Four clinical subtypes of ATL have been classified on the basis of clinical manifestation: acute, lymphoma, chronic, and smoldering (3). Among these subtypes, chronic-type ATL shows characteristic manifestations such as increased abnormal lymphocytes in peripheral blood, lactate dehydrogenase (LDH) levels up to twice the normal upper limit, and absence of hypercalcemia. Chronic-type ATL is relatively rare and its frequency is estimated to be 8% to 18% of ATL cases (3). Previous reports regard the chronic type as indolent ATL compared with acute/lymphoma types, which show an aggressive clinical course (3, 4). However, a recent study of indolent ATL demonstrated that about half of the patients with chronic-type ATL progress to acute-type ATL within approximately 18 months from diagnosis and subsequent death (4). This finding suggests that patients with chronic-type ATL also had a poor prognosis. High LDH, high blood urea nitrogen, and low albumin levels have been identified as poor prognostic factors for chronic-type ATL, and patients with chronic-type ATL with these poor prognostic factors therefore need to be treated by intensive chemotherapy as in the case of patients with aggressive ATL (5).

Disruptions of *CDKN2A*, *CDKN2B*, and *TP53* have been reported as candidate genes that play important roles in acute

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transformation of chronic-type ATL (6–12). However, these acute transformation–related genetic alterations have been identified only by focusing on genes that were previously shown to be involved in tumor progression of other malignancies. Therefore, these genetic alterations may be indicative of acute transformation in some cases, although the molecular mechanism of acute transformation remains to be fully elucidated. Identification of the molecular characteristics of chronic-type ATL using unbiased and genome-wide methods can provide further insights to elucidate the acute transformation mechanisms in chronic-type ATL. However, the molecular pathogenesis of chronic-type ATL has long remained unknown due to its rarity (13).

In the present study, high-resolution oligo-array comparative genomic hybridization (aCGH) and gene expression profiling (GEP) were applied to 27 cases of chronic-type ATL in an effort to determine the molecular pathogenesis. The same approaches were used with 35 cases of acute-type ATL, and we then compared the molecular characteristics of chronic- and acute-type ATL to investigate the molecular mechanism of acute transformation.

Materials and Methods

Patient samples

We collected and analyzed 27 cases of chronic-type ATL and 35 cases of acute-type ATL (Table 1 and Supplementary Table S1 in Supplementary Data). These samples were obtained from patients at Imamura-Bunin Hospital (Kagoshima, Japan), Nagasaki University School of Medicine (Nagasaki, Japan), Heart Life Hospital (Nakagusukuson, Japan), and Kyushu Cancer Center (Fukuoka, Japan). In accordance with Shimoyama criteria, the diagnoses were made by expert hematologists (A. Utsunomiya, K. Tsukasaki, Y. Imaizumi, N. Taira, and N. Uike; ref. 3). Samples and medical records used in our study were approved by the Institute Review Board of the Aichi Cancer Center (Nagoya, Japan). Informed consent was obtained according to the Declaration of Helsinki from all patients. DNA and RNA used in this study were extracted from purified CD4-positive cells as previously reported (14). For the cumulative incidence of acute transformation, events were defined as acute transformation or any treatment for ATL.

Copy number analysis by aCGH and GEP

We performed aCGH analysis on all samples using 400K aCGH (Agilent, Cat. # G4448A; Agilent Technologies) and 44K aCGH (Agilent, Cat. # G4413A) slides (Supplementary Table S1). Thirteen acute-type cases analyzed in a previous study were included (14). Procedures for DNA digestion, labeling, hybridization, scanning, and data analyses were performed according to the manufacturer's protocols (www.agilent.com). Raw data were transferred to the Genomic Workbench v5.0 software (Agilent Technologies) for further analysis as described previously (14–16). Among these identified alterations, we focused on minimal common regions (MCR). MCRs are defined as alterations that encompass less than 3 protein-coding genes among all samples analyzed in this study (17). Copy number variations/polymorphisms (CNV) were identified using a database (HS_hg18_CNV-20120403, Agilent), which was obtained from Database of Genomic Variants (http://projects.tcag.ca/variation/) in April 2012 and then excluded from further analyses as described previously (16). We also performed aCGH analysis on matched normal DNA samples that were available and confirmed that the identified MCRs were not CNVs (Supplementary Fig. S1A).

For analysis of GEP, the Whole Human Genome 44K Oligo-microarray Kit (Agilent, Cat. # G4112F) was used for the hybridization of labeled RNA. The total RNA of 13 chronic samples and 21 acute samples was analyzed. The experimental protocol used reflected the manufacturer's protocol (www.agilent.com) as previously reported (15, 16). Using the results of GEP, gene set enrichment analysis (GSEA) was performed as previously described (15, 16, 18).

The detailed description of these analyses can be found in Supplementary Methods. The microarray data were submitted to ArrayExpress and assigned accession numbers E-MTAB-1808 (aCGH) and E-MTAB-1798 (GEP).

Mutation analyses of CD58 and β2-microglobulin

The exons 1–4 of *CD58* and 1 and 2 of *β2-microglobulin* (*B2M*), whose mutations were identified in peripheral T-cell lymphomas (PTCL; ref. 19), were amplified from gDNA using PCR. PCR primers used are detailed in the previous study (20). Twenty-six acute-type and 26 chronic-type ATL samples, for which adequate DNA was available, were analyzed. Direct sequencing of PCR products was performed through capillary electrophoresis using the ABI3100 sequencer (Applied Biosystems).

Flow cytometry

Analysis of cell surface CD58 in ATL cell lines was performed using anti-CD58 PE antibody (AlCD58, Beckman Coulter).

Table 1. Patient information at sampling

Subtype	No. of samples	Median age (range), y	Median WBC (range), u/L	Median LDH (range), IU/L	Median calcium (range), mg/dL	Median albumin (range), g/dL	Median BUN (range), mg/dL
Chronic type	27	61 (42–81)	1,1400 (6,000–22,100)	233 (155–465)	9.3 (8.4–10.2)	4.2 (3.0–4.8)	15.5 (7.4–26.4)
Acute type	35	57 (32–85)	2,1700 (4,100–224,800)	688 (203–2,223)	9.3 (7.7–17.4)	3.8 (2.6–4.5)	NA

Abbreviations: BUN, blood urea nitrogen; NA, not available; WBC, white blood cells.

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Analyses were performed using a FACSCalibur flow cytometer (BD Biosciences) and FlowJo Version 7.2.4 software (TreeStar). The detailed description of these analyses can be found in Supplementary Methods.

Statistical analysis

Frequencies of genomic alterations were evaluated using Fisher exact test, and cumulative acute transformation rates were analyzed using Kaplan–Meier method.

All statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing; ref. 21).

Results

Genomic alteration profiles of chronic- and acute-type ATL

To evaluate the genomic alterations of chronic- and acute-type ATL, aCGH was performed for 62 patient samples (27 cases of chronic-type and 35 cases of acute-type ATL; Table 1 and Supplementary Table S1). Figure 1A shows genomic alteration profiles of chronic- and acute-type ATL. We identified 362 MCRs (230 losses and 132 gains) among the alterations. These MCRs contained 1–3 protein-coding genes, which are most likely the candidate genes of the alterations (15, 17). Frequent alterations are supposed to especially contribute to the pathophysiology of the disease. MCRs that were found in

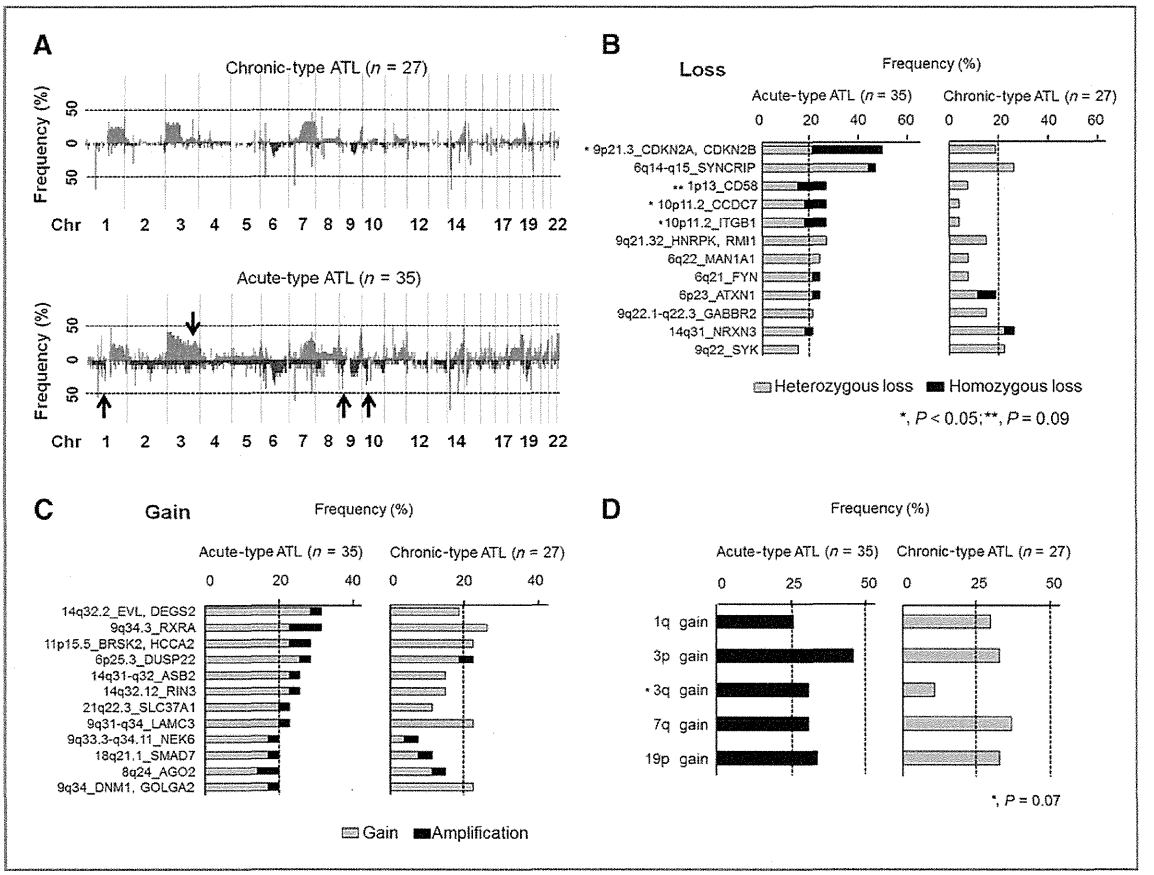

Figure 1. Genomic alteration profiles of chronic- and acute-type ATL. A, frequency of genomic alterations in chronic-type and acute-type ATL. Top, 27 cases with chronic-type ATL; bottom, 35 cases with acute-type ATL. The horizontal axis indicates each probe aligned from chromosome 1 to 22 and the short arm (p) to long arm (q). The vertical axis indicates the frequency of genomic alterations among the analyzed cases. The top area represents gain and the bottom area represents loss. Arrows represent characteristic alterations of acute-type ATL compared with chronic-type. B, MCRs encompassing 1–3 coding genes of copy number loss. MCRs found in greater than 20% of chronic-type or acute-type ATL are shown and ranked by frequency of alteration (left, acute type; right, chronic type). Among these MCRs, loss of *CDKN2A/CDKN2B* located in 9p21.3, losses of *CCDC7* and *ITGB1* located in 10p11.2 were observed more frequently in acute-type ATL. Loss of *CD58* was also found more frequently in acute type than in the chronic type (Fisher exact test; *, $P < 0.05$; **, $P = 0.09$). Frequently altered MCRs in chronic-type ATL were also recognized in the acute type. C, MCRs of copy number gain. MCRs found in greater than 20% of chronic-type or acute-type ATL are shown and ranked by frequency of alteration (left, acute type; right, chronic type). None of these MCRs were characteristic of acute-type or chronic-type ATL. D, gains of chromosomes 1q, 3p, 3q, 7q, and 19p were observed in greater than 20% of acute-type and chronic-type ATL. MCRs were not detected in any of these lesions. Gain of 3q was more frequently found in acute-type ATL than in the chronic type (*, $P = 0.07$).

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more than 20% of chronic- or acute-type ATL were therefore analyzed (Fig. 1B and C).

Genomic loss of *CDKN2A/CDKN2B* was the first most frequently altered MCR in acute-type ATL (17 of 35 cases). The second most frequently altered MCR of acute-type ATL was genomic loss of *SYNCRIP* (16 of 35 cases). On the other hand, genomic losses of *SYNCRIP* and *NRXN3* and gain of *RXRA* were most frequently altered MCRs in chronic-type ATL (7 of 27 cases). Among these identified MCRs, the losses of *CDKN2A/CDKN2B*, *CCDC7*, and *ITGB1* were significantly characteristic of acute-type ATL (Fig. 1B, $P < 0.05$). In addition, acute-type ATL tended to have a loss of *CD58* (Fig. 1B). The frequently altered MCRs in chronic-type ATL were also found in acute-type ATL (Fig. 1B and C). Gains of chromosomes 1q, 3p, 3q, 7q, and 19p were also frequently observed in acute- and chronic-type ATL, although they did not show MCRs (Fig. 1D). Among these alterations, acute-type ATL tended to have a gain of 3q ($P = 0.07$).

Frequent loss of CDKN2A/CDKN2B

Our analysis identified loss of *CDKN2A/CDKN2B* located in 9p21.3 as the most frequently and specifically altered genomic region in acute-type ATL compared with chronic-type ATL. Therefore, this loss is suggested to play an important role in the pathophysiology of acute-type ATL and acute transformation of chronic-type ATL.

Seventeen of the 35 acute-type ATL samples showed loss of 9p21.3, which was also found in 5 of the 27 chronic-type ATL samples. These losses always included *CDKN2A/CDKN2B* (Fig. 2A). Homozygous loss of *CDKN2A/CDKN2B* was observed in 10 of the 17 affected acute-type ATL samples but was never observed in chronic-type ATL. The genes whose expression was affected by copy number changes are considered candidate genes in the regions of genomic alterations (15, 22, 23). We therefore evaluated the expressions of CDKN2A and CDKN2B in acute-type and chronic-type ATL with or without loss of 9p (Fig. 2B). CDKN2A expression was much lower in acute-type ATL samples with the loss of 9p than in other samples. CDKN2B expression was not reduced in accordance with the loss of 9p. Therefore, CDKN2A is a likely candidate tumor suppressor gene located in 9p21.3.

Serial samples of a patient with chronic-type ATL showing acute transformation were analyzed in detail. The DNA and RNA samples of this patient at about 19 months before acute transformation (chronic phase, C-10) and at acute transformation (acute phase, A-15) were available. Clonality analysis of T-cell receptor gamma locus showed that clones of ATL cells at chronic and acute phases were identical to each other (Supplementary Fig. S1B). Although the chronic-phase sample showed heterozygous loss of *CDKN2A/CDKN2B*, the acute-phase sample showed homozygous loss of *CDKN2A/CDKN2B* (Fig. 2C). In addition, the expression of CDKN2A was remarkably reduced in the acute phase (Fig. 2D). Analysis of these serial samples of an identical patient also indicated that CDKN2A is the most likely candidate gene located in 9p21.3 and that the loss of *CDKN2A* is associated with acute transformation.

Frequently altered cell-cycle pathway in acute-type ATL

CDKN2A contains 2 known transcriptional variants, *INK4a* (*p16*) and *ARF* (*p14*). Both of these genes are known to be negative regulators of the cell cycle. We next evaluated the distributions of genomic alterations of *CDKN2A* with other genes that were previously reported to affect the cell cycle (Fig. 2E; ref. 24). Our analysis revealed that losses of *CDKN2A* and losses of *TP53* tended to be mutually exclusive events, and this pattern was also observed for losses of *TP53* and gains of *MDM4/RFWD2*. These alterations of cell-cycle–related genes were specifically observed in acute-type ATL compared with chronic-type ATL (80% of acute-type and 56% of chronic-type ATL, $P < 0.05$; Fig. 2F). Among chronic-type ATL cases, those with acute transformation tended to have alterations of cell-cycle–related genes (Fig. 2G). GSEA also revealed that the cell-cycle–related gene set and genes functionally associated with proliferation were significantly enriched in acute-type ATL compared with chronic-type ATL (Supplementary Fig. S1C).

These results indicated that alterations of the cell-cycle pathway, including the genomic loss of *CDKN2A*, played critical roles in the pathophysiology of acute-type ATL and acute transformation of chronic-type ATL. *In vitro* assays showed that inductions of INK4a or ARF that are encoded by *CDKN2A* caused suppression of cell proliferation, cell-cycle arrest, and apoptosis in ATL cell lines with genomic loss of 9p21.3 (Supplementary Fig. S2).

Genomic alterations of CD58 in ATL

In addition to loss of *CDKN2A/CDKN2B*, we found that losses of *CCDC7*, *ITGB1*, and *CD58* and gain of chromosome 3q were more frequently recognized in acute-type ATL than in chronic-type ATL. Alterations of cell-cycle–related genes, including *CDKN2A*, are considered important events for the transformation described above. We therefore analyzed the distributions of alterations of cell-cycle–related genes and the genes that were characteristic of acute-type ATL in each type of ATL case (Fig. 3). This analysis revealed that alterations of cell-cycle–related genes and the gene alterations characteristic of acute-type ATL mainly coexisted. A case having the loss of *CD58* or gain of 3q without alterations of cell cycle existed for each type of ATL, although all cases with losses of *ITGB1* and *CCDC7* showed the alterations of cell-cycle–related genes.

In chronic-type ATL cases without alterations of cell-cycle–related genes, a case with loss of *CD58* showed acute transformation later, although a case with gain of 3q did not exhibit the transformation without any therapy during 30 months after the diagnosis. *CD58* is a gene known to be involved in activation of natural killer (NK) cells and cytotoxic T cells (CTL; refs. 25, 26). Inactivation of *CD58* is reported to play an important role in the pathophysiology of diffuse large B-cell lymphoma (DLBCL) through the mechanism of escape from the immunosurveillance system (20). Recurrent mutation of *CD58* has also been observed recently in PTCLs (19). We therefore further analyzed *CD58* in ATL.

Analyses using aCGH revealed that 26% (9 of 35) of acute-type ATL and 7% (2 of 27) of chronic-type ATL had genomic loss of 1p13 (Figs. 1B and 4A). These losses always included *CD58* and one case showed genomic loss that only included

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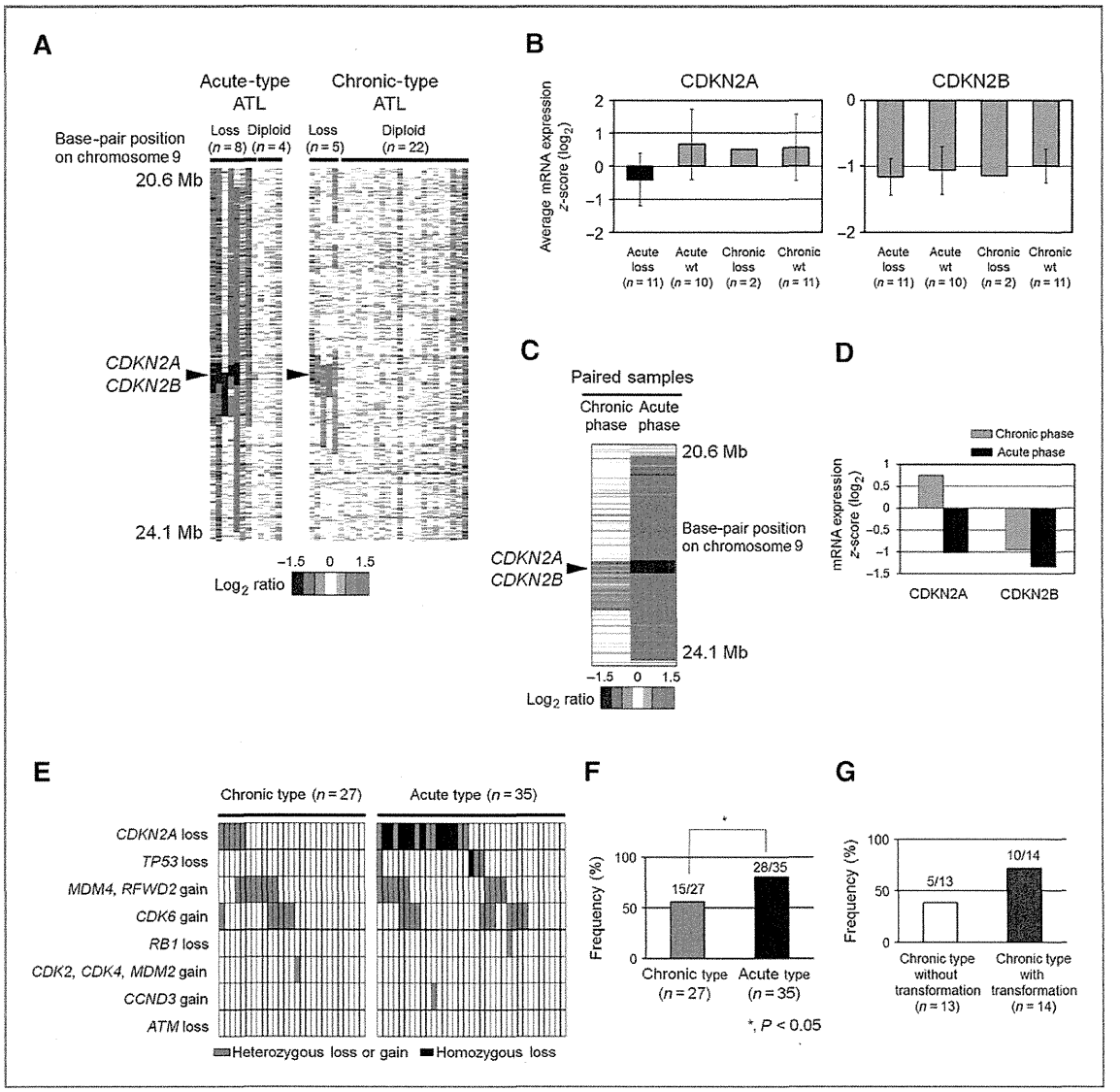

Figure 2. Loss of 9p was mainly observed in acute-type ATL and not chronic-type ATL. A, genomic alterations of chromosome 9p, including *CDKN2A/CDKN2B*. Heatmap analysis of 400K aCGH shows $\log_2$ ratios of tumor cells relative to normal controls. White, blue, and red represent diploid, loss, and gain, respectively. Arrowhead, the *CDKN2A/CDKN2B* locus. B, gene expression levels of CDKN2A and CDKN2B. Gene expression levels of CDKN2A and CDKN2B were analyzed in 13 chronic-type and 21 acute-type ATL cases by GEP. Average gene expressions and SDs are shown in cases grouped as indicated. CDKN2A expression was reduced only in acute-type ATL cases exhibiting loss of *CDKN2A/CDKN2B*. CDKN2B expression did not change in relation to genomic loss or subtype. Probes of A_23_P43484 (CDKN2A) and A_23_P216812 (CDKN2B) were used in experiments. C, genomic alteration of 9p in serial samples of a case with chronic type showing acute transformation. Left, a heatmap of the $\log_2$ ratio in the chronic phase; right, a heatmap of the ratio in the acute phase. The sample in the chronic phase indicates a heterozygous loss of the *CDKN2A/CDKN2B* locus and the loss changes to a homozygous loss for the sample in the acute phase. D, gene expressions of CDKN2A and CDKN2B in serial samples. CDKN2A expression was remarkably reduced in the acute phase, but CDKN2B expression was almost identical during transformation in this case. Gray, the chronic phase; black, the acute phase. E, alterations of cell-cycle–related genes in chronic-type and acute-type ATL. In the heatmap, rows correspond to the indicated alterations and columns represent individual ATL cases. Gray, a heterozygous loss or gain; black, a homozygous loss. Losses of *CDKN2A* and *TP53* tended to be mutually exclusive, and losses of *TP53* and gains of *MDM4/RFWD2* showed a similar tendency. F, alteration frequency of cell-cycle–related genes. Genetic alteration frequency of cell-cycle–related genes was significantly higher in acute-type ATL cases (80%) than in chronic-type ATL (56%; Fisher exact test; *, $P < 0.05$). The actual number of affected samples over the total number analyzed is shown at top of the figure. G, alteration frequency of cell-cycle–related genes among chronic-type ATL cases. The frequency of alterations of cell-cycle–related genes was higher in cases with later acute transformation than in cases without acute transformation.