Published OnlineFirst October 15, 2014; DOI: 10.1158/0008-5472.CAN-14-0643

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Cancer Res Published OnlineFirst October 15, 2014.

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

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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); 1–10. ©2014 AACR.*

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi: 10.1158/0008-5472.CAN-14-0643

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

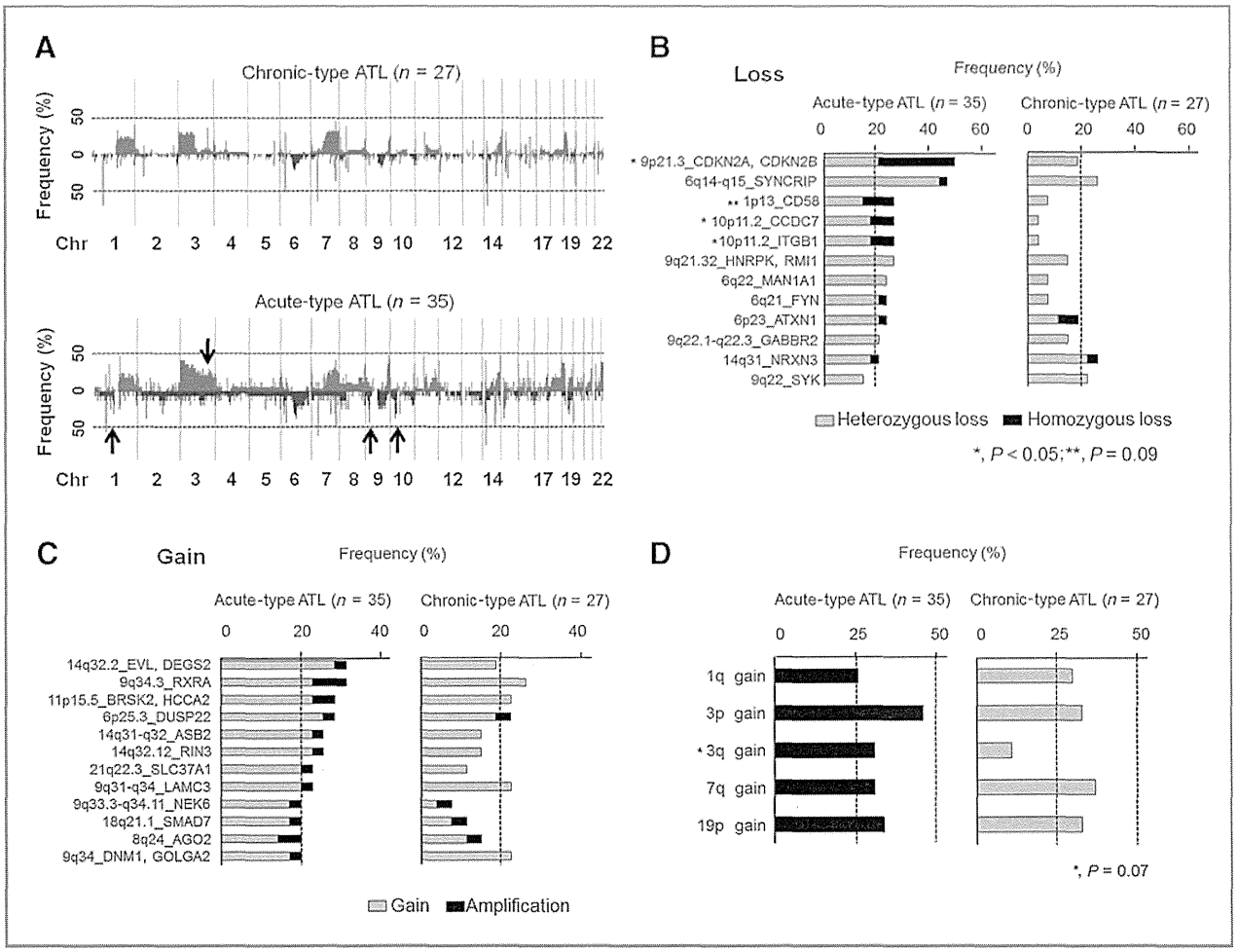

Figure 1. Genomic alteration profiles of chronic- and acute-type ATL. A, frequency of genomic alterations in chronic-type and acute-type ATL. Top, 27 cases with chronic-type ATL; bottom, 35 cases with acute-type ATL. The horizontal axis indicates each probe aligned from chromosome 1 to 22 and the short 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

Published OnlineFirst October 15, 2014; DOI: 10.1158/0008-5472.CAN-14-0643

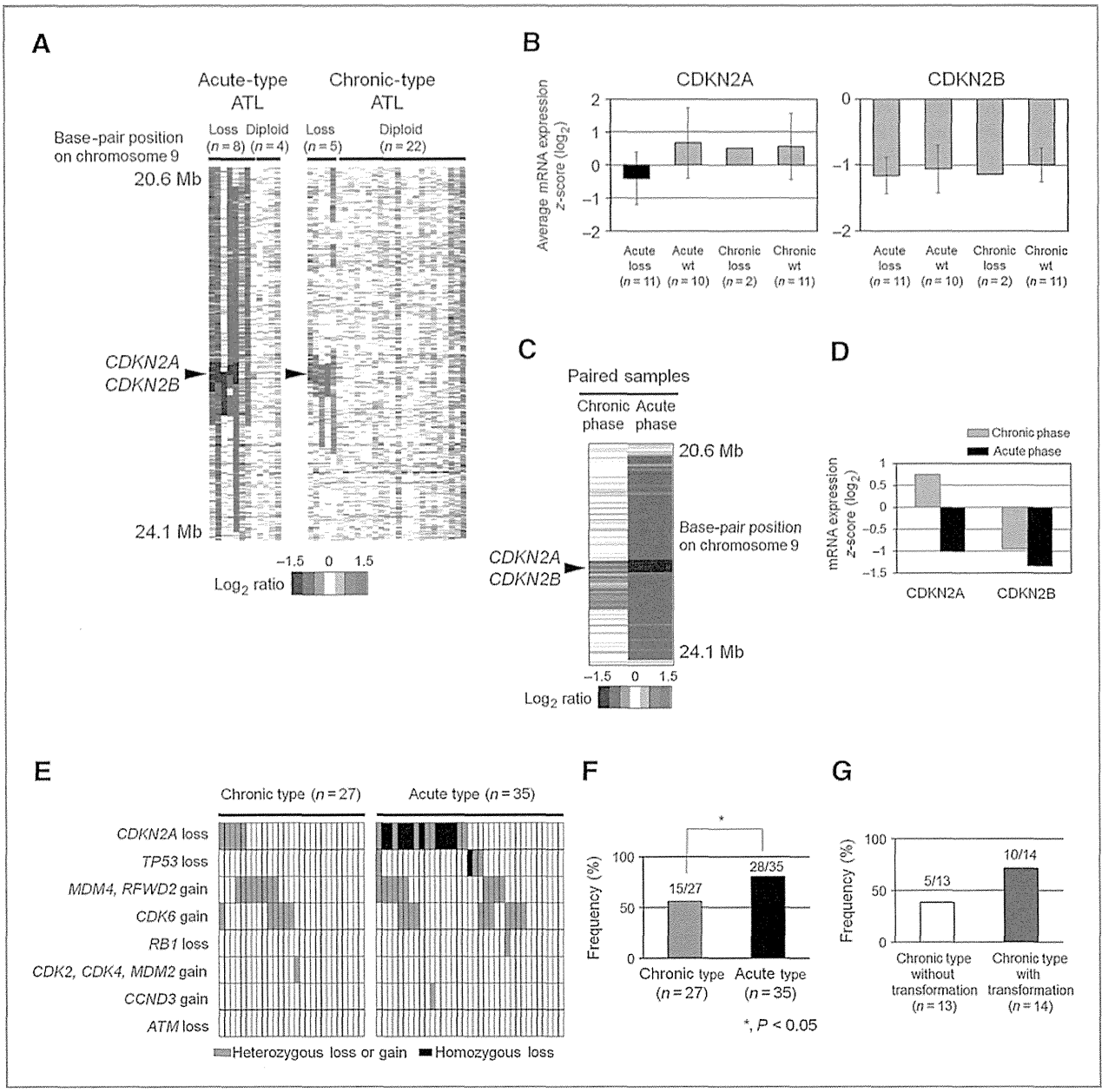

Figure 2. Loss of 9p was mainly observed in acute-type ATL and not chronic-type ATL. A, genomic alterations of chromosome 9p, including *CDKN2A/CDKN2B*. Heatmap analysis of 400K aCGH shows $\log_2$ ratios of tumor cells relative to normal controls. White, blue, and red represent diploid, loss, and gain, 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.

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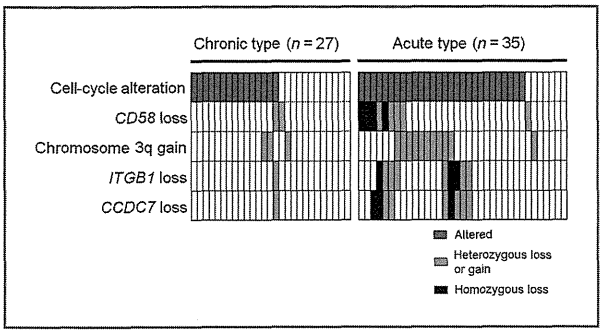

Figure 3. Distribution of genomic alterations frequently observed in acute-type ATL among ATL samples. Heatmap with rows corresponding to the indicated alterations and columns representing individual ATL cases. Gray, a heterozygous loss or gain; black, a homozygous loss. Dark gray also shows the alterations of any cell-cycle–related gene. Alterations frequently found in acute-type ATL were not mutually exclusive of the alteration of cell-cycle–related genes. Cases with losses of *ITGB1* and *CCDC7* always exhibited the alterations of cell-cycle–related genes. Most cases with loss of *CD58* or gain of 3q also exhibited the alterations of cell-cycle–related genes, but a case showing the loss of *CD58* or gain of 3q without disruption of the cell cycle existed in each type of ATL. The loss of *CD58* and gain of 3q were almost mutually exclusive, except for two cases of acute-type ATL.

CD58. Homozygous loss of *CD58* was observed only in acute-type ATL samples. Furthermore, expression of CD58 was reduced in acute-type ATL cases accompanied with the genomic loss (Fig. 4B). Flow cytometric analyses also suggested that genomic loss of *CD58* reduced the expression on the cell surfaces (Fig. 4C). Sequence analysis of *CD58* revealed a nonsense mutation in one acute-type ATL case. This mutation indicated that the 97th position of serine changed to a stop codon (p.S97X; c.290C>A; Fig. 4D). The nontumor cells of this patient showed no mutation, and we therefore regarded this mutation as a somatic mutation. One-nucleotide substitution registered as an SNP in the NCBI database (http://www.ncbi.nlm.nih.gov/gene/) was found in 7 cases (c.43A>G; rs17426456; Supplementary Table S2). Combined with the results of the genomic and mutation analyses, 29% of acute-type and 7% of chronic-type ATL had genetic alteration of *CD58*. These alterations were significantly specific to acute-type ATL compared with chronic-type ATL (Fig. 4E, $P = 0.05$).

In addition to the alteration of *CD58*, inactivation of *B2M* is also reported to play a pivotal role in the immune escape mechanism of DLBCLs (20). Among analyzed cases, only a chronic-type ATL case (C-2) had heterozygous loss of *B2M*, and this case also showed heterozygous loss of *CD58* (Supplementary Table S2). No somatic mutations of *B2M* were observed in ATL cases analyzed.

Genomic alterations predicting acute transformation of chronic-type ATL

We investigated the associations of MCRs that were characteristic of acute-type ATL and that were commonly found in more than 20% of chronic- and acute-type ATL with cumulative acute transformation rates among chronic-type ATL cases (Supplementary Table S3).

Cases exhibiting gain of *RXRA* and loss of *ITGB1*, *CCDC7*, or *CD58* were significantly associated with early progression to acute-type ATL ($P = 0.01$, 0.02, 0.02, and 0.04, respectively; Fig. 5A). Chronic-type ATL cases having the alterations of cell-cycle–related genes also tended to show early progressions to acute-type ATL ($P = 0.07$; Fig. 5B), although cases having only the loss of *CDKN2A* were not significantly associated with the progression (Supplementary Table S3). A chronic-type ATL case with losses of *ITGB1* and *CCDC7* had the alterations of cell-cycle–related genes, and we therefore analyzed the chronic-type ATL cases by the presence of alterations of *CD58* and/or cell-cycle–related genes. This analysis revealed that cases with these alterations were specifically associated with earlier progression to acute-type ATL ($P = 0.03$, Fig. 5C).

Discussion

We have studied 27 cases of chronic-type ATL and compared with 35 cases of acute-type ATL. Until now, only a few chronic-type ATL cases had been analyzed, and the molecular mechanisms of the transformation were investigated by focusing on the well-known tumor suppressor genes (*CDKN2A* and *TP53*; refs. 6–12). In contrast, our investigation comprehensively analyzed genomic profiles, and molecular aspects were analyzed using unbiased and whole-genome methods. Our study of chronic-type ATL represents the largest study to date that has analyzed the whole-genomic status of chronic-type ATL cases. We could identify characteristic molecular profile of chronic-type ATL and could demonstrate possible molecular mechanisms of acute transformation. This study suggested that alterations of cell-cycle–related genes and *CD58* are new predictive implications for chronic-type ATL (Fig. 5C).

Common genomic alterations in chronic- and acute-type ATL

Genomic alteration profiles of chronic- and acute-type ATL were found to be almost identical (Fig. 1). The number of genomic alterations was found to be higher in acute-type ATL than in the chronic-type, and the frequently altered regions of chronic-type ATL were also observed in the acute-type. Thus, chronic-type ATL might be a pre-acute form of the disease.

The common MCRs in chronic- and acute-type ATL included genes involving T-cell receptor signaling, such as *FYN* and *SYK* (27, 28). We also identified *SYNCRIP* as a common MCR in both types of ATL. *SYNCRIP* is a gene known to be involved in maturation of mRNA (29). *RXRA*, which has been reported to be implicated in colorectal carcinogenesis (30), is also frequently altered in both types of ATL. In addition, our analysis suggested that gain of *RXRA* is involved in acute transformation of chronic-type ATL because the chronic-type ATL possessing the gain of *RXRA* showed earlier progression to the acute-type. These MCRs may play important roles in the development of ATL coordinately with HTLV-1.

Deregulation of the cell-cycle pathway: an alteration related to acute transformation

Our analyses of genomic alterations revealed that no single genomic alteration seems to be responsible for the mechanism

Published OnlineFirst October 15, 2014; DOI: 10.1158/0008-5472.CAN-14-0643

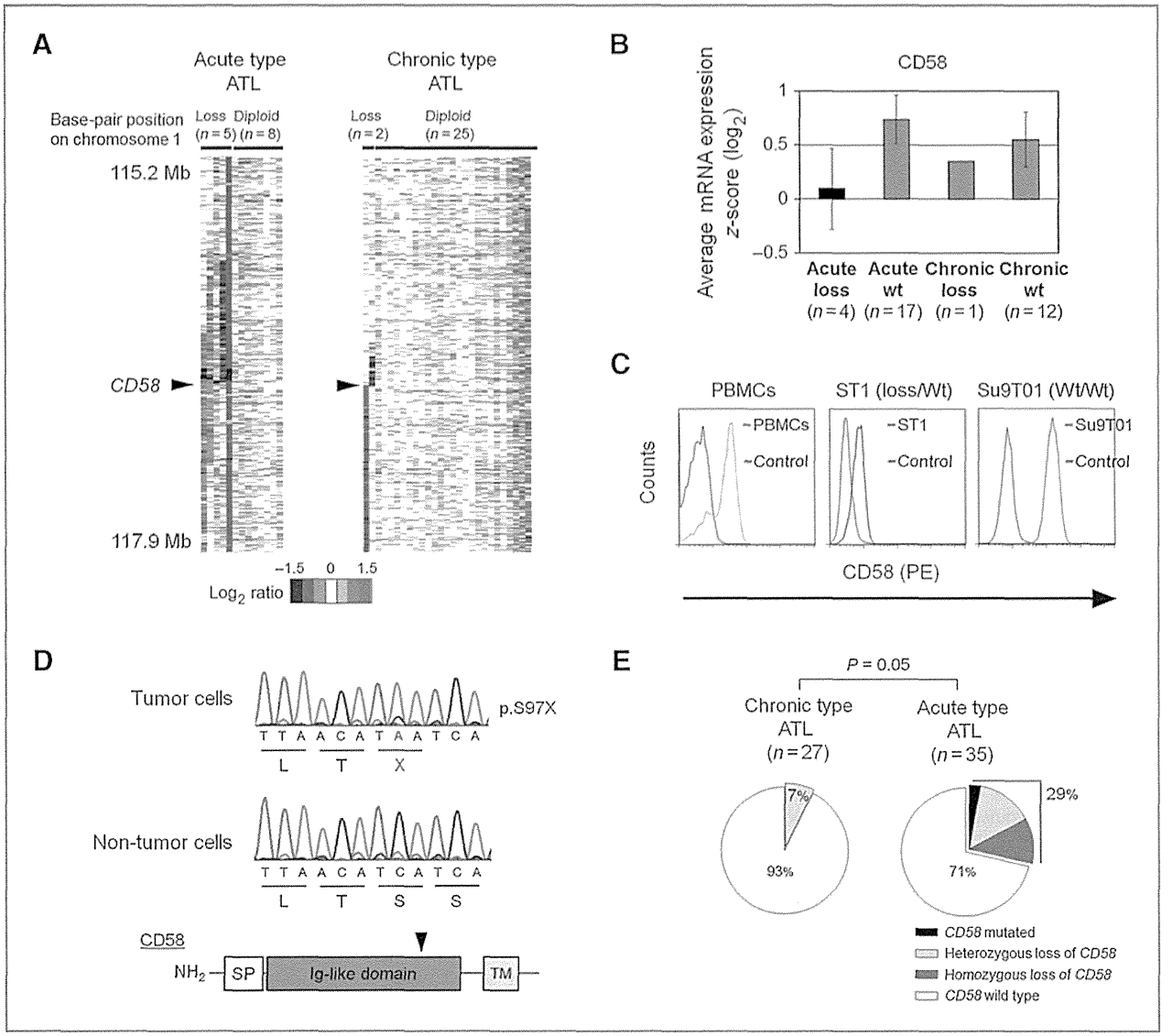

Figure 4. *Alteration of CD58 in acute transformation of chronic-type ATL. A, genomic alterations of chromosome 1p, including CD58. Heatmap analysis of 400K aCGH shows $\log_2$ ratios of ATL cases. White, blue, and red represent diploid, loss, and gain, respectively. Arrowhead, the CD58 locus. B, gene expression levels of CD58. Expression was 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. CD58 expression was reduced only in acute-type ATL cases exhibiting loss of CD58. Probe A_23_P138308 (CD58) was used in experiments. C, CD58 expressions on ATL cell lines and peripheral blood mononuclear cells (PBMC). Flow cytometric analysis of PBMCs from a healthy donor and two ATL cell lines for surface CD58 expression (orange line, PBMCs; blue line, ST1; red line, Su9T01). ST1 with heterozygous loss of CD58 had the low expression. The gray lines represent the cell lines with the isotype control antibody. D, DNA sequencing chromatogram of an acute-type ATL case (A-35) showing nonsense mutation in exon 2 of CD58 (top). DNA extracted from nontumor cells (CD4-negative cells in peripheral blood of this patient) did not show the mutation (middle). Bottom, a schematic representation of the CD58 protein depicting the location of the single peptide (SP), Ig-like domain, and transmembrane domain (TM). The inverted triangle indicates the position of the mutation. E, characterization of CD58 alteration in ATL. Seven percent of chronic-type ATL cases showed genomic loss of CD58, whereas 29% of acute-type ATL cases showed genomic alteration of CD58, with one case exhibiting mutation (Fisher exact test; $P = 0.05$).*

of acute transformation, and various genomic alterations and combinations of alterations exist in this mechanism (Fig. 3). We found that deregulation of the cell cycle, including genomic loss of *CDKN2A*, might be an important event in the transformation. Genomic loss of *CDKN2A* was also reported to play a crucial role in the transformation of chronic lymphocytic leukemia known as Richter syndrome (31, 32).

Although previous studies using Southern blot analysis revealed that 11% to 17% of acute-type ATL had the homozygous loss of *CDKN2A* (7, 9), our analyses using unbiased and whole-genome methods were able to reveal the frequency of the loss in greater detail. We found that approximately 30% of acute-type ATL cases showed a homozygous loss of the *CDKN2A/CDKN2B* locus, and 50% of acute-type ATL cases

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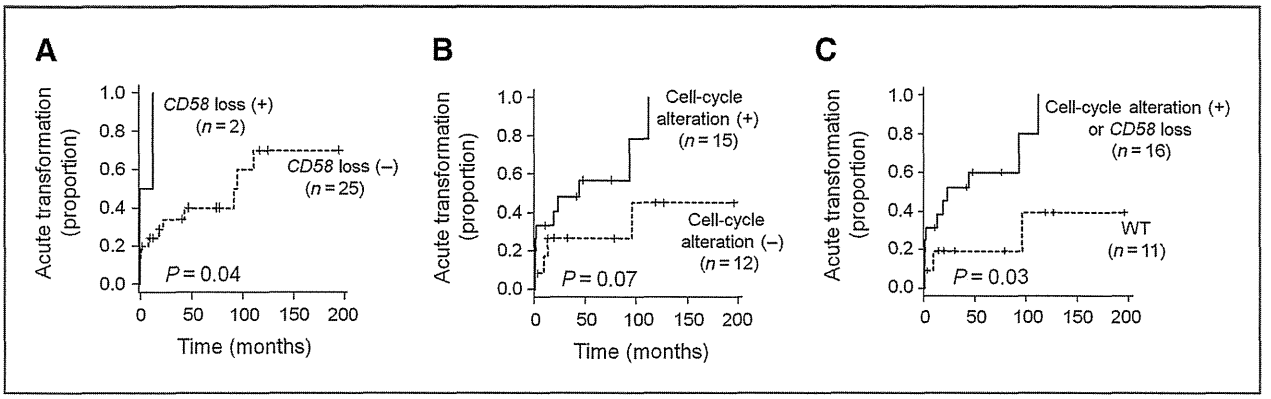

Figure 5. Genomic alterations associated with acute transformation in chronic-type ATL. A, genomic loss of *CD58* was significantly associated with earlier acute transformation ($P = 0.04$). B, chronic-type ATL cases with alterations of cell-cycle–related genes tended to exhibit earlier progression to acute-type ATL ($P = 0.07$). C, cases with either *CD58* loss or alterations of cell-cycle–related genes showed a much shorter time to acute transformation within chronic-type ATL cases ($P = 0.03$).

exhibited the homozygous or heterozygous loss of this locus. Yamagishi and colleagues used high-resolution aCGH analyses and found that this loss was frequently found in ATL samples (33). We also found that 5 of 27 chronic-type ATL cases had heterozygous loss of *CDKN2A*. Three of the 5 cases with *CDKN2A* loss progressed to the acute type, but 11 of the 22 cases without *CDKN2A* loss also showed acute transformation. Because of this finding, *CDKN2A* loss was not significantly associated with the earlier acute transformation in our study (Supplementary Table S3). Although previous studies revealed that approximately 5% of chronic-type had this loss (7, 9, 10), these previous studies did not show the cumulative acute transformation rate according to *CDKN2A* loss.

CDKN2A expression was reduced in acute-type ATL samples exhibiting genomic loss of the *CDKN2A* locus. A portion of acute-type ATL cases without the genomic loss showed a low expression level of CDKN2A, suggesting that methylation of the gene might affect the expression in these samples (11, 12). However, we consider that the genomic loss of *CDKN2A* has a greater influence on the expression of the gene than the methylation because the CDKN2A expression levels were remarkably reduced in accordance with the genomic loss (Fig. 2B and D).

Alterations of both *CDKN2A* and *TP53* were previously reported to be mutually exclusive (34), and our results showed the same trend. In addition, loss of *TP53* and gains of *MDM4*/*RFWD2* tended to be mutually exclusive in our acute-type ATL samples. Because these genes are involved in the TP53 pathway, our findings indicate that the TP53 pathway may also play a pivotal role in the pathophysiology of acute-type ATL. In fact, 80% of acute-type ATL had the alterations of cell-cycle–related genes, including *CDKN2A* and *TP53*. On the basis of this finding, we found that the alterations of cell-cycle–related genes might be predictive factors for acute transformation in chronic-type ATL cases (Fig. 5B).

Disruption of the immunosurveillance system in acute transformation of chronic-type ATL

The combined analyses of aCGH and sequencing revealed that 19% of ATL cases (7% of chronic-type and 29% of acute-type ATL) exhibited the *CD58* alteration. One acute-type ATL case showed somatic mutation, and the other cases showed genomic loss of the *CD58* locus. The alteration of *B2M* was a rare event in ATL compared with DLBCL (20). CD58 is a ligand of the CD2 receptor that is expressed on CTLs and NK cells and contributes to adhesion and activation of these cells. Previous reports showed that CTLs and NK cells could not recognize and injure target cells when treated with monoclonal CD58 antibody (35, 36). It is important to note that immune escape mechanism by CD58 inactivation was proven in DLBCL by Challa-Malladi and colleagues (20). The genomic loss and nonsense mutation of CD58 were for the first time demonstrated in ATL in this study and were suggested to be a predictive marker for acute transformation in chronic-type ATL. Therefore, the immune escape mechanism by the CD58 inactivation is likely to be involved in the pathophysiology of ATL as shown in DLBCL although detailed analysis is needed in the future.

Administration of immunosuppressive drugs to HTLV-1 carriers is currently considered a risk factor for early development of ATL (37, 38). It has been also suggested that immune escape from CTLs is induced by inactivation of the Tax protein derived from HTLV-1 in ATL (39–41). In addition, a report also suggested that immune escape from NK cells played an important role in ATL development (42). These findings suggest the presence of an immune escape mechanism in the pathophysiology of ATL. The present result regarding the significance of *CD58* alteration as a predictive factor for acute transformation in chronic-type ATL should be validated in more number of cases in the future study. Further studies are also needed regarding the protein expressions of CD58, B2M, and human leukocyte antigen class I.

In conclusion, our comparison of the molecular characteristics of chronic-type and acute-type ATL revealed that deregulation of the cell cycle and escape from the immune system are likely to be involved in acute transformation of chronic-type ATL. Development of ATL is thought to involve accumulation of several genomic alterations (43). The alterations of both pathways discovered in this study might be the late events following viral infection in the pathophysiology of ATL. These alterations could serve as biomarkers for patients with

Published OnlineFirst October 15, 2014; DOI: 10.1158/0008-5472.CAN-14-0643

chronic-type ATL. Furthermore, the presence of genomic alterations related to immune escape should be considered in the development of immunotherapeutic approaches for ATL.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Conception and design: N. Yoshida, A. Utsunomiya, K. Tsukasaki, A. Umino, M. Seto

Development of methodology: K. Karube

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): N. Yoshida, A. Utsunomiya, K. Arita, M. Suguro, S. Tsuzuki

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Utsunomiya, N. Uike, T. Kinoshita, M. Seto

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Acknowledgments

The outstanding technical assistance of Yumiko Kasugai, Seiko Sato, and Kyoko Hirano is very much appreciated. The authors thank Drs. Takashi Miyagi, Sivasundaram Karnan, Harumi Kato, Kiyoko Yamamoto, Fang Liu, Tatsuo Kakiuchi, and Taishi Takahara for their critical discussions and constructive suggestions. N. Yoshida also thanks Drs. Akira Sakai, Yuta Katayama, Hideki Asaoku, and Tatsuo Ichinohe for their encouragement throughout this study.

Grant Support

This work was supported in part by a grant-in-Aid from the Ministry of Health, Labor and Welfare of Japan, the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Japan Society for the Promotion of Science (N. Yoshida, K. Karube, S. Tsuzuki, and M. Seto), a grant-in-Aid for Cancer Research from the Ministry of Health, Labor and Welfare of Japan (M. Seto), and a grant-in-Aid from the Takeda Science Foundation (M. Seto).

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Received March 6, 2014; revised June 30, 2014; accepted July 22, 2014; published OnlineFirst October 15, 2014.

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Published OnlineFirst October 15, 2014; DOI: 10.1158/0008-5472.CAN-14-0643

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Cancer Science
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Heat shock protein 90 inhibitor NVP-AUY922 exerts potent activity against adult T-cell leukemia–lymphoma cells

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Key words
Adult T-cell leukemia–lymphoma, HSP90 inhibitors, NF-κB, NVP-AUY922, PIM kinases

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Funding Information
The Japan Society for the Promotion of Science.

Received May 1, 2014; Revised September 16, 2014; Accepted September 19, 2014

Cancer Sci **105** (2014) 1601–1608

doi: 10.1111/cas.12540

Adult T-cell leukemia–lymphoma (ATL), an aggressive neoplasm etiologically associated with HTLV-1, is a chemoresistant malignancy. Heat shock protein 90 (HSP90) is involved in folding and functions as a chaperone for multiple client proteins, many of which are important in tumorigenesis. In this study, we examined NVP-AUY922 (AUY922), a second generation isoxazole-based non-geldanamycin HSP90 inhibitor, and confirmed its effects on survival of ATL-related cell lines. Analysis using FACS revealed that AUY922 induced cell-cycle arrest and apoptosis; it also inhibited the growth of primary ATL cells, but not of normal PBMCs. AUY922 caused strong upregulation of HSP70, a surrogate marker of HSP90 inhibition, and a dose-dependent decrease in HSP90 client proteins associated with cell survival, proliferation, and cell cycle in the G_1 phase, including phospho-Akt, Akt, IKKα, IKKβ, IKKγ, Cdk4, Cdk6, and survivin. Interestingly, AUY922 induced downregulation of the proviral integration site for Moloney murine leukemia virus (PIM) in ATL cells. The PIM family (PIM-1, -2, -3) is made up of oncogenes that encode a serine/threonine protein kinase family. As PIM kinases have multiple functions involved in cell proliferation, survival, differentiation, apoptosis, and tumorigenesis, their downregulation could play an important role in AUY922-induced death of ATL cells. In fact, SGI-1776, a pan-PIM kinase inhibitor, successfully inhibited the growth of primary ATL cells as well as ATL-related cell lines. Our findings suggest that AUY922 is an effective therapeutic agent for ATL, and PIM kinases may be a novel therapeutic target.

Heat shock protein 90 is involved in folding and functions as a chaperone for multiple client proteins, many of which are important in tumorigenesis. In contrast to normal cells, tumor cells contain an abundance of catalytically active HSP90, which is found in multichaperone complexes. Therefore, HSP90 has emerged as a target of interest in cancer therapy.[1] Inhibition of HSP90 leads to misfolding of client proteins and degradation through the ubiquitin proteasome pathway. Heat shock protein 90 inhibitors target tumor cells on mutated or amplified oncoproteins, such as transmembrane tyrosine kinases (human epidermal growth factor receptor 2, epidermal growth factor receptor, c-Met, insulin-like growth factor 1 receptor), metastable signaling proteins (Akt, Raf-1, IKK), mutated signaling proteins (p53, Kit, Flt-3, v-Src), chimeric signaling proteins (nucleophosmin/anaplastic lymphoma kinase, BCR-ABL), steroid receptors (androgen, estrogen, progesterone receptors), and cell cycle regulators (CDK4, CDK6). The HSP90 inhibitor 17-AAG, derived from geldanamycin, has shown potent antitumor activity against ATL.[2,3] However, geldanamycin derivatives have several limitations, including poor solubility, formulation difficulties, and severe hepatotoxicity in clinical settings,[4–6] which have prompted development of next generation synthetic HSP90 inhibitors including NVP-AUY922 (AUY922), a second generation isoxazole-based non-geldanamycin HSP90 inhibitor that inhibits the ATPase activity of HSP90.[7,8] AUY922 has shown nanomolar efficacy against a wide range of human cancer cells *in vitro* and also inhibits progression of a variety of tumors *in vivo*.[7–11] Furthermore, in a phase I clinical trial of AUY922 in patients with advanced solid tumors, the agent showed acceptable tolerability.[12]

Adult T-cell leukemia–lymphoma is a chemoresistant malignancy with a CD4-positive T-lymphocyte origin etiologically associated with HTLV-1.[13] In ATL, activation of NF-κB, AP-1, and PI3K/Akt results in upregulation of expression of a large number of cellular genes involved in cell proliferation and survival.[14–16] Adult T-cell leukemia–lymphoma is generally classified into four clinical subtypes: acute, chronic, smoldering, and lymphoma. Although several approaches have been reported, combination chemotherapy is still the treatment of

choice for newly diagnosed aggressive ATL. Patients with aggressive ATL have a median survival time of 13 months, indicating limitations in present treatment strategies.[17] However, agents that interrupt a variety of signal transduction pathways such as HSP90 inhibitors are thought to be potential treatment options for the disease. In this study, we examined the effects of AUY922 on ATL cells *in vitro* and explored a novel therapeutic target by investigating its molecular mechanisms.

Materials and Methods

Cells and ATL-related cell lines. The ATL-derived cell lines KK1, KOB, SO4, ST1, and LM-Y1, were obtained from ATL patients and established in our laboratory.[18–21] KK1, KOB, SO4, and LM-Y1 were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated FBS and 0.5 U/mL interleukin-2 (kindly provided by Takeda Pharmaceutical Company, Ltd., Osaka, Japan). ST1 and HTLV-1-infected T-cell lines, MT2[22] and HuT102[23], were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated FBS. The KOB, LM-Y1, ST1, MT2, and HuT102 cell lines possess wild-type p53, whereas KK1 and SO4 have mutant-type p53.[24] Primary leukemia cells from patients with ATL were also used. The diagnosis of ATL was based on clinical features, hematological findings, and presence of anti-HTLV-1 antibodies in serum. Monoclonal HTLV-1 provirus integration in the DNA of leukemic cells was confirmed in patients using Southern blot hybridization (data not shown). Peripheral blood mononuclear cells from patients with ATL and a normal healthy donor were isolated by Ficoll–Paque density gradient centrifugation, and washed with PBS. For enrichment of ATL cells, CD4 T cells were negatively enriched using Miltenyi CD4 T-Cell Isolation Kit II (Miltenyi Biotec, Auburn, CA, USA). Each patient sample contained more than 90% leukemia cells at the time of analysis. After receiving approval from the Ethics Committee at Nagasaki University Hospital (Nagasaki, Japan), all patient samples were obtained with informed consent.

Chemicals and cell proliferation assay. AUY922 was kindly provided by Novartis Institutes for Biomedical Research (Basel, Switzerland). 17-AAG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and SGI-1776 (Santa Cruz Biotechnology) were obtained, and dissolved in DMSO. The effect of AUY922 on cell proliferation was examined using the cell viability agent provided in a CellTiter 96 AQueos Cell Proliferation Assay kit (Promega, Madison, WI, USA). Briefly, the cell lines (2–5 × 10^5/mL) and PBMCs (1 × 10^6/mL) were separately incubated in 96-well plates in the presence or absence of various concentrations of AUY922. After 72 h, the reagent was added and incubation was continued for 2–4 h, then absorbance at 492 nm was measured using an automated microplate reader. All experiments were carried out in triplicate. Error bars represent the standard error in each experiment. Non-parametric statistical analysis (Mann–Whitney *U*-test) was carried out using GraphPad Prism version 6.00 software (GraphPad

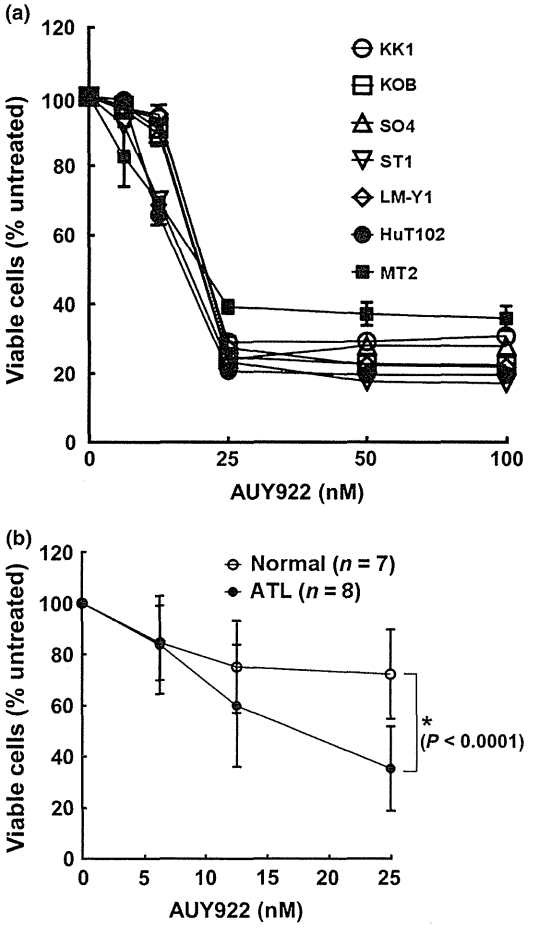

Fig. 1. Growth inhibition effects of heat shock protein 90 inhibitor AUY922. Inhibitory effects of AUY922 on cell survival of adult T-cell leukemia–lymphoma-related cell lines (a), and primary adult T-cell leukemia–lymphoma cells (n = 8) and normal PBMCs (n = 7) (b). Cells were incubated in the presence of various concentrations of AUY922 for 72 h and *in vitro* survival was determined using an MTS assay. A relative viability of 100% was designated as the total number of cells that survived after 72 h in the absence of AUY922. The relative viability of cultured cells was determined from triplicate cultures and is presented as the mean ± SD (bars). *P < 0.0001.

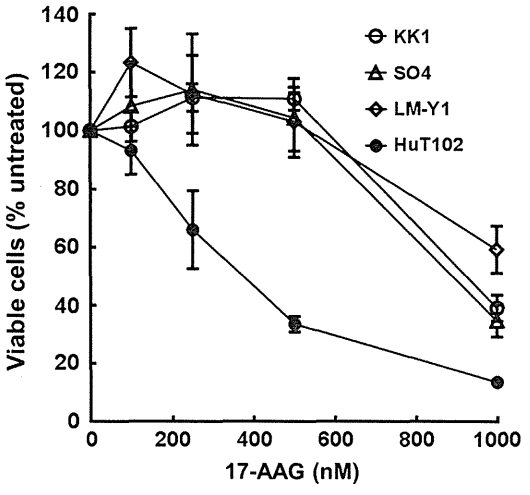

Fig. 2. Growth inhibition effects of heat shock protein 90 inhibitor 17-AAG. Inhibitory effects of 17-AAG on cell survival of adult T-cell leukemia–lymphoma-related cell lines. Cells were incubated in the presence of various concentrations of 17-AAG for 72 h and *in vitro* survival was determined using MTS assay. The relative viability of cultured cells is presented as the mean determined from triplicate cultures. A relative viability of 100% was determined based on the total number of cells that survived after 72 h in the absence of 17-AAG. The relative viability of cultured cells was determined from triplicate cultures and is presented as the mean ± SD (bars).

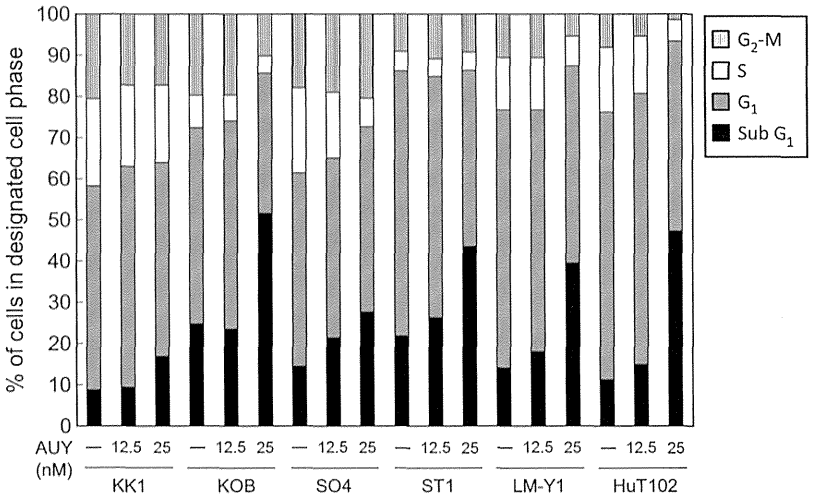

Fig. 3. Effects of heat shock protein 90 inhibitor AUY922 on cell cycle. Adult T-cell leukemia–lymphoma-related cell lines were incubated in the absence (–) or presence of AUY922 (12.5 or 25.0 nM) for 48 h and stained with propidium iodide, then DNA content was assayed using flow cytometry. The percentage of cells in various phases of the cell cycle was determined.

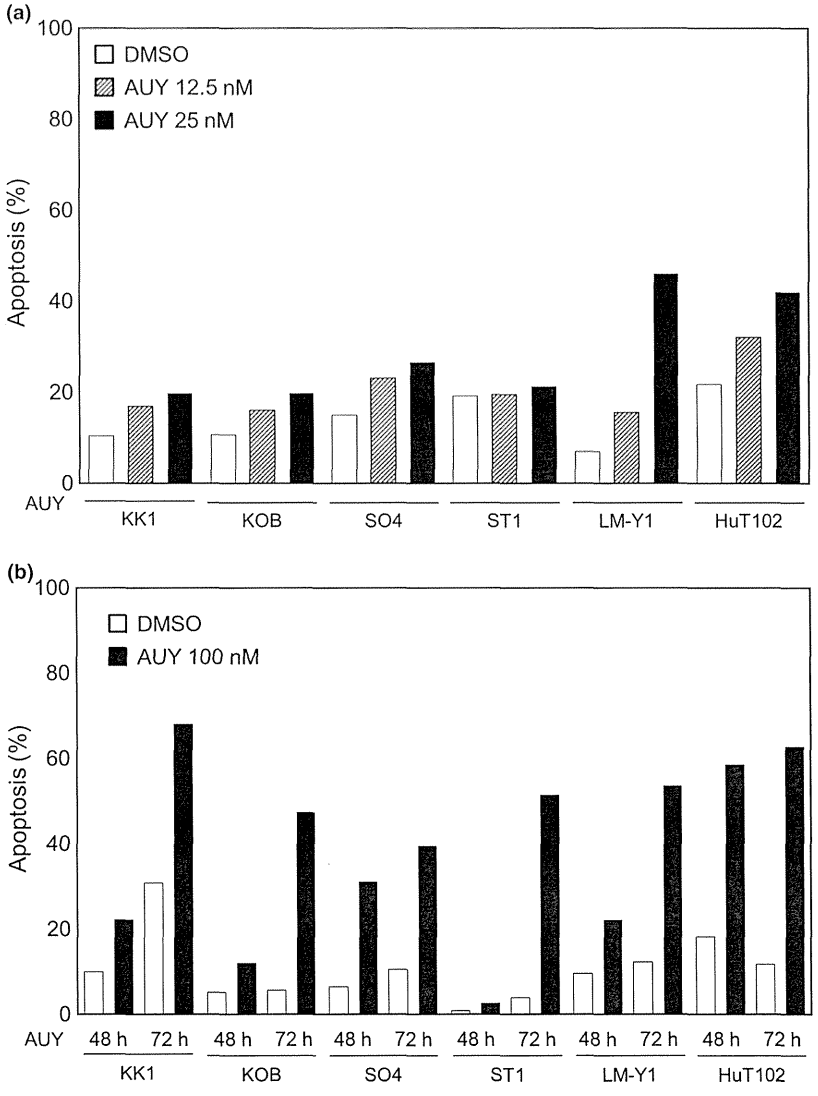

Fig. 4. Effects of heat shock protein 90 inhibitor AUY922 on apoptosis. Adult T-cell leukemia–lymphoma-related cell lines were treated with or without AUY922 (12.5 or 25.0 nM [a], or 100 nM [b]) for 48 h, then harvested, stained with annexin V–propidium iodide, and analyzed using flow cytometry. Data shown represent the percentages of apoptotic cells among untreated and AUY922-treated cells.

Software, San Diego, CA, USA). *P*-values <0.05 were regarded as significant.

Flow cytometric analysis (apoptosis assays and cell cycle analysis). To evaluate apoptotic changes, we used annexin V and a PI Kit (Bender Medsystems, Vienna, Austria). Cell cycle was analyzed using a Cycletest Plus DNA reagent kit (BD Biosciences, San Jose, CA, USA). In brief, 10^6 cells were washed with a buffer solution containing sodium citrate, sucrose, and

dimethyl sulfoxide suspended in a solution containing RNase A, and stained with 125 μg/mL PI for 10 min. All experiments were carried out using a FACSCanto II flow cytometer and FACSDiva software (BD Biosciences).

Western blot analysis and antibodies. Cells were harvested after treatment and washed, then homogenized at 4°C in lysis buffer (0.1% SDS, 1% Igepal CA-630, 0.5% sodium deoxycholate) and a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Cell lysates (20–50 μg) were resolved by electrophoresis on polyacrylamide gels and transferred to PVDF membranes. After blocking the membranes in 5% nonfat dry milk or 5% FBS and 0.1% Tween-20 in Tris-buffered saline for 1 h at room temperature, the blots were hybridized overnight at 4°C with primary antibodies. After hybridization with secondary antibodies conjugated with HRP, immunocomplexes were visualized using an enhanced chemiluminescence kit (GE Healthcare, Chalfont St. Giles, UK). Analyses were carried out with antibodies to HSP90, PIM-1 (Santa Cruz Biotechnology), HSP70, Cdk4, Cdk6, Akt, p-Akt, IκBα, IKKα, IKKβ, IKKγ, Bcl-2, survivin, PIM-2, PIM-3 (Cell Signaling Technology, Beverly, MA, USA), and β-actin (Sigma-Aldrich).

DNA microarray analysis. Gene expression profiling of ATL-related cell lines was examined. KK1, SO4, LM-Y1, and HuT102 cells with or without exposure to 100 nM AUY922 for 24 h were harvested. Total RNA was extracted using ISOGEN (Nippon Gene, Toyama, Japan) and purified with an RNeasy Mini Kit (Qiagen, Germantown, MD, USA), then total purified RNA was amplified with a one-color Low Input Quick Amp Labeling Kit (Agilent Technologies, Santa Clara, CA, USA). Cyanine 3–labeled fragmented cRNA was hybridized to a SurePrint G3 Human GE 8 × 60 K Microarray Kit (Agilent Technologies) covering 27 958 Entrez Gene RNAs. The microarrays were washed and scanned with a High-Resolution Microarray Scanner (Agilent Technologies). Data were processed using a quantile normalization method. Significant functions were calculated by Ingenuity Pathways Analysis (Ingenuity Systems, Redwood, CA, USA) with DAVID software (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA; available from http://david.abcc.ncifcrf.gov/) from a list of genes showed a 1.5-fold increase or decrease following treatment with AUY922.

Results

AUY922 inhibits growth of ATL-related cell lines and primary ATL cells. First, we analyzed the effects of AUY922 on proliferation of ATL-related cell lines. Incubation with AUY922 at various concentrations (0–100 nM) for 72 h inhibited cellular proliferation in a dose-dependent manner in a range from 0 to 25 nM, while a plateau was reached at concentrations >25 nM, as assessed by an MTS assay (Fig. 1a). The concentrations of AUY922 required to inhibit cellular proliferation of ATL-related cell lines by 50% (IC_{50}) varied from 12.5 to 25.0 nM. Importantly, AUY922 was effective regardless of the presence of wild-type or mutant p53. We also assessed AUY922-induced cellular inhibition of PBMCs obtained from both normal subjects and patients with ATL. Importantly, primary ATL cells were more susceptible to AUY922 than normal PBMCs, and the difference was statistically significant at 25 nM (Fig. 1b). Also, when compared directly with 17-AAG, AUY922 was between 20- and 50-fold more active at inhibiting growth of ATL-related cell lines (Fig. 2).

AUY922 induces sub-G_1/G_1 phase arrest of ATL-related cell lines. Next, we examined the effect of AUY922 on cell cycle progression in the tested cell lines. Cells were incubated with the control, AUY922 at 12.5 nM, or AUY922 at 25.0 nM for 48 h, then cell cycle distribution was analyzed using flow cytometry. Faint increases of G_1 and G_2–M cell populations were seen in KK1 and KOB, and SO4 cells, at 12.5 nM AUY922, respectively. In all of the tested cell lines, the sub-G_1 cell population increased in a dose-dependent manner, indicating apoptotic cell death (Fig. 3).

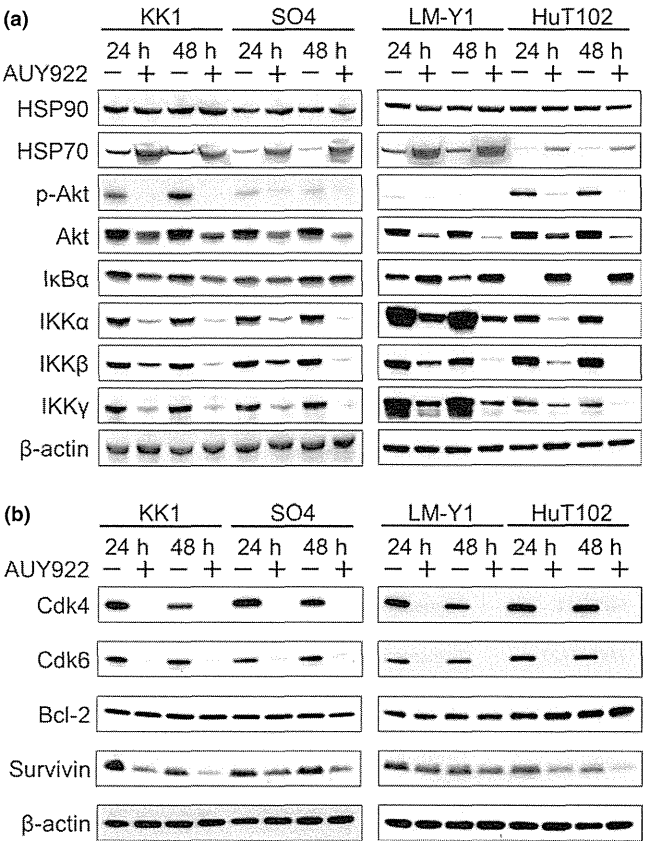

Fig. 5. Effects of heat shock protein (HSP) 90 inhibitor AUY922 on HSP90, HSP70, and HSP90 client proteins. Western blot analysis revealed that AUY922 treatment led to strong upregulation of HSP70, a surrogate marker of HSP90 inhibition. In addition, dose-dependent decreases in HSP90 client proteins associated with cell survival, proliferation, and cell cycle, including phospho-Akt (p-Akt), Akt, IκB kinase (IKK)α, IKKβ, IKKγ (a), and Cdk4, Cdk6, and survivin (b), were seen.

AUY922 induces apoptosis of ATL-related cell lines. To examine whether induction of apoptosis accounted for the inhibition of proliferation observed in ATL-related cell lines, cells were treated with the control, 12.5 nM AUY922, or 25.0 nM AUY922 for 48 h, or 100 nM AUY922 for 48–72 h, then examined using the annexin V–PI method. Annexin V binds to cells that express phosphatidylserine on the outer layer of the cell membrane, a characteristic finding in those entering apoptosis. AUY922 increased the proportion of cells positive for annexin V in all cell lines in a dose-dependent manner (Fig. 4a). Moreover, 100 nM AUY922 increased the proportion of cells positive for annexin V in all cell lines in a time-dependent manner (Fig. 4b). We carried out additional apoptosis assays using the non-HTLV-1 related T-cell lines Jurkat and Molt4. Those results were similar to the results obtained with ATL-related cell lines (Fig. S1).

AUY922 affects induction of HSP70 and depletion of oncogenic proteins through inhibition of HSP90 activity. To verify the

Table 1. Microarray analysis of adult T-cell leukemia–lymphoma-related cell lines treated with heat shock protein 90 (HSP90) inhibitor AUY922

		Fold change (log2 ratio)				
Gene symbol	Gene	KK1	SO4	LM-Y1	HuT102	Average
I. Genes upregulated in AUY922-treated ATL-related cell lines						
LGR4	Leucine-rich repeat containing G protein-coupled receptor 4	4.72	5.51	4.81	2.93	4.5
CLU	Clusterin	3.03	4.75	2.67	3.84	3.6
HSPA1B	Heat shock 70 kDa protein 1B	2.58	4.05	2.31	4.14	3.3
RGS2	Regulator of G-protein signaling 2	2.41	1.97	2.78	5.86	3.3
PDZK1	PDZ domain containing 1	3.34	4.70	2.06	1.22	2.8
MXD4	MAX dimerization protein 4	2.91	3.20	2.06	2.91	2.8
HSP90AA1	Heat shock protein 90 kDa alpha, class A member 1	2.90	3.04	1.86	2.59	2.6
BAG3	BCL2-associated athanogene 3	1.04	1.57	2.75	3.29	2.2
NQO1	NAD(P)H dehydrogenase, quinone 1	2.10	2.42	1.15	2.80	2.1
CREBBP	CREB binding protein	2.85	2.09	1.20	1.87	2.0
DEDD2	Death effector domain containing 2	1.67	1.81	1.65	2.78	2.0
BST2	Bone marrow stromal cell antigen 2	2.09	2.28	1.64	1.76	1.9
SPHK2	Sphingosine kinase 2	2.46	2.63	1.44	1.02	1.9
HSPD1	Heat shock 60 kDa protein 1	1.98	2.08	1.30	1.56	1.7
HDAC4	Histone deacetylase 4	2.00	2.13	1.19	1.48	1.7
CEBPA	CCAAT/enhancer binding protein, alpha	2.38	1.74	1.47	1.09	1.7
SAP30BP	SAP30 binding protein	2.09	2.08	1.00	1.45	1.7
SQSTM1	Sequestosome 1	1.59	1.21	1.56	2.10	1.6
B9D2	B9 protein domain 2	2.16	1.87	1.00	1.37	1.6
TMEM127	Transmembrane protein 127	1.78	1.76	1.24	1.56	1.6
CLN3	Ceroid-lipofuscinosis, neuronal 3	1.66	1.89	1.37	1.35	1.6
II. Genes downregulated in AUY922-treated ATL-related cell lines						
CCL3L3	Chemokine (C-C motif) ligand 3-like 3	−4.16	−4.35	−7.43	−4.41	−5.1
OSM	Oncostatin M	−2.72	−3.16	−4.38	−3.21	−3.4
PIM1	Pim-1 oncogene	−3.72	−4.15	−1.95	−1.53	−2.8
CYP1A1	Cytochrome P450, family 1, subfamily A, polypeptide 1	−2.35	−5.03	−1.02	−2.17	−2.6
IL13	Interleukin 13	−2.02	−3.06	−2.71	−2.72	−2.6
PLAUR	Plasminogen activator, urokinase receptor	−2.73	−2.06	−2.18	−2.95	−2.5
VEGFA	Vascular endothelial growth factor A	−2.29	−2.78	−2.63	−1.60	−2.3
CAMK1D	Calcium/calmodulin-dependent protein kinase ID	−2.32	−2.70	−2.69	−1.34	−2.3
ADAMTSL4	ADAMTS-like 4	−2.73	−2.75	−2.17	−1.14	−2.2
HBEGF	Heparin-binding EGF-like growth factor	−2.00	−2.95	−1.98	−1.84	−2.2
DMC1	DMC1 dosage suppressor of mck1 homolog, meiosis-specific homologous recombination (yeast)	−2.09	−1.22	−1.81	−3.54	−2.2
PTPN6	Protein tyrosine phosphatase, non-receptor type 6	−2.65	−2.89	−1.06	−1.96	−2.1
CEBPB	CCAAT/enhancer binding protein (C/EBP), beta	−2.48	−3.16	−1.30	−1.52	−2.1
LIF	Homo sapiens leukemia inhibitory factor	−1.39	−1.22	−3.94	−1.75	−2.1
KLF11	Kruppel-like factor 11	−2.12	−2.21	−2.23	−1.60	−2.0
TERT	Telomerase reverse transcriptase	−1.29	−2.04	−2.72	−2.09	−2.0
TNFRSF12A	Tumor necrosis factor receptor superfamily, member 12A	−2.38	−2.41	−2.30	−1.01	−2.0
TRIB3	Tribbles homolog 3 (Drosophila)	−2.41	−2.94	−1.40	−1.19	−2.0
BIRC3	Baculoviral IAP repeat containing 3	−1.97	−2.01	−1.37	−2.54	−2.0
BNIP3L	BCL2/adenovirus E1B 19 kDa interacting protein 3-like	−1.82	−2.65	−1.20	−2.07	−1.9
CAMK2B	Calcium/calmodulin-dependent protein kinase II beta	−1.36	−1.25	−3.19	−1.80	−1.9
TNFAIP3	Tumor necrosis factor, α-induced protein 3	−1.66	−2.19	−1.33	−2.15	−1.8
TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10	−1.65	−1.53	−1.73	−2.34	−1.8
CDKN2D	Cyclin-dependent kinase inhibitor 2D (p19, inhibits CDK4)	−2.29	−1.50	−1.62	−1.80	−1.8
ANG	Angiogenin, ribonuclease, RNase A family, 5	−2.48	−2.05	−1.33	−1.09	−1.7
PIM3	Pim-3 oncogene	−1.98	−1.71	−1.66	−1.58	−1.7
DTL	Denticleless homolog (Drosophila)	−1.82	−1.25	−1.72	−2.11	−1.7
IL2RA	Interleukin 2 receptor, alpha	−1.67	−2.10	−1.42	−1.62	−1.7
MXI1	MAX interactor 1	−1.14	−1.42	−1.54	−2.71	−1.7
NEK6	NIMA (never in mitosis gene a)-related kinase 6	−1.44	−2.19	−1.39	−1.56	−1.6
E2F7	E2F transcription factor 7	−2.22	−1.38	−1.61	−1.33	−1.6
PRIM1	Primase, DNA, polypeptide 1	−1.90	−1.18	−1.37	−1.90	−1.6
SEPT8	Septin 8	−1.43	−1.02	−1.77	−2.06	−1.6
KLF10	Kruppel-like factor 10	−1.92	−1.23	−1.76	−1.20	−1.5

To determine which molecules play important roles in AUY922-induced ATL-cell death, gene expression profiling was carried out using DNA microarray analysis. Among genes with changes in average expression of at least 1.5-fold (log2 ratio) in either direction in the four tested cell lines, we selected those with known functions related to apoptosis, cell cycle, and cell proliferation. The results showed upregulation of HSP70 in those, which was consistent with the results of our Western blot analysis. We also noted upregulation of HSP90, although the protein level of HSP90 was not changed. Interestingly, decreases in two of the Moloney murine leukemia virus (PIM) kinases, PIM-1 and PIM-3, were commonly found.

molecular mechanisms of the effects of AUY922 on survival and apoptosis of ATL-related cell lines, we examined the expressions of HSP90, HSP70, and several intracellular regulators of cell proliferation, cell cycle, and apoptosis, including p-Akt, Akt, IκBα, IKKα, IKKβ, IKKγ, Cdk4, Cdk6, Bcl-2, and survivin. AUY922 treatment led to induction of HSP70, a surrogate marker for inhibition of HSP90 function, but did not influence the protein level of HSP90 itself. HSP90 and its co-chaperones modulate tumor cell apoptosis, and much of their activity seems to be mediated through effects on the PI3K/Akt pathway and NF-κB function. Suppression of HSP90 function by AUY922 decreases the level of Akt, resulting in a reduction of activated p-Akt. The IKK complex, composed of IKKα, IKKβ, and IKKγ, is a positive regulator of NF-κB. In general, a decrease in the IKK complex inhibits phosphorylation of IκBα, resulting in its increased level. In the present study, AUY922 treatment decreased expression of the IKK complex in all tested cell lines. Among the apoptosis-related proteins examined, we found a decrease in survivin. Overall, we found similar changes in HSP90 client proteins regardless of the presence of wild-type or mutant p53 (Fig. 5).

Downregulation of PIM kinases in ATL-related cell lines treated by AUY922. To determine which molecules play important roles in AUY922-induced ATL-cell death, gene expression profiling was carried out using DNA microarray analysis. Among genes with changes in average expression of at least 1.5-fold (log2 ratio) in either direction in the four tested cell lines, we selected those with known functions related to apoptosis, cell cycle, and cell proliferation. Our results showed upregulation of HSP70 in those cells, which was consistent with the results of our WB analysis, and we also noted upregulation of HSP90, although the protein level of HSP90 was not changed. Interestingly, decreases in two of the PIM kinases, PIM-1 and -3, were commonly found (Table 1). PIM has multiple cellular functions related to cell survival, proliferation, differentiation, apoptosis, and tumorigenesis, and its expression is also correlated with poor prognosis in most hematopoietic malignancies, although its role in ATL remains unclear. Therefore, to investigate this, we examined the protein expression levels of PIM kinases using WB in ATL-related cell lines treated by AUY922. Although the protein levels of PIM kinases varied in each of the cell lines when untreated, the protein expression levels of PIM-1, -2, and -3 were universally decreased in all treated cell lines (Fig. 6).

SGI-1776 inhibits cell proliferation by blocking PIM kinases. To confirm the importance of PIM kinases in ATL cells, we evaluated the inhibitory effect of SGI-1776 on those, as well as proliferation of ATL-related cell lines and primary ATL cells.

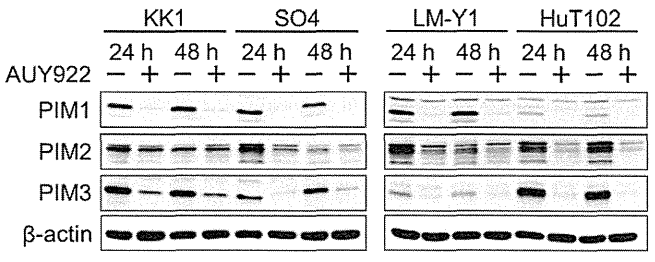

Fig. 6. Effects of heat shock protein 90 inhibitor AUY922 on Moloney murine leukemia virus (PIM) kinases in adult T-cell leukemia–lymphoma. Western blot analysis revealed that AUY922 induced downregulation of PIM-1, -2, and -3 in adult T-cell leukemia–lymphoma-related cell lines.

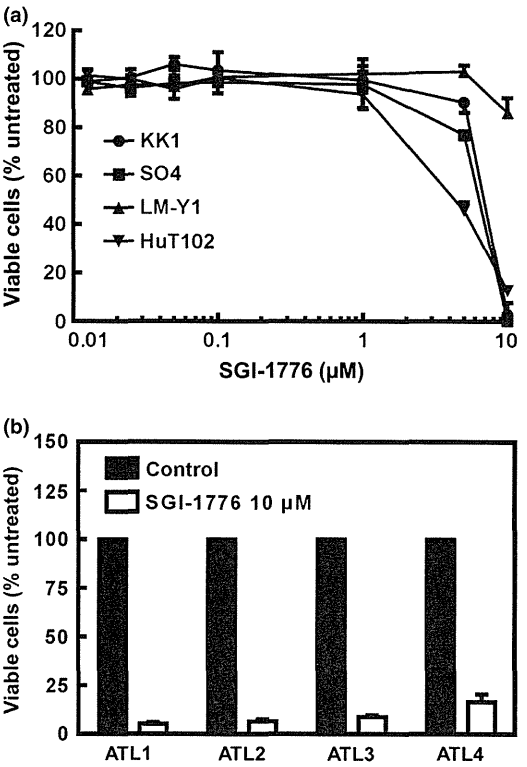

Fig. 7. Growth inhibitory effects of SGI-1776 in adult T-cell leukemia–lymphoma. SGI-1776, a pan-PIM kinase inhibitor, inhibited cellular survival suppression in adult T-cell leukemia–lymphoma-related cell lines in both dose- and cell-dependent manners (a). Furthermore, SGI-1776 inhibited cellular survival in primary adult T-cell leukemia–lymphoma cells (b).

When ATL-related cell lines were cultured with various concentrations (0–10 μM) of SGI-1776 for 72 h, cellular proliferation was inhibited in both dose- and cell-dependent manners (Fig. 7a). In primary ATL cells, SGI-1776 at 10 μM inhibited cellular proliferation (Fig. 7b). Together, these results suggest that PIM kinases may be a novel therapeutic target for treatment of ATL.

Discussion

In cancer cells, HSP90 client proteins play a major role in multiple oncogenic processes, such as cell proliferation and anti-apoptosis. HSP90 inhibitors are promising therapeutic agents for variable cancer, and phase I/II studies of AUY922 with advanced solid tumors and hematological malignancies are underway.[25]

We observed that AUY922 has very high cytotoxicity toward ATL-related cell lines and primary ATL cells. We also found that the inhibitory effect of AUY922 was superior to that of 17-AAG and 17-DMAG,[2,3] and our results confirmed previous reports noting that AUY922 showed potent cell inhibition in a low nanomolar range.[7–9] Moreover, we also showed that ATL-related cell lines and primary ATL cells were more susceptible to inhibition of proliferation by treatment with AUY922 than normal PBMCs. The difference between normal and cancer cells in regard to ATP-binding affinity with HSP90 likely contributed to this selectivity of effect.[26]

We found that the inhibitory effect of AUY922 on ATL cells was due to the induction of cell cycle arrest and apoptosis. Our results showed that AUY922 induced G_1 arrest due to decreased protein levels of CDK4 and CDK6, which have been identified as HSP90 client proteins that are important for cell cycle G_1 phase progression.[27] Survivin has also been identified as an HSP90 client protein[27] and reported to be overexpressed in ATL cells.[28] Our findings showed that AUY922 induced apoptosis associated with reduction of survivin in ATL-related cell lines. In addition, treatment with AUY922 decreased the IKK complex proteins (IKKα, IKKβ, and IKKγ). HSP90 is a regulator of NF-κB signaling through IKK activation and a reduction in the IKK complex inhibits IκBα phosphorylation followed by a reduction in NF-κB activity.[29] Among apoptosis-related proteins, we found a decrease in survivin and no change in Bcl-2, known as an NF-κB target, following treatment with AUY922. These findings suggest that typical Bcl-2 family members are not involved in AUY922-induced apoptosis. Furthermore, NF-κB activity may contribute to induction of cell cycle arrest and apoptosis of ATL-related cell lines.

AUY922 also induced Akt degradation, which resulted in a reduction of p-Akt. It has been reported that PI3K/Akt plays a role in activation of pro-survival pathways in HTLV-1-infected T-cell lines and primary ATL cells.[16,30–32] In those studies, Akt was shown to be a molecular target in ATL, and it has also been identified as an HSP90 client protein and shown to be sensitive to HSP90 inhibitors.[33,34]

Although the relationship between the p53 mutation and chemosensitivity in ATL remains unknown, Tawara *et al.* and Nishimura *et al.*[35,36] noted a tendency for the median survival periods of patients with the p53 mutation and/or loss of heterozygosity of that region to be shorter as compared to patients without a p53 aberration. Importantly, we found that AUY922 had effects on ATL-related cell lines irrespective of their p53 status.

Based on the present DNA microarray results, we focused on the role of PIM kinases in ATL and are the first to present those results. *PIM* is an oncogene encoding a serine/threonine protein kinase family comprised of PIM-1, -2, and -3; PIM kinases have multiple functions involved in cell proliferation, survival, differentiation, apoptosis, and tumorigenesis.[37,38] Elevated levels of PIM-1 and PIM-2 have been mostly found in hematologic malignancies and prostate cancer, and increased PIM-3 expression has been observed in solid tumors.[39,40] In addition, PIM expression is correlated with poor prognosis in some hematopoietic malignancies.[41–44] Our results indicated an anti-ATL activity of AUY922, which was mediated by degradation of PIM kinases. Those kinases are induced by activation of transcriptional factors downstream of growth factor signaling pathways, such as the Janus kinase and signal transducer and activator of transcription (JAK-STAT) and NF-κB pathways. Therefore, it is possible that the decrease in PIM kinases induced by AUY922 was due to a reduction in NF-κB activity.[45] The present results are the first to show an inhibitory effect of SGI-1776 on ATL-related cell lines and primary ATL cells. We concluded that PIM kinases are partly responsible for cell survival in ATL.

SGI-1776 has been shown to induce apoptosis in cells related to human acute myeloid leukemia and chronic lymphocytic leukemia.[46,47] Although a phase 1 clinical trial of SGI-1776 in patients with castration-resistant prostate cancer and refractory non-Hodgkin's lymphoma was started, evaluation of this compound was halted due to cardiac toxicity.[48] Our findings suggest that PIM kinases are a novel therapeutic target for treatment of ATL, indicating that a new generation of PIM kinase inhibitors with reduced toxicity in clinical settings is needed.

Heat shock protein 90 mediates protection of PIM kinases from proteosome degradation and PIM-1 was previously reported to be an HSP90 client protein.[49] However, it is not known whether PIM-2 and -3 are also such client proteins. In our WB analysis of SGI1776, even though it was not determined whether PIM-2 and/or -3 directly interact with HSP90, the results suggest that they are HSP90 client proteins in ATL.

In summary, our findings show that AUY922 may be potentially useful as a chemotherapeutic agent and PIM kinases a novel therapeutic target for treatment of ATL.

Acknowledgments

The authors would like to thank Novartis for providing NVP-AUY922. This study was supported in part by a Grant-in-aid for Scientific Research (23590641) from the Japan Society for the Promotion of Science.

Disclosure Statement

The authors have no conflicts of interest.

Abbreviations

AP-1	activator protein-1
ATL	adult T-cell leukemia–lymphoma
HSP90	heat shock protein 90
IKK	IκB kinase
NF-κB	nuclear factor-κB
p-Akt	phospho-Akt
PI	propidium iodide
PI3K	phosphatidylinositol 3-kinase
PIM	proviral integration site for moloney murine leukemia virus
WB	Western blot

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Effects of heat shock protein 90 inhibitor AUY922 on apoptosis in non-HTLV-1 related T-cell lines. The non-HTLV-1 related T-cell lines Jurkat and Molt4 were treated with or without 100 nM AUY922 for 48 or 72 h, then harvested, stained, with annexin V–propidium iodide, and analyzed using flow cytometry. Data shown represent the percentages of apoptotic cells among untreated and AUY922-treated cells.