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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

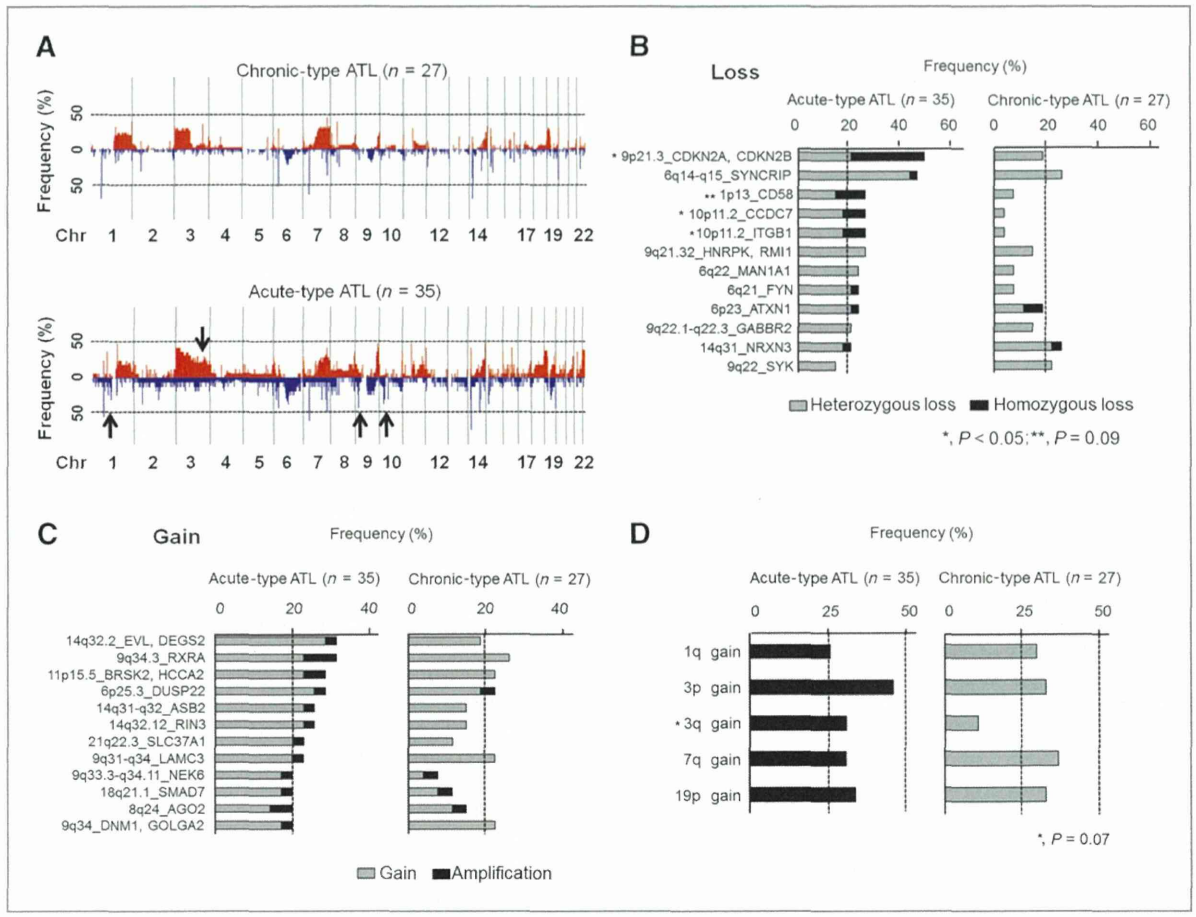

Figure 1. Genomic alteration profiles of chronic- and acute-type ATL. A, frequency of genomic alterations in chronic-type and acute-type ATL. Top, 27 cases with chronic-type ATL; bottom, 35 cases with acute-type ATL. The horizontal axis indicates each probe aligned from chromosome 1 to 22 and the short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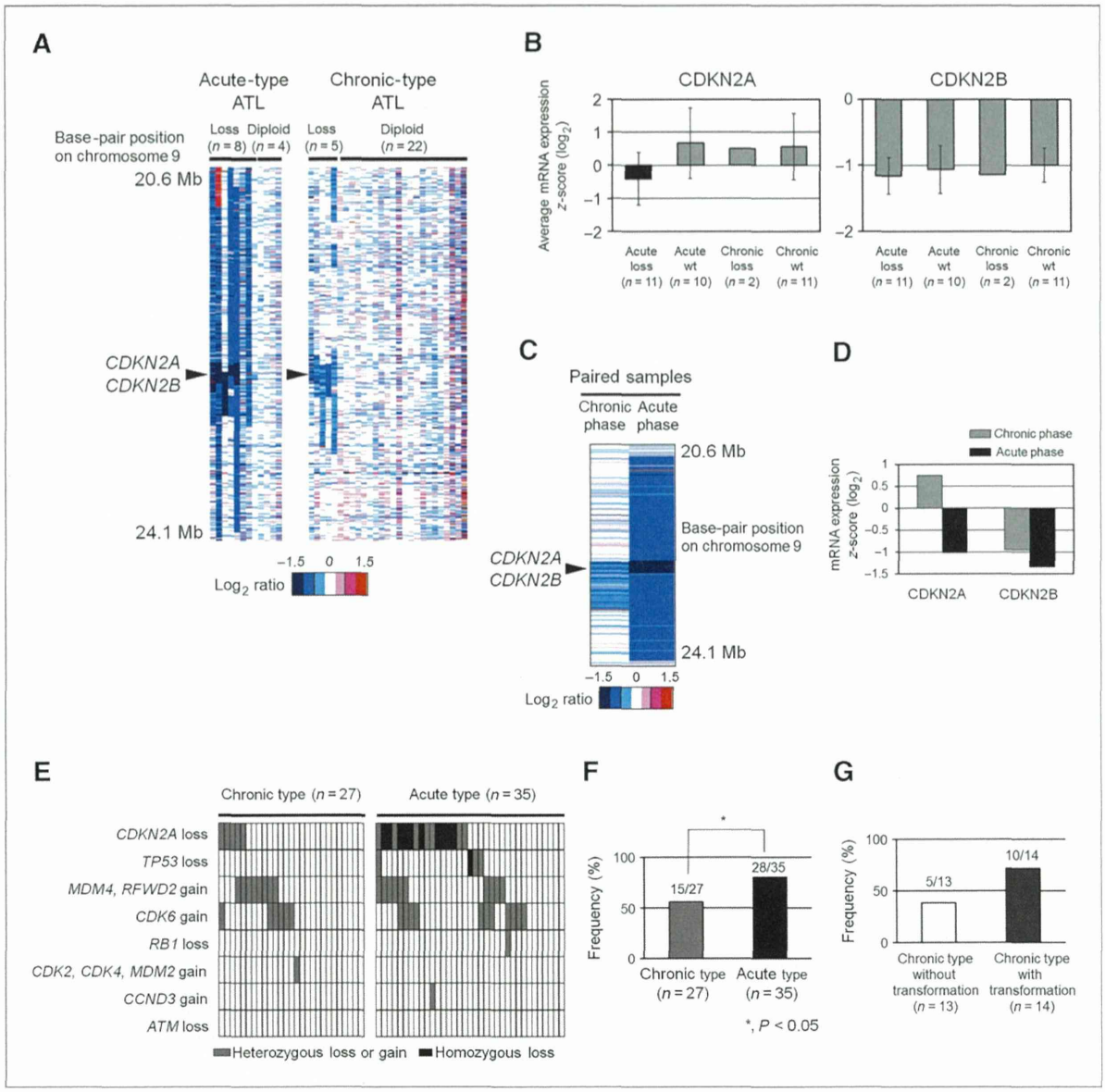

Figure 2. Loss of 9p was mainly observed in acute-type ATL and not chronic-type ATL. A, genomic alterations of chromosome 9p, including *CDKN2A/CDKN2B*. Heatmap analysis of 400K aCGH shows $\log_2$ ratios of tumor cells relative to normal controls. White, blue, and red represent diploid, loss, and gain,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

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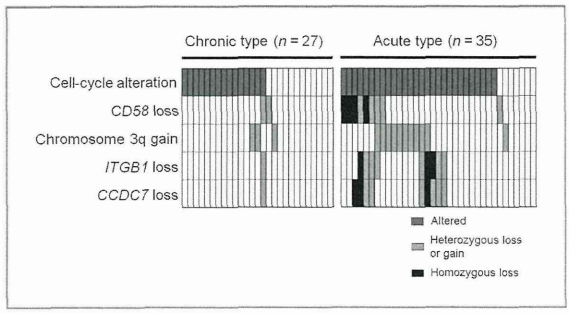

Figure 3. Distribution of genomic alterations frequently observed in acute-type ATL among ATL samples. Heatmap with rows corresponding to the indicated alterations and columns representing individual ATL cases. Gray, a heterozygous loss or gain; black, a homozygous loss. Dark gray also shows the alterations 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

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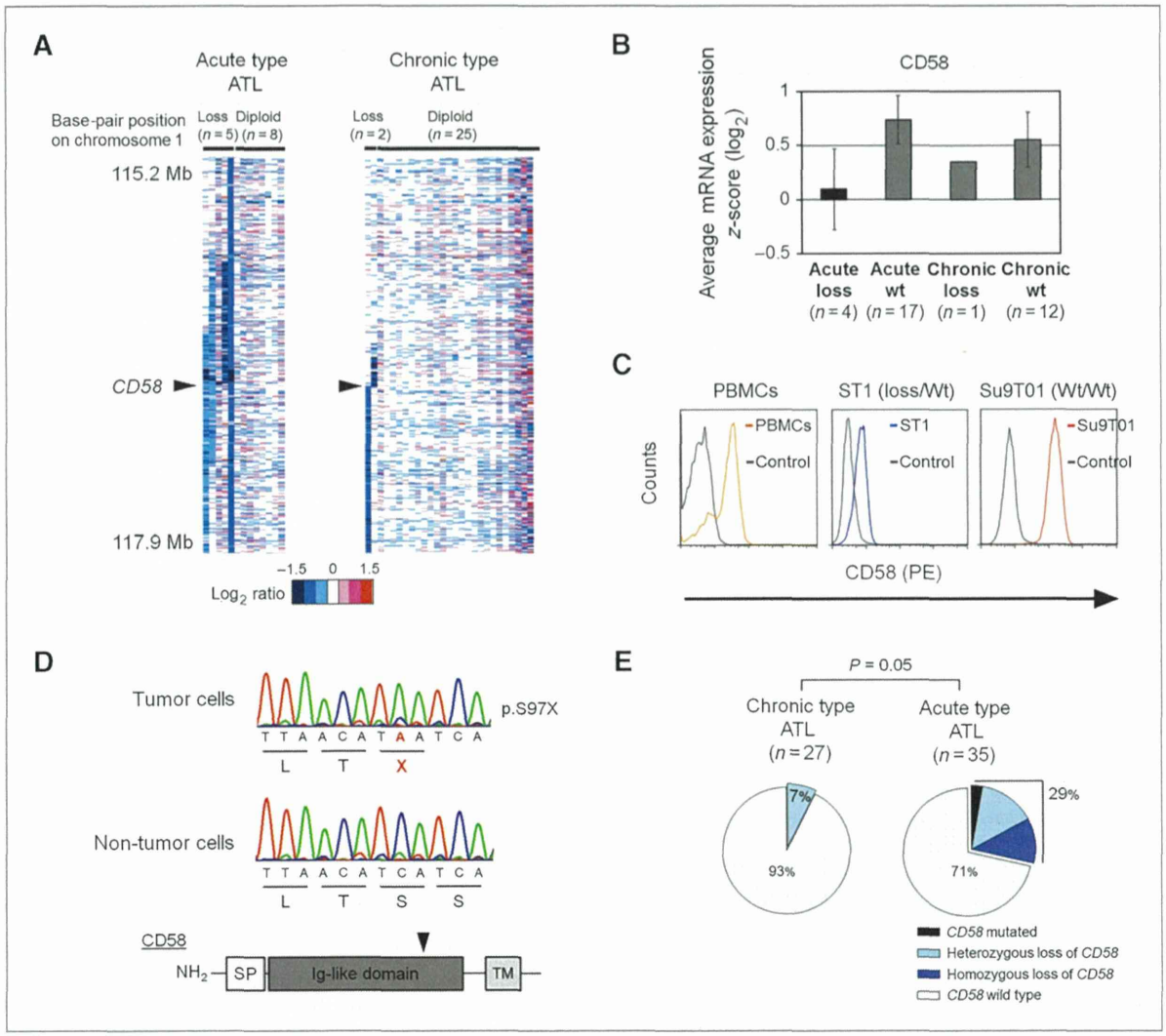

Figure 4. Alteration of *CD58* in acute transformation of chronic-type ATL. A, genomic alterations of chromosome 1p, including *CD58*. Heatmap analysis of 400K aCGH shows $\log_2$ ratios of ATL cases. White, blue, and red represent diploid, loss, and gain, respectively. Arrowhead, the *CD58* locus. B, 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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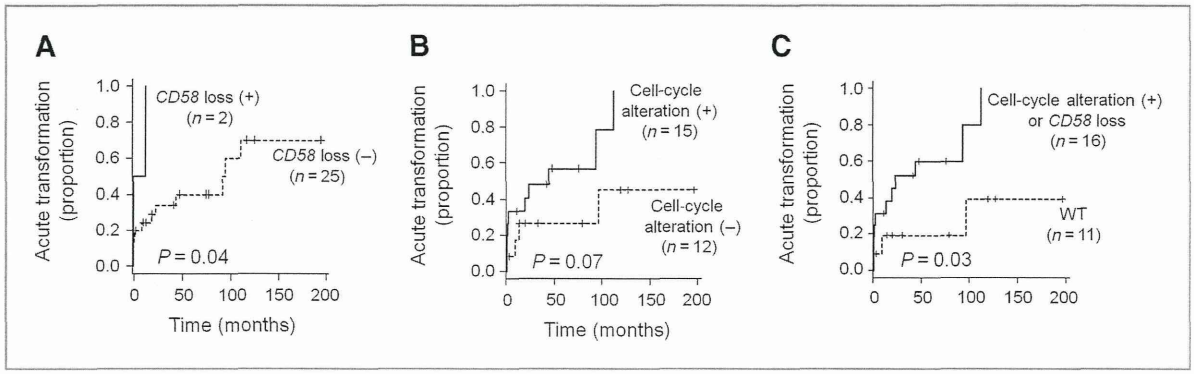

Figure 5. Genomic alterations associated with acute transformation in chronic-type ATL. A, genomic loss of *CD58* was significantly associated with earlier acute transformation ($P = 0.04$). B, chronic-type ATL cases with alterations of cell-cycle–related genes tended to exhibit earlier progression to 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

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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.

Authors' Contributions

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

Development of methodology: K. Karube

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N. Yoshida, A. Utsunomiya, K. Tsukasaki, Y. Imaizumi, N. Taira, K. Arita, S. Tsuzuki, K. Ohshima

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

Writing, review, and/or revision of the manuscript: N. Yoshida, K. Karube, A. Utsunomiya, K. Tsukasaki, T. Kinoshita, M. Seto

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

Study supervision: K. Karube, M. Seto

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