Cancer Research

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); 6129–38. ©2014 AACR.*

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Introduction

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

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

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.

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

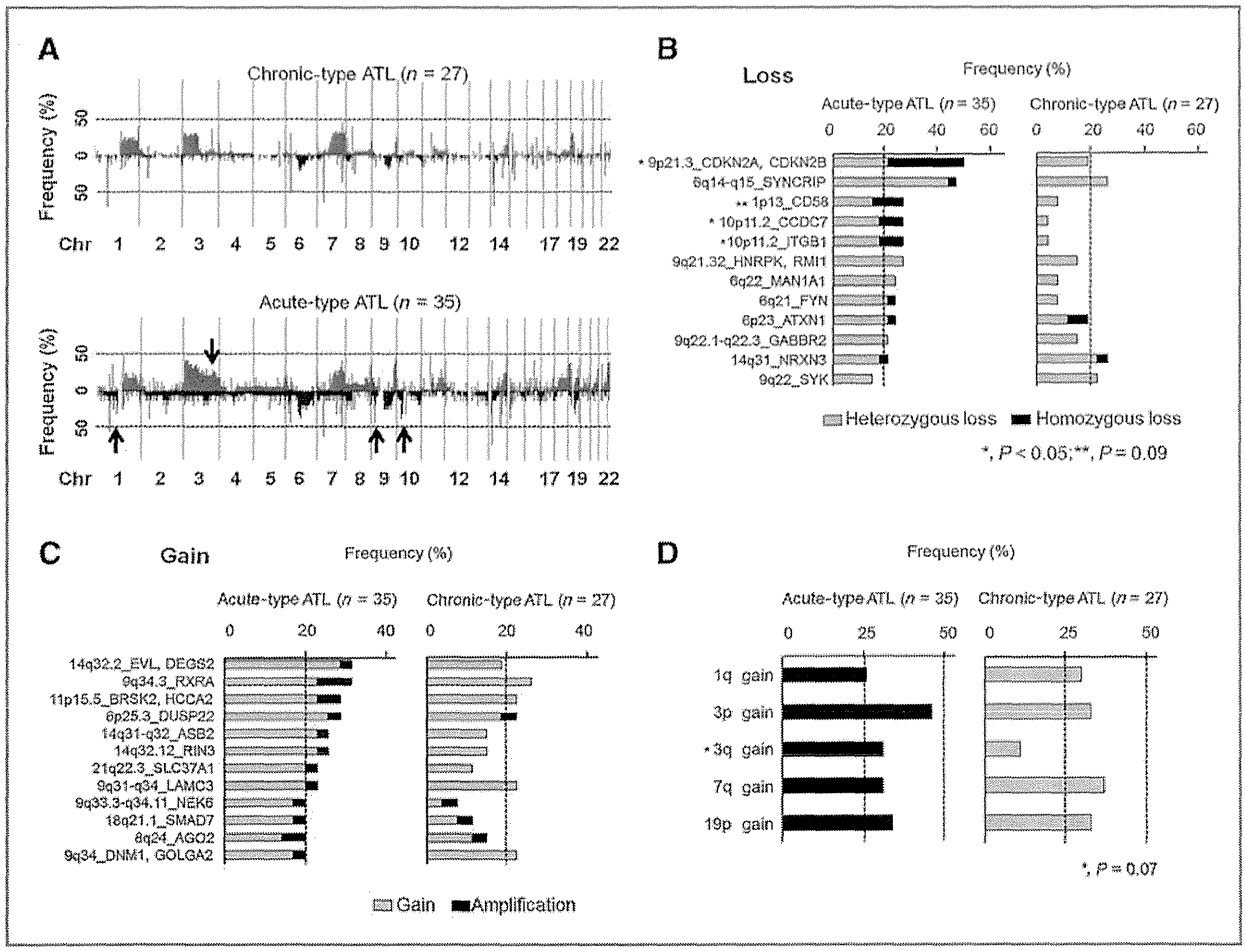

Figure 1. Genomic alteration profiles of chronic- and acute-type ATL. A, frequency of genomic alterations in chronic-type and acute-type ATL. Top, 27 cases with chronic-type ATL; bottom, 35 cases with acute-type ATL. The horizontal axis indicates each probe aligned from chromosome 1 to 22 and the short 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 (*, $P = 0.07$).

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

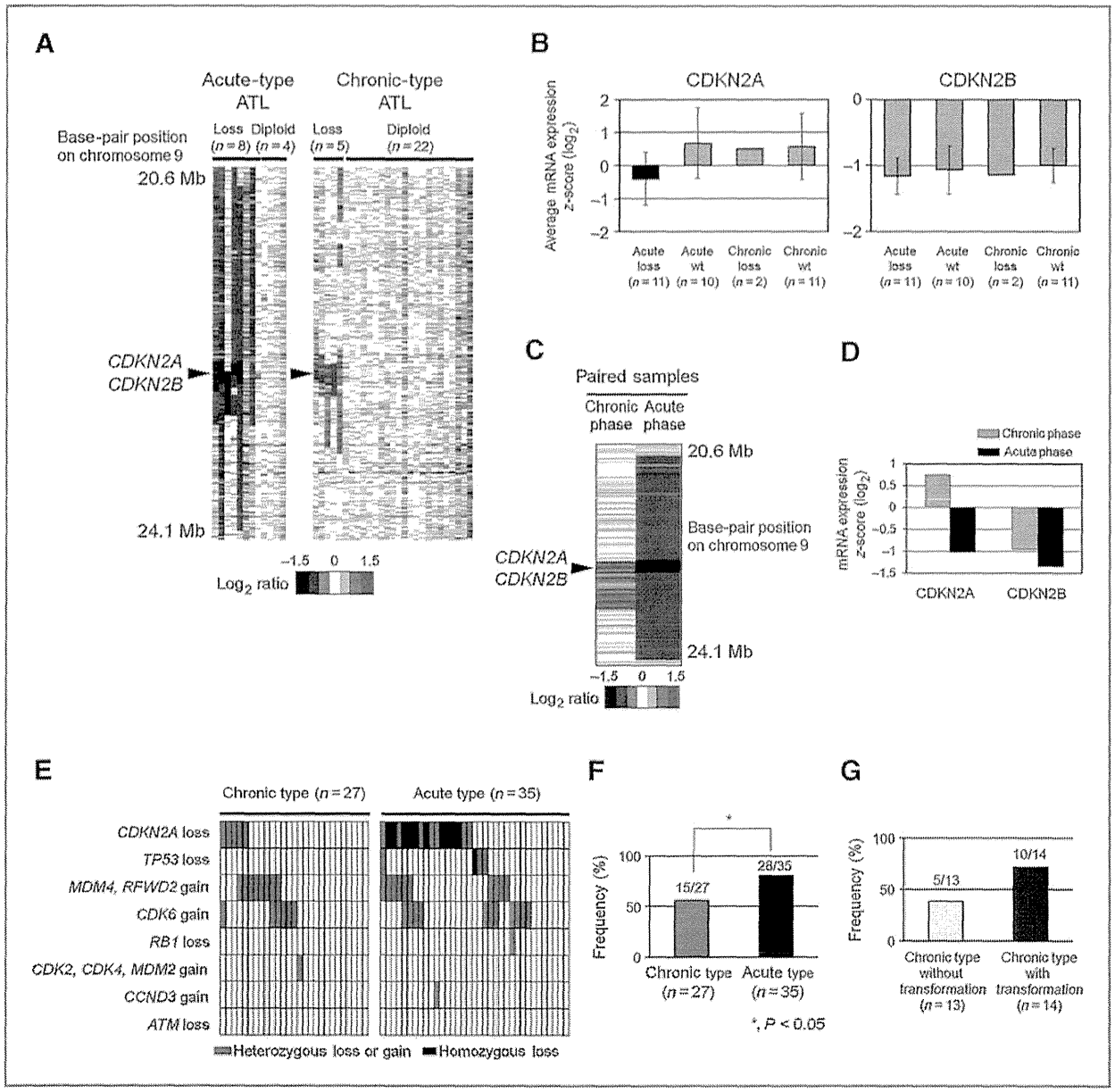

Figure 2. Loss of 9p was mainly observed in acute-type ATL and not chronic-type ATL. A, genomic alterations of chromosome 9p, including *CDKN2A/CDKN2B*. Heatmap analysis of 400K aCGH shows $\log_2$ ratios of tumor cells relative to normal controls. White, blue, and red represent diploid, loss, and gain,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

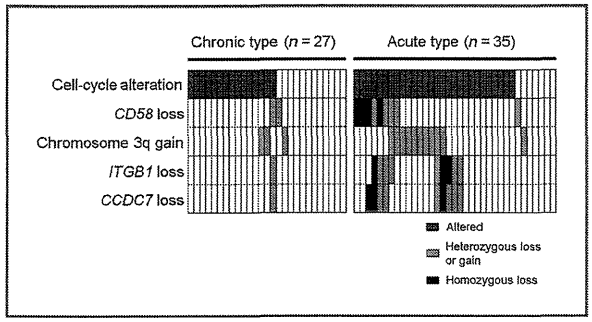

Figure 3. Distribution of genomic alterations frequently observed in acute-type ATL among ATL samples. Heatmap with rows corresponding to the indicated alterations and columns representing individual ATL cases. Gray, a heterozygous loss or gain; black, a homozygous loss. Dark gray also shows the alterations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

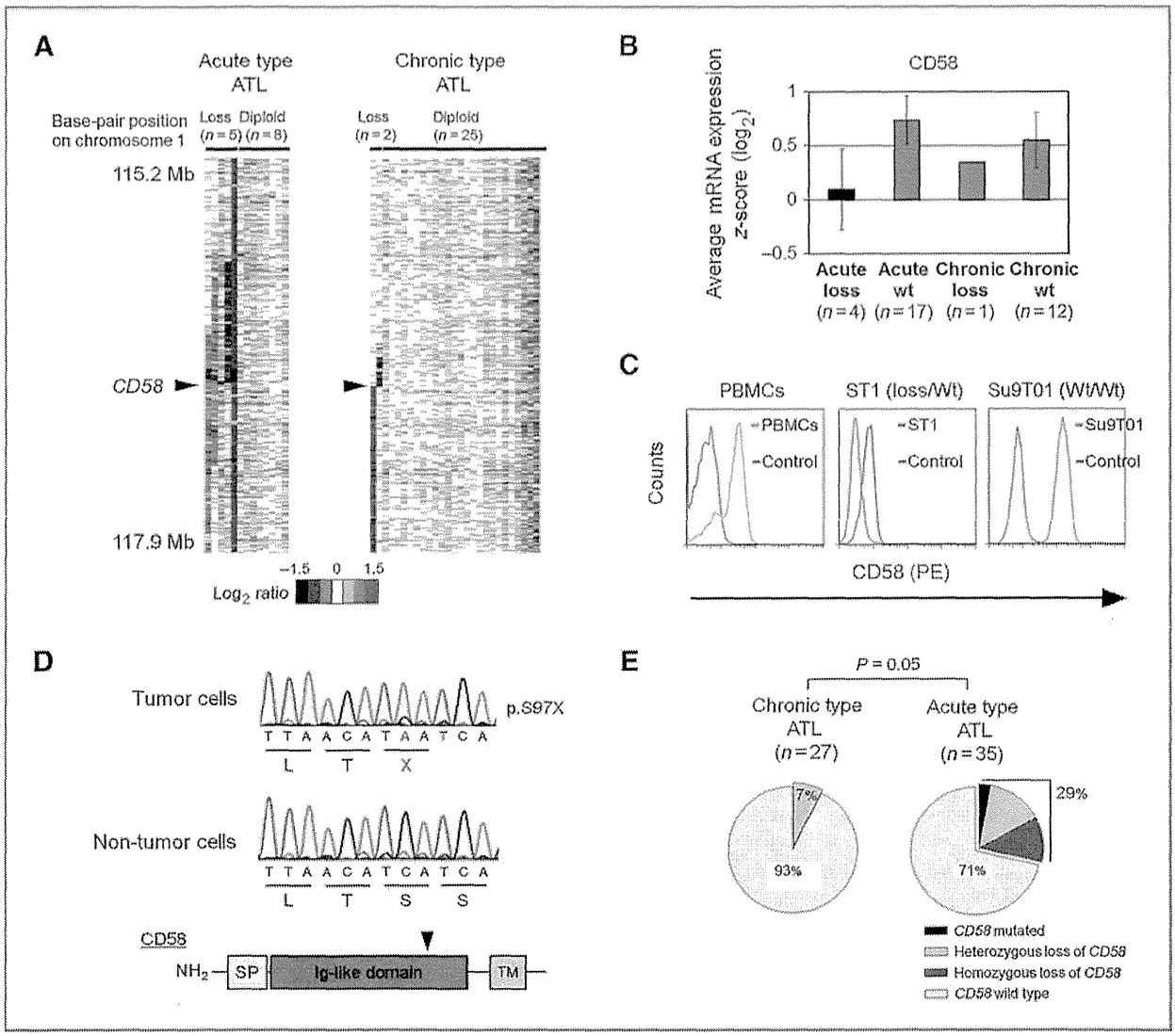

Figure 4. Alteration of *CD58* in acute transformation of chronic-type ATL. A, genomic alterations of chromosome 1p, including *CD58*. Heatmap analysis of 400K aCGH shows $\log_2$ ratios of ATL cases. White, blue, and red represent diploid, loss, and gain, respectively. Arrowhead, the *CD58* locus. B,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

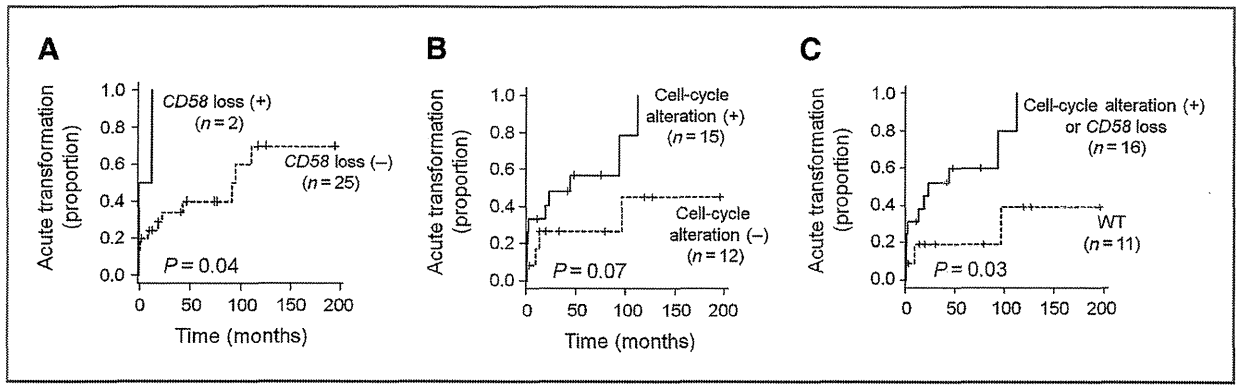

Figure 5. Genomic alterations associated with acute transformation in chronic-type ATL. A, genomic loss of *CD58* was significantly associated with earlier acute transformation ($P = 0.04$). B, chronic-type ATL cases with alterations of cell-cycle–related genes tended to exhibit earlier progression to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

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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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

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High expression of MAGE-A4 and MHC class I antigens in tumor cells and induction of MAGE-A4 immune responses are prognostic markers of CHP-MAGE-A4 cancer vaccine☆

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ABSTRACT

Purpose: We conducted a cancer vaccine clinical trial with MAGE-A4 protein. Safety, clinical response, and antigen-specific immune responses were analyzed and the prognostic factors by vaccination were investigated.
Experimental design: Twenty patients with advanced esophageal, stomach or lung cancer were administered MAGE-A4 vaccine containing 300 μg protein subcutaneously once every 2 weeks in six doses. Primary endpoints of this study were safety and MAGE-A4 immune responses.
Results: The vaccine was well tolerated. Fifteen of 20 patients completed one cycle of vaccination and two patients showed SD. A MAGE-A4-specific humoral immune response was observed in four patients who had high expression of MAGE-A4 and MHC class I on tumor cells. These four patients showed significantly longer overall survival than patients without an antibody response after vaccination ($p = 0.009$). Patients with tumor cells expressing high MAGE-A4 or MHC class I antigen showed significantly longer overall survival than those with low expression. Induction of CD4 and CD8T cell responses was observed in three and six patients, respectively, and patients with induction of MAGE-A4-specific IFNγ-producing CD8T cells, but not CD4T cells, lived longer than those without induction.
Conclusions: The CHP-MAGE-A4 vaccine was safe. Expression of MAGE-A4 and MHC class I in tumor tissue and the induction of a MAGE-A4-specific immune response after vaccination would be feasible prognostic markers for patients vaccinated with MAGE-A4.

1. Introduction

The expression of cancer/testis (CT) antigens is normally limited to human germ line cells in the testis and to various types of human cancers [1,2]. Among CT antigens, the melanoma-associated antigen gene (MAGE) family is also known to show such unique expression and to induce spontaneous humoral and cellular immune responses in MAGE-expressing cancer patients [3,4], with the result that they are feasible targets for tumor immunotherapy.

Numerous cancer vaccine strategies are under development and some patients have experienced clinical benefits after vaccination. Among the MAGE family, a phase II cancer vaccine trial with MAGE-A3 protein in non-small-cell lung cancer patients showed 8% reduction of the recurrence rate [5]. Based on the outcome of this phase II study, a randomized double-blind phase III study (MAGRIT trial) with MAGE-A3 protein vaccination was performed [6].

MAGE-A4 is also reported to be expressed in a wide variety of tumors, e.g., 60% esophageal cancer, 50% head and neck cancer, 24% non-small-cell lung cancer, 33% gastric tumor, and 21% Hodgkin's

☆ The study was registered in the University hospital Medical Information Network Clinical Trials Registry (UMIN-CTR) Clinical Trial (Unique trial number: UMIN000003188) on February 15, 2010 (UMIN-CTRURL: http://www.umin.ac.jp/ctr/index.htm).

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disease but not in normal tissues besides the testis. MAGE-A4 elicits spontaneous humoral or cellular immune responses in patients with MAGE-A4-expressing non-small-cell lung cancer, head and neck cancer and adult T cell leukemia/lymphoma [3,4,7,8]. High expression of MAGE-A4, as well as other CT antigens, in tumors was correlated with the poor prognosis of patients with bladder cancer, ovarian cancer, non-small-cell lung cancer and head and neck cancer [9–14]. Many MAGE-A4 epitope peptides recognized by CD4 and CD8 T cells in the context of human leukocyte antigen (HLA) class I and class II have been identified, e.g., HLA-A0201 [15,16], HLA-A2401 [17], HLA-B3701 [18], HLA-DP0501, and HLA-DR1403 [19].

Because tumor-specific T cells are considered to be a direct effector of tumor immunity, the expression level of MHC class I on cancer cells is crucial for the prognosis of cancer patients, especially in the case of an immune therapy such as a cancer vaccine. It is reported that deficient MHC class I surface expression is associated with reduced patient survival in colon cancer, gastric cancer and non-small-cell lung cancer [20–23], and is considered to be one of the causes of the immune escape of tumor cells [24,25]. In patients vaccinated with tumor antigens, some papers reported the effect of the expression level of MHC class I on cancer cells on the clinical effect of vaccinated patients, but there are few reports on their prognosis after vaccination [26,27].

In this study, we conducted a cancer vaccine clinical trial with a complex of MAGE-A4 protein and cholesteryl pullulan (CHP) nanoparticles in advanced cancer patients. We monitored and analyzed the safety, clinical effect, humoral and cellular immune responses and expression of antigens in these patients.

2. Materials and methods

2.1. CHP-MAGE-A4 vaccine

The complex of cholesterol-bearing hydrophobized pullulan (CHP) and MAGE-A4 protein (CHP-MAGE-A4) was provided by ImmunoFrontier, Inc. (Tokyo, Japan) [28]. The synthesis, production, formulation and packaging of the investigational agent were performed in accordance with current Good Manufacturing Practices (cGMP) and met the applicable criteria for use in humans. The toxicity of the drug products was assessed using animal models, and stability was monitored during the clinical trial using representative samples of the investigational drug product.

2.2. Study design

A phase I, open-label, single-institutional clinical trial of the CHP-MAGE-A4 vaccine was designed to evaluate the safety, immune response and clinical response. Patients eligible for entry were those who had advanced cancers that were refractory to standard therapy and expressed MAGE-A4 antigen as assessed by immunohistochemistry (IHC). The CHP-MAGE-A4 vaccine containing 300 μg MAGE-A4 protein was administered subcutaneously once every 2 weeks in six doses. Two weeks after the last administration, the safety, immune response and clinical response were evaluated. Thereafter, the vaccine was administered additionally. Clinical response was assessed according to the Response Evaluation Criteria in Solid Tumors (RECIST ver1.1) [29]. Safety was evaluated according to the National Cancer Institute Common Terminology Criteria for Adverse Events ver.3.0 (NCI-CTCAE ver.3.0) [30]. The protocol was approved by the Ethics Committee of Osaka Universities according to the Declaration of Helsinki. Written informed consent was obtained from each patient before enrolling in the study. The study was conducted in compliance with Good Clinical Practice and was registered in the University hospital Medical Information Network Clinical Trials Registry (UMIN-CTR) Clinical Trial (Unique trial number: UMIN000003188) on February 15, 2010 (UMIN-CTRURL: http://www.umin.ac.jp/ctr/index.htm).

2.3. MAGE-A4 protein and peptides

For ELISA, recombinant N-His-tagged MAGE-A4 protein was given by Mie University. For Western blot analysis, the MAGE-A4 open reading frame was given by Hokkaido University and was cloned into pGEX-HT plasmid given by Dr. J. Takagi (Osaka University, Osaka, Japan). N-GST-His-tagged MAGE-A4 protein was expressed in M15 *Escherichia coli* cells and purified by Glutathione Sepharose 4B. Finally, recombinant MAGE-A4 protein without a His-tag was purified by TEV protease [31]. For in vitro stimulation of T cells, the following series of 31 MAGE-A4 overlapping peptides spanning the protein was synthesized: 1–20, 11–30, 21–40, 31–50, 41–60, 51–70, 61–80, 71–90, 81–100, 91–110, 101–120, 111–130, 121–140, 131–150, 141–160, 151–170, 161–180, 171–190, 181–200, 191–210, 201–220, 211–230, 221–240, 231–250, 241–260, 251–270, 261–280, 271–290, 281–300, 291–310, and 300–317.

2.4. ELISA

Recombinant protein (0.4 μg/ml) in coating buffer was adsorbed onto 96-well plates and incubated overnight at 4 °C. Plates were washed with phosphate-buffered saline (PBS) and blocked with 1% bovine serum albumin (BSA). 100 μl of serially diluted serum was added to each well and incubated for 2 h at room temperature. Horseradish peroxidase (HRP)-conjugated goat anti-human IgG (Medical & Biological Laboratories, Nagoya, Japan) was added to the wells. Ovalbumin (OVA, albumin from chicken egg white; Sigma, St. Louis, MO) was used as the control protein in each assay. The cut-off value of the antibody reaction was 0.47 O.D., calculated from the results of 47 healthy donors with the average + 2 SD.

2.5. Immunohistochemistry (IHC)

IHC was performed using formalin-fixed paraffin-embedded cancer specimens obtained from all patients enrolled in this trial and 57 esophageal cancer patients who had received surgical treatment. Monoclonal antibodies were anti-MAGE-A4 protein (57B), anti-HLA class I (EMR 8–5) and anti-CD8 (clone C8/144B). The reaction was evaluated as +++ (>50% stained cells), ++ (25–50%), + (5–25%), ± (1–5%) and − (<1%) for MAGE-A4 and HLA class I expression.

2.6. In vitro stimulation of CD4 and CD8 T cells

CD8 and CD4T cells were purified from peripheral blood mononuclear cells (PBMCs) using CD8 Microbeads and a CD4+ T Cell Isolation Kit (Miltenyi Biotec). The remaining cells were used as antigen-presenting cells (APCs) after pulsing with a mixture of 31 MAGE-A4 overlapping peptides. Then, 5×10^5 CD4 or CD8T cells were cultured with 10×10^5 APCs after irradiation with IL-2 (10 U/mL; Roche Diagnostics) and IL-7 (20 ng/mL; R&D Systems) for 21 days or 8 days, respectively. CD4 or CD8T cells harvested were re-stimulated with T-APCs pulsed with a mixture of 31 MAGE-A4 overlapping peptides or HIV (p17, 39–51) peptide as the control for 6 h [32].

2.7. IFNγ intracellular staining (ICS)

ICS was performed with an ICS kit (BD Biosciences) according to the manufacturer's instructions followed by treatment with GolgiStop reagent containing monensin (BD Biosciences) for 1 h. Cells

Table 1
Immune responses and clinical responses following CHP-MAGE-A4 vaccination.

Patient ID	Immunization	MAGE-A4-specific immune response						Clinical response	OS (days)
		Antibody[a]		CD4[b]		CD8[b]			
		Pre	Post	Pre	Post	Pre	Post		
P-1	16	–	+	–	+	–	+	PD	218
P-2	13	–	–	–	–	–	–	PD	254
P-3	5	+	nd	nd		nd		NE	(74)
P-4	6	–	–	–	–	–	–	PD	82
P-5	7	+	+	–	–	–	+	PD	206
P-6	15	–	–	–	–	–	–	SD	228
P-7	31	–	++	–	+	–	+	PD	436
P-8	2	–	nd	nd		nd		NE	(42)
P-9	16	–	–	–	–	–	–	PD	340
P-10	7	–	–	–	–	–	–	PD	90
P-11	5	+	nd	nd		nd		NE	(81)
P-12	35	–	+	–	+	–	+	SD	767
P-13	7	–	–	–	–	–	–	PD	129
P-14	9	–	–	–	–	–	–	PD	179
P-15	7	–	–	–	–	–	+	PD	96
P-16	40	–	++	–	–	–	+	PD	1029
P-17	4	–	nd	nd		nd		NE	(63)
P-18	4	–	nd	nd		nd		NE	(66)
P-19	6	–	–	–	–	–	–	PD	92
P-20	7	+	+	–	–	–	–	PD	116

OS: overall survival; PD: progressive disease; SD: stable disease; NE: not evaluated; nd: not done.

[a] Antibody response was determined by ELISA. Antibody response shown here represents O.D. for MAGE-A4 protein: ++ ≥1.0; + >1.0 to ≥0.47; – >0.47.

[b] CD4 and CD8 T cell responses were determined by IFNγ intracellular cytokine staining with those cells stimulated in vitro once. IFNγ-positive cells: +++ >10%; ++ >5% to ≤10%; + >1% to ≤5%; – ≤1%.

were stained with CD8-V450 (clone RPA-T8; BD Biosciences), CD4-V450 (clone RPA-T4; BD Biosciences), CD3-Alexafluor 700 (clone UCHT1; BD Biosciences), eFluor 780-fixable viability dye (eBioscience, San Diego, CA) and IFNγ-FITC (clone 4S.B3; BD Biosciences).

2.8. Western blot

Recombinant protein (20 ng) in sample buffer was boiled for 5 min and subjected to SDS-PAGE with 10–20% polyacrylamide Bio-Rad Ready-Gels (Bio-Rad). After electrophoresis, the membrane was blocked with 5% FCS/PBS and then incubated with patients' sera diluted 1:100 for 1 h at room temperature. Horseradish peroxidase (HRP)-conjugated goat anti-human IgG (MBL) was added to the membrane. Signals were developed with a 5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium chromogenic substrate kit (Bio-Rad). Anti-MAGE-A4 monoclonal antibody (57B) used as the positive control at 1:200 dilution was given by Dr G.C. Spagnoli (University Hospital Basel, Basel, Switzerland).

2.9. Activated regulatory T cells in PBMC

Activated regulatory T cells (Treg) were analyzed by a flow cytometer using CD3-PerCPCy5.5 (clone OKT3; eBioscience), CD4-Alexafluor 700 (clone RPA-T4; eBioscience), CD8-V500 (clone RPA-T8; BD Biosciences), CD45RA-FITC (clone HI100; BD Biosciences), eFluor 780-fixable viability dye (eBioscience) and FoxP3-PE (clone 236A/E7; eBioscience). The details of the assay and the definition of activated Tregs were described previously [33].

2.10. Statistics analysis

Rates of the immune responses were compared by Fisher's exact test, and the survival curve was estimated using the Kaplan–Meier method and compared by the log-rank test. All analyses were performed using the SPSS statistical package, version 15.0 (SPSS Inc., Chicago, IL).

3. Results

3.1. Patient characteristics

Twenty advanced cancer patients were enrolled: 18 patients with esophageal cancer, a patient with lung cancer and a patient with gastric cancer expressing MAGE-A4 antigen (Supplementary Table). They received 2–40 immunizations and 15 patients completed a cycle of vaccination (Table 1).

3.2. Safety

Grade 1 fever and Grade 1 injection site reactions, e.g., skin redness or pruritus, were observed in 4 and 13 patients, respectively, after vaccination, and improved without any treatment (Supplementary Table). No severe adverse event was observed.

3.3. Clinical response

All patients underwent image analysis and routine physical checks during and after vaccination. An SD response was observed in two esophageal cancer patients, P-6 and P-12, out of 15 patients who completed vaccination (Table 1). In patient P-6, relapsed lymph node metastasis in the right neck after radical esophagectomy showed a 9% increase in its diameter after 6 immunizations with CHP-MAGE-A4. In patient P-12, although the main tumor disappeared after chemotherapy, metastasis in the left lung was observed with a 15% increase in its diameter after a cycle of vaccination. Both patients received additional cycles of CHP-MAGE-A4 vaccination; however, these target lesions showed rapid enlargement after the second cycle.

3.4. Monitoring of humoral immune response

MAGE-A4 antibody in sera obtained from all patients at baseline and 15 vaccine-completed patients two weeks after the final immunization were analyzed by ELISA. Four patients, P-3, P-5, P-11

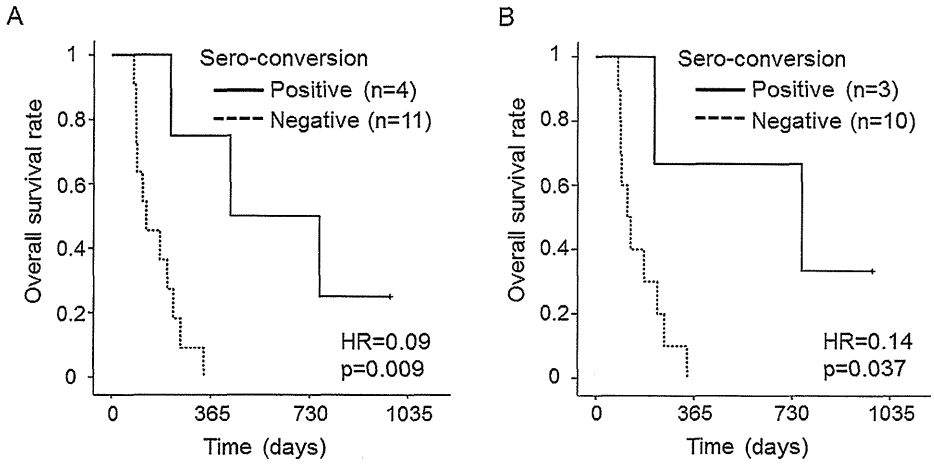

Fig. 1. Antibody production and prognosis. Overall survival of 15 patients and 13 esophageal cancer patients who completed 1 cycle of vaccination and the antibody response determined by ELISA were analyzed. Kaplan–Meier curves illustrate the duration of overall survival of sero-converted patients (solid line) and patients without an antibody response (dotted line) in 15 patients (A) and 13 esophageal cancer patients (B). The hazard ratio (HR) and log-rank *P* value for overall survival comparing patients with positive against negative antibody responses were calculated.

and P-20, showed the production of MAGE-A4 antibody at baseline (sero-positive) while others did not (sero-negative) (Table 1). After vaccination, 4 of 13 sero-negatives among 15 vaccine-completed patients showed increased O.D. values by ELISA and were considered positive serological responses (Supplementary Fig. 1A). No increased response was observed with sera from two sero-positives. These sero-conversions were observed just after a cycle of vaccination in all four patients. Anti-MAGE-A4, but not anti-His-tag, antibody responses in sera from patients P-1, P-12 and P-16 were analyzed by Western blot analysis using recombinant MAGE-A4 protein without any tags (Supplementary Fig. 1B).

Then, the overall survival after the first immunization in sero-conversion positives and negatives was analyzed in 15 vaccine-completed patients. The four sero-converted patients showed prolonged overall survival, significantly longer than that of patients without an antibody response after vaccination (Fig. 1A). When the analysis was limited to esophageal cancer patients, the overall survival of the three sero-converted patients was also significantly longer than that of patients without a MAGE-A4 antibody response (Fig. 1B).

3.5. Immunohistochemical analysis of MAGE-A4, MHC class I and CD8

Expression of MAGE-A4 and MHC Class I antigens on tumor cells was analyzed by IHC using formalin-fixed paraffin-embedded tumor tissues obtained from all enrolled patients (Supplementary Table). Among 15 vaccine-completed patients, high expression of MAGE-A4 (>25% tumor cells) and MHC class I (>5% tumor cells) was observed in tumor tissues from 12 and 12 patients, respectively. Then, we analyzed whether there is any relation between the expression of MAGE-A4 and MHC class I antigens on tumor cells and the induction of immune responses by CHP-MAGE-A4 vaccination. Four of eight patients with high expression of MAGE-A4 or MHC class I antigen on tumor cells showed an antibody response while no patients with low expression of either antigen on tumors showed an antibody response. High expression of both MAGE-A4 and MHC class I antigens was observed on tumor cells from sero-converted patients (Fig. 2A and B). Next, we analyzed whether there is any relation between the expression of those antigens and overall survival by CHP-MAGE-A4 vaccination. Patients with tumor cells expressing high MAGE-A4 or MHC class I antigen showed significantly longer overall survival than those with lower expressions (Fig. 2C and D).

3.6. Induction of MAGE-A4-specific CD4 and CD8 T cell responses

MAGE-A4-specific CD4 and CD8 T cell responses were analyzed by ICS assay using PBMCs obtained from 15 vaccine-completed patients at baseline and 2 weeks after the 6th immunization (Supplementary Fig. 2). MAGE-A4-specific IFNγ-producing CD4 and CD8 T cells were observed in no patient at baseline. After vaccination, induction of a CD4 T cell response was observed in three patients, P-1, P-7, P-12, who showed sero-conversion, and induction of a CD8 T cell response was observed in six patients, P-1, P-5, P-7, P-12, P-15, P-16, who showed antibody production (Table 1). Patients with induction of MAGE-A4-specific IFNγ-producing CD8 T cells, but not CD4 T cells, lived longer than those without induction (Supplementary Fig. 3).

3.7. Impact of CD4+ FoxP3 high+ regulatory T cells on overall survival

The ratio of CD4+ Foxp3 high+ cells in CD3+ T cells was analyzed using PBMCs obtained at baseline from 15 vaccine-completed patients. When the patients were divided by the mean of the ratio, the two SD patients, P-6 and P-12, belonged in the low ratio group (Supplementary Fig. 4A and B). Patients with a low ratio of CD4+ Foxp3 high+ cells in CD3+ T cells showed longer overall survival than patients with a high ratio after vaccination, although it was not significant (Supplementary Fig. 4C).

4. Discussion

We showed that the induction of MAGE-A4-specific immune responses correlated well with the prognosis of patients vaccinated with CHP-MAGE-A4. In our previous study of cancer vaccines with NY-ESO-1 protein [34–39], NY-ESO-1f peptide [40] and NY-ESO-1 overlapping peptide [41], feasible clinical responses were observed in several patients; however, we could not confirm the effects of NY-ESO-1 vaccines on the good prognosis of enrolled patients. There are several reports of successful cancer vaccines which prolonged the overall survival of vaccinated patients [42,43], and some studies revealed that patients with the induction of an antigen-specific CD8 T cell response, but not an antibody response,

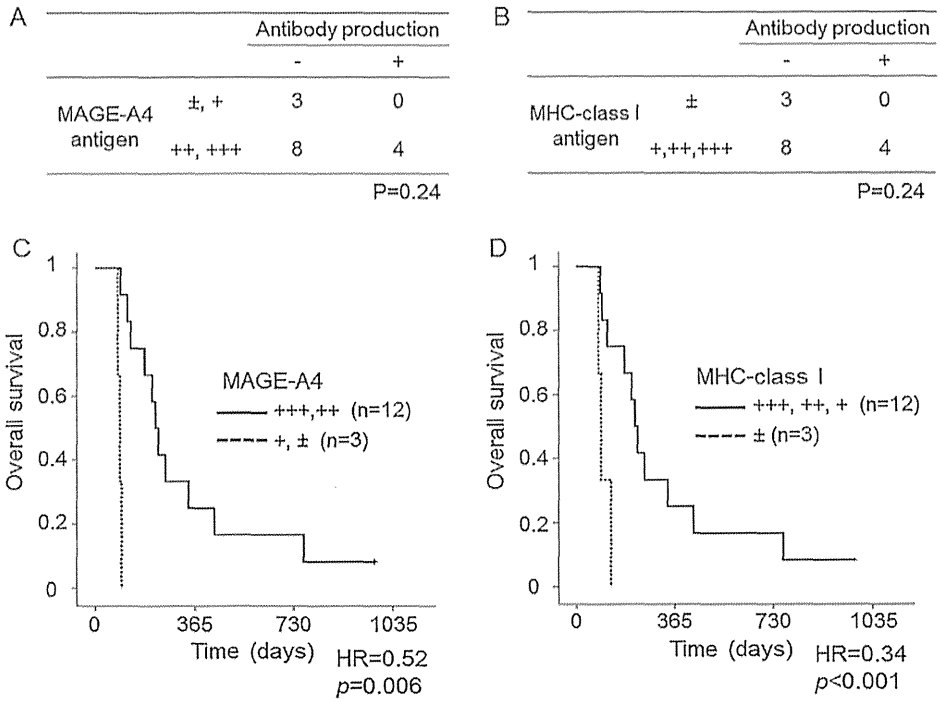

Fig. 2. MAGE-A4 and MHC-class antigen expression and prognosis. MAGE-A4 and MHC-class I were analyzed by immunohistochemical analysis with monoclonal antibodies; anti-pan-MAGE protein (57B), anti-human leukocyte antigen (HLA) class I (EMR 8–5). The reaction was evaluated as +++ (>50% stained cells), ++ (25–50%), + (5–25%), ± (1–5%) and – (<1%) for MAGE-A4 and HLA class I expression. Among 15 vaccine-completed patients, tumor tissues from the four MAGE-A4 sero-converted patients showed higher expression of both MAGE-A4- and MHC-class I-antigens of tumor cells (A and B). The patients with tumor cells expressing higher MAGE-A4 or MHC-class I antigen showed a significantly longer overall survival than those with lower expressions (C and D).

survived longer [44]. Our results of immune monitoring indicated that the induction of not only MAGE-A4 CD8 T cells but also MAGE-A4 antibody responses could be a marker for predicting the good prognosis of patients vaccinated with MAGE-A4 protein. Detection of an antibody response is considered to be a useful tool for monitoring cancer vaccines with protein because it is easy to analyze with sera using ELISA [35]. In our previous studies of NY-ESO-1 antigen, specific humoral and cellular responses were spontaneously induced in patients with NY-ESO-1-expressing tumors, and elicited much more frequently and earlier in patients vaccinated with NY-ESO-1 than MAGE-A4 [39–41]. CT antigens, among tumor antigens, are known to have better immunogenicity because of their unique expression pattern [1]. However, MAGE-A4 antigen might not possess such strong immunogenicity as other CT antigens, for example, NY-ESO-1. Although it is not easy to determine and explain the intent of the immunogenicity of antigenic molecules, one possibility for determining immunogenicity is the immune competition by other molecules. The existence of ubiquitous expressions of other MAGE family members might interfere with the immune response of MAGE-A4 as a tumor antigen [3]. Another possibility is the stability of MHC and antigenic peptide complexes. It is reported that MAGE-A4 epitope peptide combined with HLA-A2 is less stable than Tax10 or influenza matrix epitope peptides but is consistent with common sets of A2-complexes determined by thermal denaturation measurements [15]. Nevertheless, the induction of a MAGE-A4 antibody response was a good marker of the long survival of patients vaccinated with MAGE-A4 protein, indicating that the immunogenicity of MAGE-A4 might be adequate to induce immune responses which can be used for immune monitoring to predict the prognosis of vaccinated patients.

To investigate which factors induce a humoral immune response by MAGE-A4 vaccine, the expression of MAGE-A4 and MHC class I antigens in tumor tissues was analyzed by IHC, and it was shown that the four sero-converted patients had cancers with high expression of both MAGE-A4 and MHC class I. Moreover, overall survival was prolonged in patients with tumors with high expression of MAGE-A4 antigen, suggesting that these patients might have elicited MAGE-A4-specific immune responses to some extent by MAGE-A4 vaccination, resulting in a good prognosis. The weak band observed in sero-negative patient P-16 at baseline by Western blot analysis (Supplementary Fig. 1B) might indicate such an undetectable level of MAGE-A4 immune responses by ELISA, probably due to the property of recombinant MAGE-A4 protein or MAGE-A4 antigen itself.

Next, we tried to find direct immunological activity against tumor cells, resulting in some clinical benefit, e.g., OS, progression-free survival, or tumor shrinkage. In two SD patients, while one showed sero-conversion but not the other, seromics analysis showed the antigen spreading among CT antigens in both patients after vaccination (Supplementary Fig. 5). In addition, activated regulatory T cells were abundantly observed in PBMC from both SD patients, although they did not influence OS. In our previous study of patients vaccinated with NY-ESO-1, antigen spreading was also observed [37], and Tregs were not increased after vaccination [41]. Antigen spreading of CTL against tumor-specific antigens after cancer vaccine with MAGE-1, 3 was also reported, indicating its contribution to tumor regression [45]. P-16 underwent resection of lung metastasis before and after vaccination, and both specimens were available for IHC analysis (Supplementary Fig. 6). Although the expressions of MAGE-A4 and MHC class I were consistent, the number of tumor-infiltrating CD8+ T cells after vaccination was twice as many as at the baseline.

In summary, CHP-MAGE-A4 vaccine was safe and two SD patients were observed. High expression of MAGE-A4 and MHC class I antigens in tumor cells and the induction of MAGE-A4 humoral and cellular immune responses would be feasible prognostic markers for patients vaccinated with MAGE-A4 protein.

Conflict of interest statement

All authors have declared that there are no financial conflicts of interest in regard to this work, but Hiroshi Shiku is a stockholder of ImmunoFrontier, Inc. CHP-MAGE-A4 reagent used in this study was supplied by ImmunoFrontier, Inc.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2014.09.002.

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Antibody response to cancer/testis (CT) antigens: A prognostic marker in cancer patients

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Keywords: cancer/testis (CT) antigen, NY-ESO-1, XAGE1 (GAGED2a), overall survival, spontaneous antibody response

Immune responses to tumor antigens have been reported in cancer patients. However, the relevance of such spontaneous immune responses to the clinical course has not been studied extensively. We showed that the overall survival of patients with antibodies against NY-ESO-1 or XAGE1 (GAGED2a) antigen was prolonged in gastric or lung cancer patients, respectively.

Cancer patients respond to their own tumors immunologically, and antibody responses, or CD4 and CD8 T-cell responses, against tumor cells or tumor cell products have been recognized (**Fig. 1**). Using such antibodies or T cells as probes, many tumor antigens have been identified in various human tumors over the past 2 decades. Those include mutated, differentiation, over-expressed and cancer/testis (CT) antigens. More recently, T-cell responses to various mutated peptides have been detected by whole exome analysis. The findings clearly indicate the presence of tumor antigens and the occurrence of immune responses to them in cancer patients. However, the relevance of such spontaneous immune responses to the clinical course has not been studied extensively. The reason for this appears to be the difficulty of detecting anti-tumor immune responses in patients because of the generally weak antigenicity of the tumor antigens together with the lack of a reliable methodology. However, some CT antigens, including NY-ESO-1 and XAGE1 (GAGED2a), have been shown to be highly immunogenic. Besides CT antigens, the tumor suppressor gene product p53 is also known to be strongly immunogenic. A previous study on spontaneous NY-ESO-1 immune responses in cancer patients revealed that antibody responses and CD4 and CD8 T-cell responses occur concomitantly as an integrated immune response.[1] Because of its sensitivity and reproducibility as well as involving a simple assay procedure, an antibody response would be a useful immune biomarker to evaluate immune responsiveness in patients. Nevertheless, it should be noted that a split immune tolerance could take place depending on the antigens.[2] NY-ESO-1 antigen readily elicits a CD8 T-cell response, but p53 has been reported as a weaker elicitor, irrespective of a similar efficiency of antibody induction with these antigens.

By the detection of antibody responses against CT antigens, we have investigated the clinical relevance of such spontaneous immune responses in cancer patients. In gastric cancer patients, we previously showed that an NY-ESO-1 antibody response was present at 3–4% in stage I and II, and 20–25% in stage III and IV patients, suggesting a higher antibody response rate in more advanced stage patients.[3] Analysis of the overall survival in 310 gastric cancer patients with stages I to IV showed no difference between antibody-positive and -negative patients. However, in the 126 patients in stages III and IV, the overall survival of antibody positive patients was prolonged, although not significantly. It should be noted that NY-ESO-1 expression itself is tumorigenic, and patients with NY-ESO-1-antigen-positive tumors showed a shorter survival compared to patients with antigen-negative tumors in various cancer types.[4] In our recent prospective study on advanced stage (stages IIIB and IV) lung adenocarcinoma patients, patients with antibody responses to XAGE1 (GEGED2a) showed a significantly prolonged overall survival compared to patients with no antibody responses.[5] XAGE1 (GAGED2a) antigen expression was a worse predictor in patients with EGFR-mutated tumors. The XAGE1 (GAGED2a) antibody frequency in the advanced lung adenocarcinoma patients was similar (approximately 20%) to the frequency of NY-ESO-1 antibody responses in advanced gastric cancer patients. However, the patient cohort is more restricted at advanced stages in lung cancer compared to gastric cancer patients. Daudi, et al.[6] recently reported that ovarian cancer patients with antibody responses to any of the MAGE family antigens showed a shorter survival compared to patients without antibody responses. In their study, patients with all stages were included, and so the shorter survival of advanced cancer patients should be carefully considered, as they discussed. A stage-controlled study is necessary to elucidate the effect of the antibody response on the clinical course. A similar overall survival-shortening effect of the antibody response was observed in patients with antibody against p53.[7] Caution, as described in Daudi's report,

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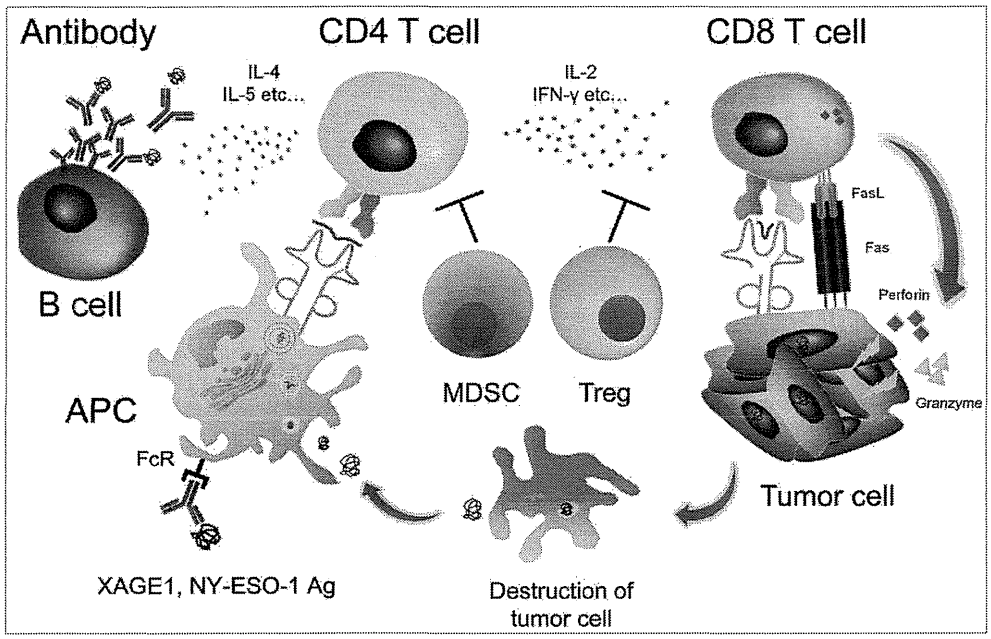

Figure 1. Spontaneous immune responses to tumors in cancer patients. The cancer/testis (CT) antigens NY-ESO-1 and XAGE1 (GAGED2a) are strongly immunogenic, and an integrated immune response, consisting of an antibody response and CD4 and CD8 T-cell responses is frequently elicited spontaneously. The antibody response is a useful biomarker of immune responses because of its sensitivity and reproducibility, as well as involving a simple assay procedure. CD4 T-cell responses to CT antigens would be enhanced by the antigen-presenting cells (APC) that efficiently internalize the antigen/antibody complex via the Fc receptor (FcR) and promote the antibody response via IL-4 and IL-5 cytokines, and the CD8 T-cell response via IL-2 and IFNγ. CD8 T cells lyse tumor cells via Fas-FasL, perforin, and/or granzyme. Tregs and MDSCs suppress CD4 and CD8 T cells.

should also be exercised when interpreting the results for p53.

Thus, in order to evaluate the clinical significance of the spontaneous antibody response in cancer patients, the clinical relevance of antigen expression should be carefully examined. The CT antigen expression itself generally worsens survival. Furthermore, it should be noted that the antibody response rate generally increases according to the stage. Therefore, the clinical benefit of the spontaneous antibody response should be evaluated in a patient cohort with restricted stages and antigen-positive tumors.

The presence of circulating NY-ESO-1-specific T-cells has been shown to be correlated with a favorable prognosis in melanoma patients.[8] Conversely, the presence of MDSCs has been shown to be correlated with a poor prognosis.[8] The clinical relevance of Foxp3-positive Treg infiltration is controversial, being correlated with poor survival in some cancers, but with better survival in others.[9] In the latter, the contribution of non-specific inflammation associated with Treg infiltration to the favorable prognosis has been suggested. Although such findings suggest an association of the immune phenotype with the clinical course in cancer patients, findings linking spontaneous immune responses to the clinical benefit are still limited. Our study, showing a link between NY-ESO-1 and XAGE1 (GAGED2a) antibody responses and prolonged overall survival, sheds light on the role of naturally occurring immune responses in cancer patients. Furthermore, we observed that genetic variants of immunoglobulin γ and κ chains influence the XAGE1 (GAGED2a) antibody response.[10] The present findings should be confirmed with different patient cohorts and extended to other antigens in other cancers to establish a firm basis for immunotherapy.

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