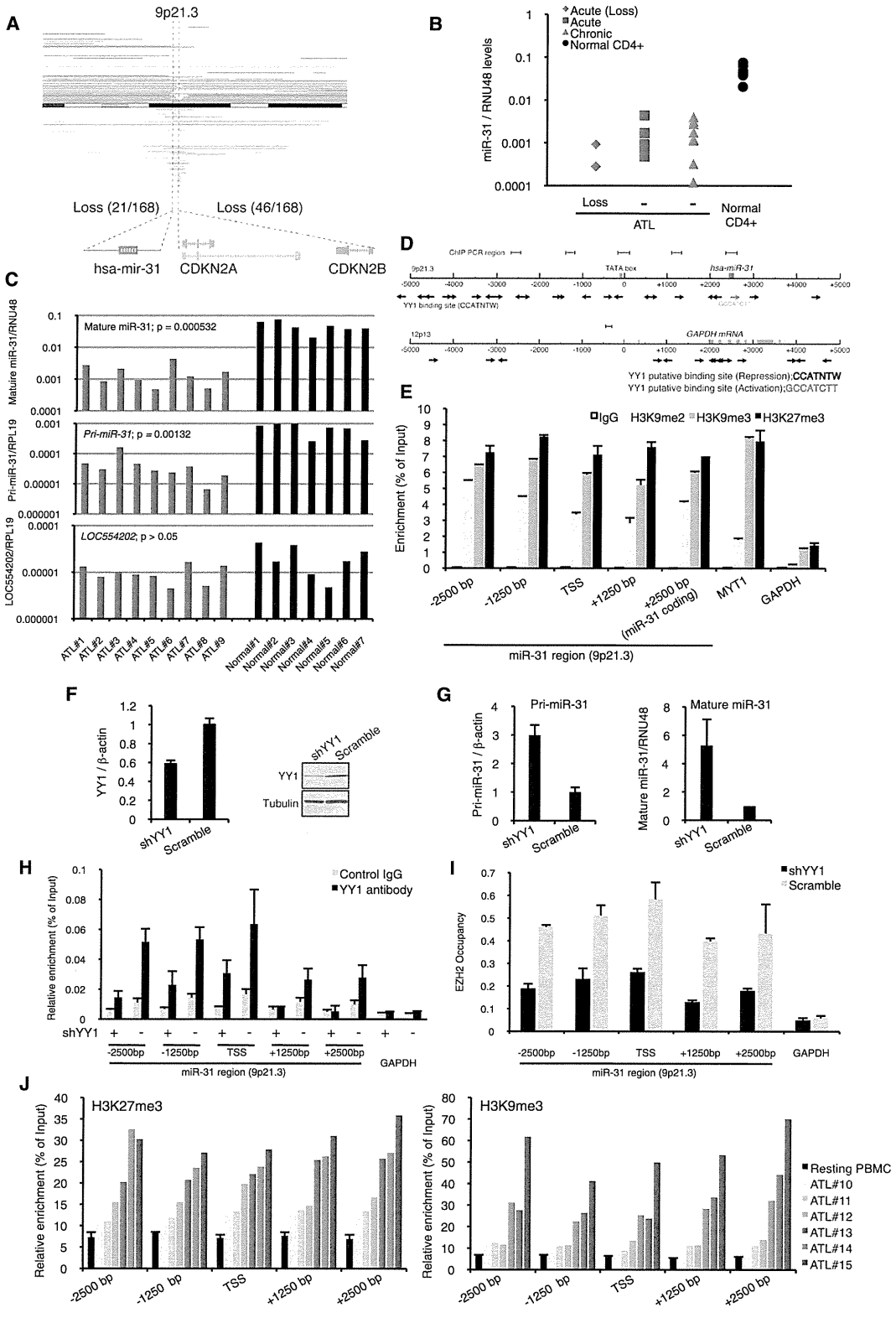

A
9p21.3
Loss (21/168)
Loss (46/168)
hsa-mir-31
CDKN2A
CDKN2B
B
Acute (Loss)
Acute
Chronic
Normal CD4+
miR-31 / RNU48 levels
Loss
ATL
Normal CD4+
C
Mature miR-31; p = 0.000532
Pri-miR-31; p = 0.00132
LOC554202; p > 0.05
Mature miR-31/RNU48
Pri-miR-31/RPL19
LOC554202/RPL19
ATL#1 ATL#2 ATL#3 ATL#4 ATL#5 ATL#6 ATL#7 ATL#8 ATL#9
Normal#1 Normal#2 Normal#3 Normal#4 Normal#5 Normal#6 Normal#7
D
ChIP PCR region
9p21.3
TATA box
hsa-miR-31
YY1 binding site (CCATNTW)
12p13
GAPDH mRNA
YY1 putative binding site (Repression):CCATNTW
YY1 putative binding site (Activation):GCCATCTT
E
IgG H3K9me2 H3K9me3 H3K27me3
Enrichment (% of Input)
-2500 bp
-1250 bp
TSS
+1250 bp
+2500 bp (miR-31 coding)
MYT1
GAPDH
miR-31 region (9p21.3)
F
YY1 / β-actin
shYY1
Scramble
YY1
Tubulin
G
Pri-miR-31
Mature miR-31
Pri-miR-31 / β-actin
Mature miR-31/RNU48
H
Control IgG
YY1 antibody
Relative enrichment (% of Input)
shYY1
-2500bp
-1250bp
TSS
+1250bp
+2500bp
GAPDH
miR-31 region (9p21.3)
I
shYY1
Scramble
EZH2 Occupancy
J
H3K27me3
H3K9me3
Relative enrichment (% of Input)
Resting PBMC
ATL#10
ATL#11
ATL#12
ATL#13
ATL#14
ATL#15

ChIP assays detected higher levels of trimethylated H3K27 and EZH2 occupancy in cells showing lower expression levels of miR-31 (Figure S4H). Furthermore, knockdown of EZH2 or SUZ12 restored miR-31 transcription in MDA-MB-453 and MCF7 cells (Figures 5F and 5G; Figure S4K, respectively), which are consistent with the results obtained with ATL cells. These results indicate a link between Polycomb-mediated epigenetic regulation and miR-31 transcription in ATL and breast cancer cell lines.

Polycomb Group Regulates NF-κB Pathway by Controlling miR-31 Expression

Based on our findings, we considered an aspect of the biological communication between epigenetic silencing and the NF-κB pathway through miR-31 regulation. The microarray data sets showed positive correlations between PRC2 components and miR-31 target gene, *NIK* expression (Figure 6A). The results also suggested that these factors tend to show higher levels in the aggressive subtype (acute type) than in the indolent subtypes (chronic and smoldering types), implying that these genes may play important roles in the clinical phenotype and prognosis of ATL. To examine this notion, we performed PRC2 knockdown in ATL cell lines. Western blots of these cells demonstrated decreased levels of NIK, p52, and phospho-IκBα (Figure 6B; Figure S5A), suggesting suppression of both canonical and noncanonical NF-κB cascade and activity (Figure 6C; Figures S5B and S5C). These results are consistent with those of miR-31 overexpression (Figures 3C–3F). Then, we tested whether exogenous manipulation of miR-31 could restore the effect of PRC2 loss. Anti-miR-31 treatment rescued impaired NF-κB activity in PRC2-disrupted cells (Figure 6D). On the other hand, overexpression of EZH2 induced NF-κB activation, which was partially canceled by the introduction of miR-31 precursor (Figure 6E; Figure S5D). These results suggest that Polycomb-mediated miR-31 suppression leads to NF-κB activation. Indeed, knockdown of the PRC2 complex led to reduced levels of cell proliferation and greater sensitivity to serum deprivation in ATL cells (Figure 6F; Figure S5E). In addition, PRC2 disruption showed a reduction in cell migration (Figure S5F).

To gain further insight into this general network, we studied the functions of miR-31 and the PRC2 complex in breast cancer cell lines. NF-κB activity was downregulated by knockdown of PRC2 components in MDA-MB-453 cells (Figure 6G; Figures S5G and S5H), although no significant differences were observed in cell proliferation (data not shown). Repression of NF-κB activity induced by knockdown of PRC2 components was partially restored by treatment with a miR-31 inhibitor, suggesting that PRC2 knockdown-mediated relief of NF-κB repression is at least a part of the result of the miR-31 induction. In addition, knockdown of PRC2 components resulted in a reduced level of receptor-initiated accumulation of NIK in B cells (Figure 6H). Our findings indicate a common molecular mechanism comprising Polycomb-mediated epigenetic regulation, miR-31 expression and the NF-κB signaling pathway.

Regulation of NF-κB by Polycomb family may in turn control the cellular apoptosis responses. We found that lentivirus-mediated EZH2 knockdown led to increased apoptotic sensitivity in TL-Om1 cells (Figure 6I). Additional expression of NIK inhibited the cell death induced by EZH2 knockdown, suggesting the reciprocal relationship between Polycomb and NF-κB cascades. By using primary tumor cells from patient, we tested the killing effect induced by miR-31, NIK knockdown, and EZH2 knockdown (Figure 6J; Figures S5I and S5J). All tested samples showed strong death response, demonstrating that survival of ATL cells was closely associated with miR-31, NIK, and EZH2, all of which show deregulated expression in ATL cells.

By qRT-PCR we finally examined the expression levels of some genes involved in the noncanonical NF-κB pathway. As shown in Figure 6K, the results clearly demonstrated higher expression levels of positive regulators such as *NIK*, *CD40,* and *LTBR*, and lower expression levels of the negative regulators such as *BIRC2/3* (cIAP1/2), which are involved in proteasomal degradation of NIK (Zarnegar et al., 2008a). These observations are in line with a previous report on Multiple Myeloma cells (Annunziata et al., 2007). In addition to these data, we obtained convincing evidence for a molecular aspect of NIK accumulation in ATL cells. Polycomb-dependent epigenetic gene silencing may be associated with miR-31 loss, followed by NF-κB activation and other signaling pathways (Figure 7).

DISCUSSION

Constitutive activation of NF-κB contributes to abnormal proliferation and inhibition of apoptotic cell death in cancer cells,

Figure 4. Genetic and Epigenetic Abnormalities Cause miR-31 Loss in ATL Cells

(A) Genomic loss of chromosome 9p21.3 in primary ATL cells. Copy number analyses revealed tumor-associated deletion of miR-31 region (21/168) and *CDKN2* region (46/168). Recurrent genetic changes are depicted by horizontal lines based on CNAG output of the SNP array analysis.

(B) miR-31 expression in various sample sets. Expression levels were evaluated by real-time PCR.

Loss, samples with genomic loss of the miR-31 region; (–) samples without genomic loss of the miR-31 region.

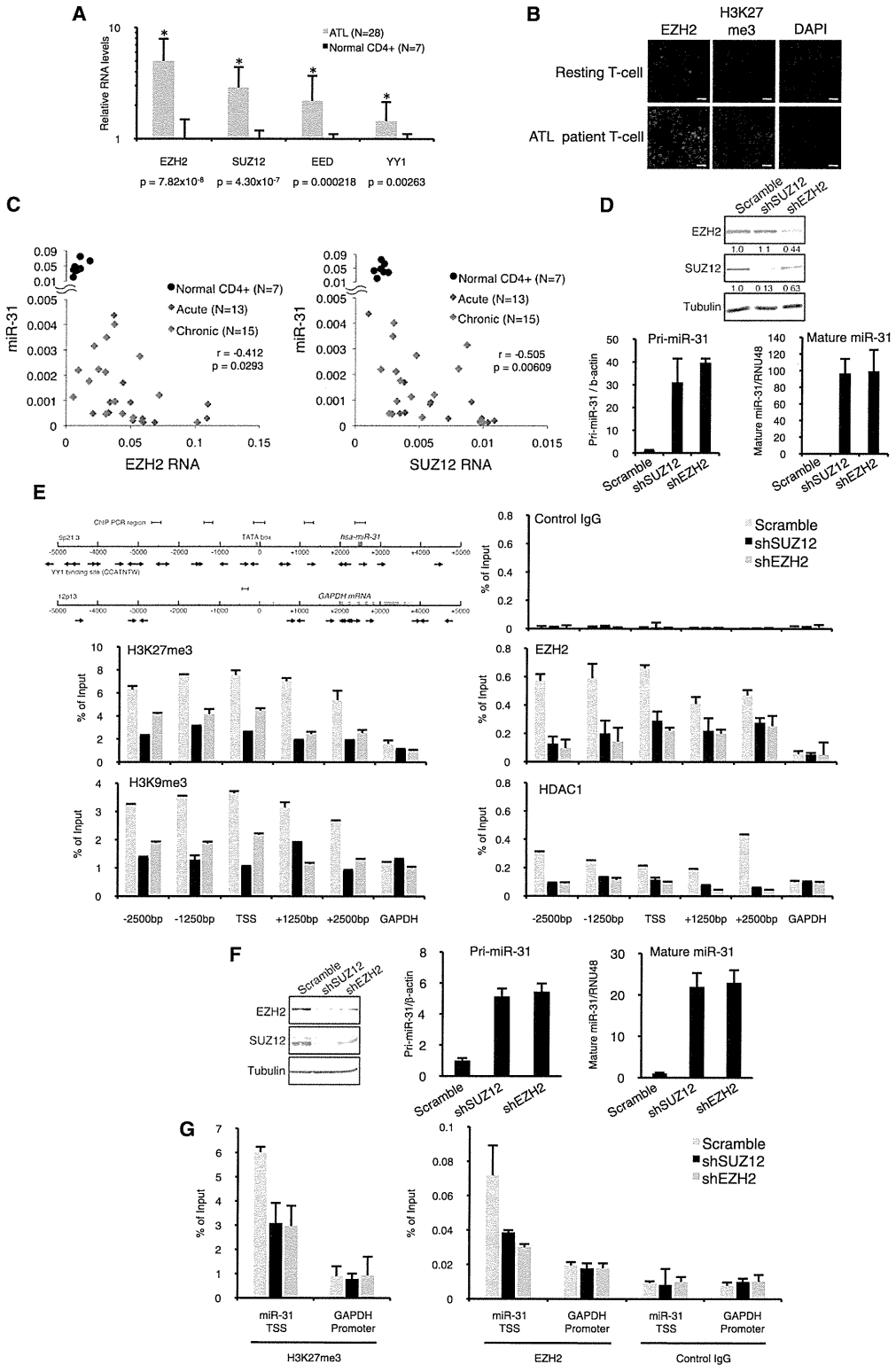
(C) PCR-based miR-31 quantifications in primary ATL samples. ATL samples without genetic loss in miR-31 region (n = 9, Figure S3B), and normal CD4+ T cells (n = 7) were tested. p values (ATL versus normal) are shown.

(D) YY1 binding motif cluster around transcriptional start site (TSS) of miR-31 region. Arrows represent positions of the motifs. Regions of PCR amplification for ChIP assay are shown.

(E) Repression-associated histone methylation in miR-31 region determined by ChIP assay (n = 3, mean ± SD). The results of relative enrichment against input control are presented and distance from miR-31 TSS is described. *MYT1* and *GAPDH* promoters are as positive or negative controls, respectively.

(F–I) YY1-dependent EZH2 occupancy in miR-31 locus. (F) YY1 knockdown in TL-Om1 cells. qRT-PCR (left, n = 3, mean ± SD) and western blotting (right) showed decreased YY1 level. (G) YY1 knockdown led to both primary and mature miR-31 restoration in TL-Om1 cells (n = 3, mean ± SD). (H) YY1 occupancy in miR-31 region analyzed by ChIP (n = 3, mean ± SD). YY1 occupancy in miR-31 locus was reduced by YY1 knockdown. (I) EZH2 occupancy in miR-31 region analyzed by ChIP (n = 3, mean ± SD). YY1 knockdown inhibited EZH2 recruitment in miR-31 region.

(J) Aberrant accumulation of repression-associated histone methylations widely in miR-31 region of primary ATL cells. PBMCs freshly isolated from ATL patients (n = 6) were analyzed by ChIP assay. PBMC from healthy adults were used for normal controls. See also Figure S3.


A
Relative RNA levels
ATL (N=28)
Normal CD4+ (N=7)
EZH2
SUZ12
EED
YY1
p = 7.82x10-8
p = 4.30x10-7
p = 0.000218
p = 0.00263
B
EZH2
H3K27 me3
DAPI
Resting T-cell
ATL patient T-cell
C
miR-31
Normal CD4+ (N=7)
Acute (N=13)
Chronic (N=15)
r = -0.412
p = 0.0293
EZH2 RNA
r = -0.505
p = 0.00609
SUZ12 RNA
D
Scramble
shSUZ12
shEZH2
EZH2
SUZ12
Tubulin
Pri-miR-31
Pri-miR-31 / b-actin
Mature miR-31
Mature miR-31/RNU48
E
Control IgG
Scramble
shSUZ12
shEZH2
% of Input
H3K27me3
EZH2
H3K9me3
HDAC1
-2500bp
-1250bp
TSS
+1250bp
+2500bp
GAPDH
F
Pri-miR-31
Mature miR-31
G
miR-31 TSS
GAPDH Promoter
H3K27me3
EZH2
Control IgG

including ATL, diffuse large B cell lymphoma (DLBCL), Hodgkin lymphoma, breast cancer, prostate cancer and others (Prasad et al., 2010). NF-κB is also essential for various cell functions, including inflammation, innate immunity, and lymphocytic development (Hayden and Ghosh, 2008). Identification of NF-κB determinants will lead to marked progress in understanding molecular pathology.

Our global analyses demonstrated an interesting miRNA expression signature as well as an aberrant mRNA expression profile, which may be associated with leukemogenesis in the primary ATL cells (Figures 1 and 6A). We revealed downregulation of tumor-suppressive miRNA including Let-7 family, miR-125b, and miR-146b, which can contribute to aberrant tumor cell signaling. Recent studies have suggested unique expression profiles of miRNAs in ATL (Yeung et al., 2008; Bellon et al., 2009), but loss of miR-31 has not been focused. Cellular amount of miRNAs may be susceptible to various environments such as transcriptional activity, maturation processing, and also epigenetic regulation. The end results appear to be affected by methodology employed and conditions and types of samples used. Our integrated expression profiling of primary ATL cells are based on a significantly larger number of samples and fruitfully provides intriguing information that may be useful in improving the understanding of T cell biology as well as in the identification of biomarkers for diagnosis.

Pleiotropy of miR-31 was first reported by Valastyan et al. (2009). The authors elegantly demonstrated the function of miR-31 in vivo and also identified several target genes that contribute to cell migration and invasiveness. In the present study, we focused on the functional significance of miR-31 in the regulation of NF-κB signaling that contributes to tumor cell survival.

Overexpression of NIK acts as an oncogenic driver in various cancers. In the present study, NIK was identified as a miR-31 target based on several lines of evidence. First, luciferase-3′ UTR reporter assay showed that *NIK* 3′ UTR sequence has a role for negative regulation (Figure S1B). By combining a specific inhibitor and mutations in miR-31-binding site, we demonstrated that miR-31 recognizes and negatively regulates the *NIK* 3′ UTR (Figures 2A and 2D). Second, by introducing a miR-31 precursor or inhibitor, we showed that amount of miR-31 inversely correlates with levels of NIK expression and downstream signaling (Figures 2E–2K). Third, genetic evidence indicated strong base pairing and biological conservation (Bartel, 2009) (Figures S1L–S1O). Our experimental approach illustrated that mmu-miR-31 regulates mouse *Map3k14* gene. Fourth, individual assessments using gene expression data clearly revealed an inverse correlation between the expression levels of miR-31 and *NIK* (Figure 3A). Collectively, we provide definitive evidence for the notion that miR-31 negatively regulates NIK expression and activity.

It is well known that the NIK level directly regulates NF-κB activity in various cell types (Thu and Richmond, 2010). We experimentally showed that miR-31 regulates noncanonical NF-κB activation stimulated by BAFF and CD40L, both of which are major B cell activating cytokines. Since signals from receptors are essential for the development and activity of B cells, the negative role of miR-31 in cytokines-induced NIK accumulation appears to be widely important in the noncanonical regulation of NF-κB in B cells and other cell types (Figures 2H–2K). Again, our findings revealed the role of NIK in the regulation of canonical NF-κB pathway. Strict regulation of NIK appears to be closely associated with the fate of lymphocytes.

The level of miR-31 was drastically suppressed in all tested primary ATL cells, and its magnitude is greater than that which has been reported in other cancers. Our results demonstrated a profound downregulation of miR-31 (fold change, 0.00403; Figure 1B) in all ATL cases, suggesting that miR-31 loss is a prerequisite for ATL development. Restoration of miR-31-repressed NF-κB activity in ATL cells, resulting in impairment of the proliferative index and apoptosis resistance (Figure 3). Furthermore, our results demonstrate that inhibition of NF-κB promotes tumor cell death in cell lines and also primary tumor cells from ATL patients (Figures 3 and 6), which are consistent with our previous observation (Watanabe et al., 2005). Since it is highly possible that miR-31 and relevant factors are pivotal in cancers, their expressions would have a great importance in view of biomarkers for the aberrant signaling and clinical outcomes.

By studying clinical samples and in vitro and ex vivo models, we obtained several biologically interesting results. First, we identified the Polycomb protein complex as a strong suppressor of miR-31. Generally, the Polycomb group constitutes a multimeric complex that negatively controls a large number of genes involved in cellular development, reproduction, and stemness (Sparmann and van Lohuizen et al., 2006). However, the key molecules involved in cancer development, progression, and prognosis are not yet fully understood. In breast and prostate cancers, oncogenic functions of EZH2 and NF-κB activation were reported independently (Kleer et al., 2003; Varambally et al., 2002; Suh and Rabson, 2004). Interestingly, these tumors show low miR-31 levels (Valastyan et al., 2009; Schaefer et al., 2010). Recently, Min et al. (2010) reported that EZH2 activates NF-κB by silencing the DAB2IP gene in prostate cancer cells.

Figure 5. Amount of PRC2 Components Epigenetically Links to miR-31 Expression in T Cells and Epithelial Cells

(A) Overexpression of PRC2 components in primary ATL cells measured by qRT-PCR (ATL, n = 28; normal, n = 7; mean ± SD). These results were supported by the data of gene expression microarray (Table S3).

(B) Escalation of EZH2 protein and trimethylated H3K27 levels in primary ATL cells illustrated by immunocytostaining (n = 4, a representative result is shown). Resting T cells were as normal control. Scale bars = 20 μm.

(C) Statistical correlation among the levels of miR-31, *EZH2*, and *SUZ12* in individual ATL samples. Correlation coefficients within ATL samples are shown in the graphs.

(D and E) Loss of PCR2 function causes chromatin rearrangement and miR-31 upregulation. (D) TL-Om1 cells expressing shSUZ12, shEZH2, and scrambled RNA were established by retroviral vector. The levels of EZH2, SUZ12, *Pri-miR-31*, and mature miR-31 were measured by western blotting and qRT-PCR (n = 3, mean ± SD). (E) Results of ChIP assays with indicated antibodies (n = 3, mean ± SD). Amounts of immunoprecipitated DNA were analyzed by region-specific PCR. *GAPDH* promoter served as a region control.

(F and G) Knockdown of Polycomb family proteins in MDA-MB-453 cells. (F) EZH2 and SUZ12 are shown by western blot. miR-31 level was examined by qRT-PCR (n = 3, mean ± SD). (G) Histone methylation and EZH2 occupancy evaluated by ChIP assay (n = 3, mean ± SD). See also Table S3 and Figure S4.

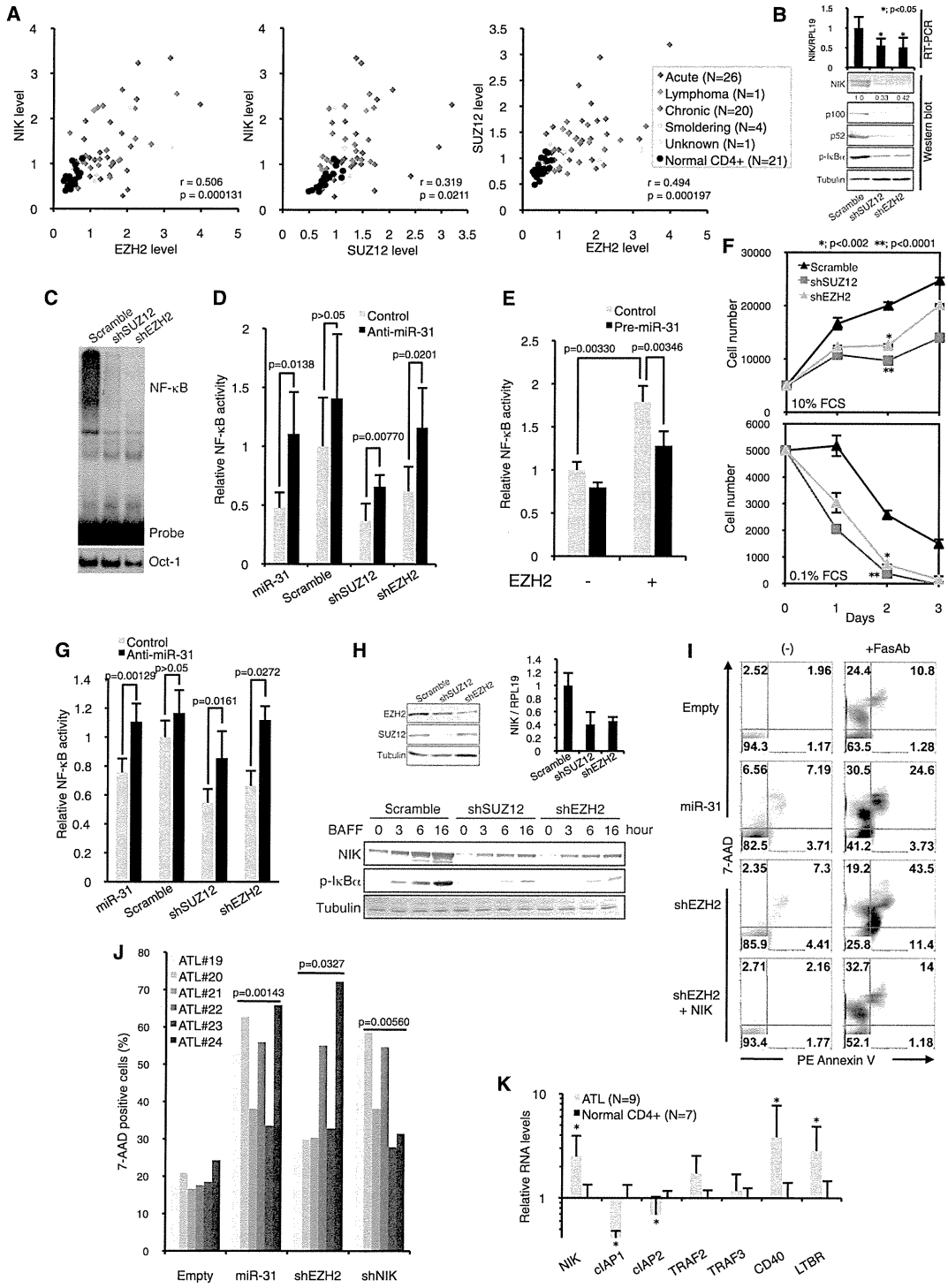


Figure 6. Epigenetic Change Driven by Polycomb Group Mediates NF-κB Signaling through miR-31 Regulation

(A) Reciprocal relationship of mRNA expression between *NIK* and Polycomb group in primary samples. Pearson's correlation coefficients among ATL samples are shown.

(B) PRC2 knockdown negatively affects NF-κB signaling in TL-Om1 cells. After establishment of PRC2 knockdown, the levels of *NIK* RNA (n = 4, mean ± SD) and proteins of NIK, p52/p100, and phospho-IκBα were examined.

(C) Downregulation of NF-κB activity in PRC2-disrupted cells detected by EMSA.

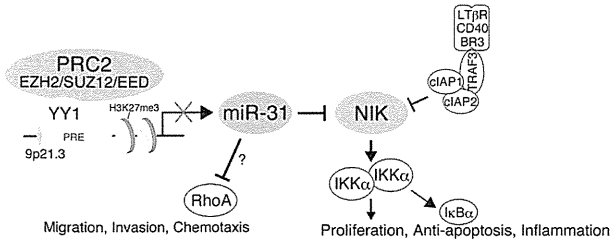


Figure 7. Proposed Model for ATL and Other Tumor Cells
Polycomb repressive factors are linked to NIK-dependent NF-κB activation via miR-31 regulation.

In the present study, we found that the Polycomb group regulates miR-31 expression and that elevated expression of EZH2 leads to NF-κB activation via NIK-miR-31 regulation in ATL and breast cancer cells (Figure 6). We also showed that restoration of miR-31 partially impaired Polycomb-mediated NF-κB operation (Figures 6D, 6E, and 6G), suggesting that miR-31 is involved in this relationship. Furthermore, a connection between NIK and PRC2 was observed in B cells (Figure 6H). Polycomb group proteins are essential in lymphocyte development and activation (Su et al., 2003, 2005). Further, given the NF-κB is a pivotal transcription regulator in normal and oncogenic functions, practical participations of epigenetic regulators and miR-31 in NF-κB signaling will increase our understanding of the molecular mechanisms of T cell functions. For generalization of the molecular axis in other cancers and normal cells, further study will be needed.

Second, YY1 is a recruiter of PRC2 to the miR-31 region. In humans, the Polycomb response element (PRE) has not been precisely identified. A good candidate for a mammalian recruiter of PRC2 is YY1, the homolog of *D. melanogaster* PHO (Simon and Kingston, 2009). We found an assembly of the YY1 binding motif in the miR-31 locus and demonstrated that YY1 knockdown dislodged EZH2 in this region (Figure 4I), which supports previous findings (Caretti et al., 2004). The detailed mechanism by which YY1 mediates recruitment of the Polycomb family may be important in the context of epigenetic regulation of orchestrated gene expression and T cell functions.

Third, Polycomb family proteins can control miRNA expression in an epigenetic fashion. The amount of PRC2 factors strongly influenced the degree of suppression of miR-31 expression (Figure 5). We speculate that, in addition to controlling the transcription, the Polycomb group can modulate translation via miRNA regulation. Furthermore, miR-101 and miR-26a are known to regulate EZH2 expression (Sander et al., 2008; Varambally et al., 2008), which is supported by our observation (Figure S4C). This signaling circuit will permit multiple gene regulation. Whereas genetic loss at the miR-31 locus is observed in some cases of ATL (Figure 4A), no genetic deletion in the miR-101-1 or miR-101-2 region was detected in ATL, which is not consistent with a previous finding in prostate cancer. Our results also suggested putative association between Let-7 family and EZH2 (Figure S4). Aberrant downregulations of these miRNAs in the primary ATL cells will be the next important questions to be addressed in efforts to improve understanding of the oncogenic signaling network.

By collaborative profiling of miRNA and mRNA expression, we identified a notable relationship between ATL subtypes and a gene cluster that contains miR-31, NIK, EZH2, and SUZ12. This finding suggests that an aberrant gene expression pattern correlates with the malignant phenotype, and this provides important clues about clinical manifestations and may help identify therapeutic targets against ATL cells (Figure 6A). Although HDAC inhibitors did not show effective responses (Figures S4I and S4J), emerging epigenetic drug such an EZH2 inhibitor (Fiskus et al., 2009) may pave a pathway leading to cures for various malignancies that involve constitutive activation of NF-κB.

In summary, we show that genetic and epigenetic loss of miR-31 is responsible for oncogenic NF-κB activation and malignant phenotypes in ATL. This provides evidence for the idea that miR-31 is an important tumor suppressor. An emerging pathway involving an epigenetic process, miR-31, and NF-κB will provide a conceptual advance in epigenetic reprogramming, inflammatory signaling, and oncogenic addiction.

EXPERIMENTAL PROCEDURES

Cell Lines and Primary ATL Cells

The primary peripheral blood mononuclear cells (PBMCs) from ATL patients and healthy volunteers used in the present work were a part of those collected with an informed consent as a collaborative project of the Joint Study on Prognostic Factors of ATL Development (JSPFAD). The project was approved by the Institute of Medical Sciences, the University of Tokyo (IMSUT) Human Genome Research Ethics Committee. Additional ATL clinical samples for copy number analysis were provided by Drs. Y. Yamada, Nagasaki University,

(D) NF-κB activity evaluated by reporter assays in the presence or absence of miR-31 inhibitor (n = 5, mean ± SD). Anti-miR-31 treatment partially rescued the NF-κB activity in PRC2 knockdowned TL-Om1 cells.
(E) Overexpressed EZH2 activates NF-κB via miR-31. Jurkat cells were transfected with an EZH2 plasmid together with miR-31 precursor or control RNA (n = 5, mean ± SD).
(F) PRC2 dysfunction changes TL-Om1 cell proliferation and response to serum starvation. Under conditions of 10% or 0.1% of FCS, cell growth curves were examined (n = 3, mean ± SD). PRC2 downregulation decreased cell growth with statistical significance.
(G) NF-κB activity in PRC2-knockdowned MDA-MB-453 cells in the presence or absence of miR-31 inhibitor were examined (n = 5, mean ± SD).
(H) PRC2 disruption inhibits BAFF-dependent NIK accumulation and IκBα phosphorylation in BJAB cells.
(I) Apoptotic cell death induced by lentivirus-mediated EZH2 knockdown in TL-Om1. Venus-positive populations were analyzed by Annexin V/7-AAD stainings (n = 3) and representative of FACS data are shown.
(J) Summary of primary tumor cell death. Lentivirus-based miR-31 expression, NIK knockdown, and EZH2 knockdown showed killing effects in six primary ATL samples. Statistical significances are shown in the graph. Results of FACS and qRT-PCR are shown in Figures S5I and S5J.
(K) Expression levels of genes involved in noncanonical NF-κB pathway in primary ATL cells (ATL, n = 9; normal, n = 7; mean ± SD). Relative expression levels were tested by qRT-PCR (*$p < 0.05$). See also Figure S5.

and K. Ohshima, Kurume University, where the projects were approved by the Research Ethics Committees of Nagasaki University and Kurume University, respectively. PBMC were isolated by Ficoll separation. ATL cells, primary lymphocytes, and all T cell lines were maintained in RPMI1640 supplemented with 10% of FCS and antibiotics. Clinical information of ATL samples is provided in Table S1.

Expression Analyses

Clinical samples for microarrays were collected by a collaborative study group, JSPFAD (Iwanaga et al., 2010). Gene expression microarray was used 4x44K Whole Human Genome Oligo Microarray (Agilent Technologies) and miRNA microarray was used Human miRNA microarray kit v2 (Agilent Technologies), respectively. Quantitative RT-PCR was performed with SYBRGreen (TAKARA). Mature miRNA assays were purchased from Applied Biosystems.

Copy Number Analyses

Genomic DNA from ATL patients was provided from the material bank of JSPFAD, Nagasaki University, and Kurume University, and was analyzed by Affymetrix GeneChip Human Mapping 250K Nsp Array (Affymetrix). Obtained data were analyzed by CNAG/AsCNAR program (Chen et al., 2008).

Oligonucleotides, Plasmids, and Retrovirus Vectors

All RT-PCR primers and oligonucleotides are described in Supplemental Experimental Procedures. miRNA precursor and inhibitor were from Applied Biosystems. Transfection of small RNA and other plasmid DNA were performed by Lipofectamine2000 (Invitrogen). For miRNA or shRNA expression, retroviral vectors (pSINsi-U6, TAKARA) were used.

3′ UTR-Conjugated miR-31 Reporter Assay

HeLa cells were cotransfected with 3′ UTR-inserted pMIR-REPORT firefly plasmid (Ambion), RSV-Renilla luciferase plasmid, and miRNA inhibitor. The cells were collected at 24 hr posttransfection, and Dual-luciferase reporter assay was performed (Promega).

Analysis of NF-κB Pathway

NF-κB activity was evaluated by EMSA and reporter assays as previously described (Horie et al., 2004). Antibodies for western blots are described in supplemental information. Cell proliferative assay was performed by Cell Counting Kit-8 (Dojindo).

Lentivirus Vectors and Apoptosis Analysis

A lentivirus vector (CS-H1-EVBsd) was provided from RIKEN, BRC, Japan. Lentivirus solution was produced by cotransfection with packaging plasmid (pCAG-HIVgp) and VSV-G- and Rev-expressing plasmid (pCMV-VSV-G-RSV-Rev) into 293FT cells. After infection of lentivirus, the apoptotic cell was evaluated by PE Annexin V / 7-AAD staining (BD PharMingen) and analyzed by FACS Calibur (Becton, Dickinson). Collected data were analyzed by FlowJo software (Tree Star).

ChIP Assay

ChIP assay was previously described (Yamagishi et al., 2009). Briefly, cells were crosslinked with 1% of formaldehyde, sonicated, and subjected to chromatin-conjugated IP using specific antibodies. Precipitated DNA was purified and analyzed by real-time PCR with specific primers (see Supplemental Experimental Procedures).

Computational Prediction

To identify miR-31 target genes, we integrated the output results of multiple prediction programs; TargetScan, PicTar, miRanda, and PITA. RNAhybrid was for secondary structure of miRNA-3′ UTR hybrid. TSSG program was for TATA box and TSS predictions. DNA methylation site was predicted by CpG island Searcher.

Statistical Analyses

Data were analyzed as follows: (1) Welch's t test for Gene Expression Microarray (p value cutoff at 10^{-6}) and miRNA Microarray (p value cutoff at 10^{-5}); (2) Pearson's correlation for two-dimensional hierarchical clustering analysis and individual assessment of microarray data sets; (3) two-tailed pared Student's t test with $p < 0.05$ considered statistically significant for in vitro cell lines and primary cells experiments, including luciferase assay, RT-PCR, ChIP assay, cell growth assay, and migration assay. Data are presented as mean ± SD.

ACCESSION NUMBERS

Coordinates have been deposited in Gene Expression Omnibus database with accession numbers, GSE31629 (miRNA microarray), GSE33615 (gene expression microarray), and GSE33602 (copy number analyses).

SUPPLEMENTAL INFORMATION

Supplemental Information includes three tables, five figures, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.ccr.2011.12.015.

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Allogeneic hematopoietic stem cell transplantation for adult T-cell leukemia-lymphoma with special emphasis on preconditioning regimen: a nationwide retrospective study

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Adult T-cell leukemia-lymphoma (ATL) is an intractable mature T-cell neoplasm. We performed a nationwide retrospective study of allogeneic hematopoietic stem cell transplantation (HSCT) for ATL in Japan, with special emphasis on the effects of the preconditioning regimen. This is the largest study of ATL patients receiving HSCT. Median overall survival (OS) and 3-year OS of bone marrow or peripheral blood transplantation recipients (n = 586) was 9.9 months (95% confidence interval, 7.4-13.2 months) and 36% (32%-41%), respectively. These values for recipients of myeloablative conditioning (MAC; n = 280) and reduced intensity conditioning (RIC; n = 306) were 9.5 months (6.7-18.0 months) and 39% (33%-45%) and 10.0 months (7.2-14.0 months) and 34% (29%-40%), respectively. Multivariate analysis demonstrated 5 significant variables contributing to poorer OS, namely, older age, male sex, not in complete remission, poor performance status, and transplantation from unrelated donors. Although no significant difference in OS between MAC and RIC was observed, there was a trend indicating that RIC contributed to better OS in older patients. Regarding mortality, RIC was significantly associated with ATL-related mortality compared with MAC. In conclusion, allogeneic HSCT not only with MAC but also with RIC is an effective treatment resulting in long-term survival in selected patients with ATL. (*Blood*. 2012;120(8):1734-1741)

Introduction

Adult T-cell leukemia-lymphoma (ATL) is an aggressive peripheral T-cell neoplasm caused by human T-cell lymphotropic/leukemia virus type-1. It has a very poor prognosis.[1-4] A recent phase 3 trial for previously untreated patients with aggressive ATL (acute, lymphoma, or unfavorable chronic type) aged 33 to 69 years demonstrated that the dose-intensified multidrug regimen VCAP-AMP-VECP resulted in a median overall survival (OS) and OS at 3 years of 12.7 months and 24%, respectively. The OS plot for this treatment did not reach a plateau.[5] Alternatively, based on a meta-analysis, Bazarbachi et al proposed that zidovudine (AZT) and interferon (IFN)–α should be considered the standard for first-line therapy in patients with acute, chronic, or smoldering types of ATL. They reported median OS and 5-year OS for acute-type ATL treated with AZT/IFN-α to be 9 months and 28%, respectively, whereas these values were 7% and 0%, respectively, for lymphoma-type ATL.[6] These results indicate that conventional chemotherapeutic agents alone, even including AZT/IFN-α, yield few or no long-term remissions or potential cures in ATL patients.

Although early experience in myeloablative chemoradiotherapy together with autologous hematopoietic stem cell rescue for ATL was associated with a high incidence of relapse and fatal toxicities,[7] allogeneic hematopoietic stem cell transplantation (HSCT) has been explored as a promising alternative treatment that can provide long-term remission in a proportion of patients with ATL.[8-10] Therefore, we previously performed a nationwide retrospective study of ATL patients who received allogeneic HSCT in Japan before December 31, 2005, with special emphasis on the effect of the graft source: 296 patients received bone marrow (BM) and/or peripheral blood stem cells (PBSCs) and 90 received cord blood.[11] We concluded that allogeneic HSCT using currently available sources is an effective treatment in selected patients with ATL, although greater effort is warranted to reduce treatment-related

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mortality (TRM). In addition, the use of unrelated cord blood as a stem cell source was associated with lower survival, with a median OS and unadjusted 3-year probability of OS of 2.6 months and 17% (95% confidence interval [CI], 9%-25%), respectively. Because the results suggested that allogeneic BM and PBSCs could be considered to be the more standard donor forms, rather than unrelated cord blood, for transplantation in ATL, as a next step, here we report results of a nationwide retrospective study of Japanese ATL patients receiving allogeneic HSCT, especially focusing on bone marrow transplantation (BMT) and peripheral blood stem cell transplantation (PBSCT), with special emphasis on the effects of the preconditioning regimen. Our current analysis included the previous cohort[11] (January 1996–December 2005) with updated clinical information as well as data on one patient who received allogeneic HSCT in February 1992 and patients who received allogeneic HSCT after December 2005. It is thought that allogeneic HSCT with reduced intensity conditioning (RIC) depends more on donor cellular immune effects after transplantation and less on the cytotoxic effects of the conditioning regimen to eradicate residual tumor cells than conventional myeloablative conditioning (MAC). In this context, RIC might be suitable for ATL because several reports have suggested the existence of graft-versus-T-cell lymphotropic/leukemia virus type-1 or graft-versus-ATL effects.[12-18] In addition, RIC might be associated with reduced TRM, which has represented a significant obstacle to successful allogeneic HSCT for ATL patients.[11] Furthermore, ATL has a long latency and occurs in older individuals at a median age of nearly 60 years.[19,20] There is the possibility that HSCT with RIC can provide clinical benefits for those older patients who hardly benefit from allogeneic HSCT with MAC. Here, we performed multivariate analyses of OS and treatment-related or ATL-related mortality after allogeneic BMT and PBSCT and have identified factors influencing transplantation outcomes in ATL patients.

Methods

Collection of data

Data on patients with ATL who had received their first allogeneic BMT, PBSCT, or BMT + PBSCT between February 1992 and December 2009 were collected from nationwide survey data of the Japan Society for Hematopoietic Cell Transplantation (JSHCT). Cases with missing preconditioning or survival data were excluded, with the result that 586 patients were included in the analysis. Data collected for analysis included the patients' clinical characteristics such as age at transplantation, sex, disease status at transplantation, date of transplantation, time from ATL diagnosis to transplantation, performance status (PS) according to the Eastern Cooperative Oncology Group criteria at transplantation, source of stem cells, relationship between recipient and donor, ATL clinical subtype,[1] preconditioning regimens, date alive at last follow up, date and cause of death, and incidence and severity of acute graft-versus-host disease (GVHD). When serologic or molecular typing for HLA-A, HLA-B, and HLA-DR were identical between the recipient and the related donor, we determined the relationship as HLA-matched related. As a control, data on patients with ATL who had received their first unrelated cord blood transplantation (CBT) between March 2001 and December 2009 were collected from the nationwide survey data of the JSHCT. Cases with missing survival data were excluded, resulting in the inclusion of 174 patients in the present study. The study was approved by the data management committees of the JSHCT, as well as by the institutional ethics committee of Nagoya City University Graduate School of Medical Sciences.

Definitions

OS was defined as the time from transplantation until death, and patients who remained alive at the time of the last follow-up were censored. For analysis, patients were divided into 2 age groups, either $>$ or $\leq$ 55 years, because the Japanese Clinical Oncology Group is currently conducting a phase 2 study of strategies including allogeneic HSCT other than CBT with MAC for ATL patients aged 20 to 55 years (UMIN000004147). Reported causes of death were reviewed and categorized into ATL-related or TRM. ATL-related mortality was defined as death caused by relapse or progression of ATL in patients who survived for at least 1.0 month after transplantation based on the judgment of each institution. TRM was defined as any death other than ATL-related mortality. Acute GVHD was diagnosed and graded using traditional criteria[21] by the physicians who performed transplantations at each institution. Patients undergoing allogeneic BMT or PBSCT were divided into 2 groups based on the preconditioning regimens, with 1 group being MAC and the other group RIC. MAC or RIC was defined according to the proposals by Giralt et al[22] and Bacigalupo et al,[23] with a slight modification. In the present study, MAC was defined as any regimen that includes (1) $\geq$ 5 Gy of total body irradiation (TBI) as a single fraction or $\geq$ 8 Gy fractionated, (2) busulfan (BU) $>$ 8 mg/kg orally or the intravenous equivalent, or (3) melphalan (Mel) $>$ 140 mg/m^2. All other regimens were classified as RIC. MAC was further subdivided into 4 groups as follows: TBI (n = 208), BU (n = 46), Mel (n = 21), and other types (n = 3). RIC also was subdivided into 3 groups: fludarabine (Flu) + BU (n = 165), Flu + Mel (n = 86), and other types (n = 49).

Statistical analysis

Descriptive statistics were used for summarizing variables related to patient demographics and transplant characteristics. Comparisons among the groups were performed by Fisher exact test as appropriate for categorical variables. The probability of OS was estimated according to the Kaplan-Meier method. The Cox proportional hazard model was used for multivariate analyses for OS using all independent variables in the model and then using a stepwise selection method by minimizing the Akaike Information Criterion (AIC). The AIC penalizes overparametrization, and variables are retained only when the model improves enough to balance the number of parameters. The lower the AIC, the better the predictive model fits the data.[24] Our inspection of plots of OS estimates versus follow-up time indicated that the assumption of proportional hazards for all variables used seemed to be valid. In the Cox proportional hazard model, incidence and severity of acute GVHD was treated as a time-varying covariate[25] as described previously.[12] Fine and Gray proportional hazard modeling was used to estimate the effect of the same variables used in multivariate analysis of OS on the cumulative incidence of TRM and ATL-related mortality, respectively.[26,27] All analyses including competing risk analysis[28,29] were performed using the cmprsk package of R Version 2.9.0 for Windows statistics software. Statistical significance was set at $P < .05$.

Results

Patients' characteristics

Among 586 ATL patients who received allogeneic BMT or PBSCT (mean age, 52 years; median, 53 years; range, 15-72 years), 280 received MAC (mean age, 48 years, median, 49 years; range, 15-69 years) and the remaining 306 received RIC (mean age, 56 years; median, 57 years; range, 28-72 years). Characteristics of these ATL patients are shown in Table 1. In comparison with MAC recipients, significantly more RIC recipients belonged to the older age group (56-72 years), more often received PBSCs as the stem cell source and more frequently had a related donor transplantation. There was no significant difference between MAC and RIC recipients regarding PS distribution from 0 to 4, but unknown PS was observed in significantly more MAC recipients than RIC recipients. There were no significant differences between MAC and

Table 1. Characteristics of ATL patients receiving allogeneic HSCT

Characteristic	MAC	RIC	*P*
Total patients, no. (%)	280	306	
Age range at transplantation, y			< .001
15-55	248 (89)	124 (41)	
56-72	32 (11)	182 (59)	
Sex			.135
Female	120 (43)	151 (49)	
Male	160 (57)	155 (51)	
Disease status at transplantation			.206
CR	96 (34)	112 (37)	
Non-CR	160 (57)	179 (58)	
Unknown	24 (9)	15 (5)	
Year.month of transplantation			.473
1992.2-2004.12	71 (25)	78 (25)	
2005.1-2006.11	69 (25)	77 (25)	
2006.11-2008.5	76 (27)	68 (22)	
2008.5-2009.12	64 (23)	83 (27)	
Time from diagnosis to transplantation, mo			.569
0.5-4.9	74 (26)	72 (24)	
4.9-6.9	66 (24)	79 (26)	
6.9-10.1	74 (26)	71 (23)	
≥10.1	65 (23)	81 (26)	
PS at transplantation			.004
0	102 (36)	119 (39)	
1	121 (43)	143 (47)	
2	29 (10)	25 (8)	
3	4 (1)	12 (4)	
4	3 (1)	2 (1)	
Unknown	21 (8)	5 (2)	
Source of stem cells			< .001
BM	212 (76)	186 (60)	
Peripheral blood	68 (24)	118 (39)	
BM + peripheral blood	0 (0)	2 (1)	
Relationship between recipient and donor			.019
HLA-matched related	96 (34)	117 (38)	
HLA-mismatched related	21 (8)	42 (14)	
HLA-unknown related	1 (0)	1 (0)	
Unrelated	162 (58)	146 (48)	
ATL clinical subtype			.253
Chronic, smoldering	10 (4)	6 (2)	
Acute	163 (58)	170 (56)	
Lymphoma	79 (28)	87 (28)	
Unknown	28 (10)	43 (14)	

RIC recipients regarding sex, disease status at transplantation (in complete remission [CR], not in CR, or unknown), and ATL clinical subtypes (chronic/smoldering, acute, lymphoma, or unknown). There were also no significant differences between MAC and RIC recipients regarding the date of transplantation and time from diagnosis to transplantation, both of which were equally distributed in quartiles among the 586 cases.

The 174 ATL patients who received unrelated CBT were aged 54 years, on average, with a median of 55 years and range of 27 to 79 years. There were 69 females and 105 males, with an ATL status at transplantation of CR (n = 50), not in CR (n = 115), and unknown (n = 9).

As for infectious complications, 145 of the 280 MAC recipients had bacterial infection, and 94 did not. Information on bacterial infection was missing for the remaining 41 MAC recipients. As for fungal infection, 23 and 219, respectively, did and did not have fungal infection; no such information was available on 38 patients. As to viral infection, 65 and 177, respectively, did and did not experience a viral infection, with such data missing on the remaining 38 patients. When we examined data on infectious complications in the RIC recipients, we found that of the 306 RIC recipients 134 had bacterial infection and 121 did not, with data unavailable for the remaining 51 patients. Twenty-three RIC recipients had fungal infection and 232 did not; no such information was available for 51 patients. As to viral infection, 57 and 199 patients, respectively, had and did not have viral infection; no information was available on the remaining 50 patients.

OS of patients receiving allogeneic HSCT

The unadjusted 3-year probability of OS was 36% (95% CI, 32%-41%) in the 586 ATL patients receiving allogeneic BMT or PBSCT and 21% (95% CI, 15%-29%) in the 174 patients receiving unrelated CBT. The median OS of the former was 9.9 months (95% CI, 7.4-13.2 months) and of the latter, 4.3 months (95% CI, 3.2-6.5 months; Figure 1A).

The unadjusted 3-year probability of OS was 39% (95% CI, 33%-45%) in the 280 ATL patients receiving MAC and 34% (95% CI, 29%-40%) in the 306 patients receiving RIC. The median OS of the former was 9.5 months (95% CI, 6.7-18.0 months), and of the latter 10.0 months (95% CI, 7.2-14.0 months; Figure 1B).

Multivariate analysis of factors influencing OS in ATL patients receiving allogeneic BMT or PBSCT

Of the 586 ATL patients receiving allogeneic HSCT other than unrelated CBT, 4 were excluded because of lack of data on the time from diagnosis to transplantation, 2 were excluded because of receiving BMT and PBSCT together, and 2 were excluded because of lack of data on HLA. Multivariate analysis of OS was therefore conducted on a total of 578 patients (Table 2). The following 10 variables were analyzed: age (15-55 or 56-72 years), sex, disease status (CR, not CR, or unknown), date of transplantation (1992.2-2004.12, 2004.12-2006.10, 2006.10-2008.4, or 2008.4-2009.12), time

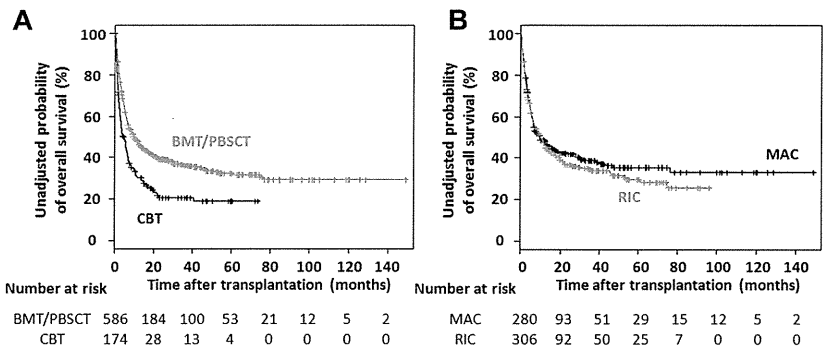

Figure 1. OS of ATL patients receiving allogeneic HSCT. (A) Kaplan-Meier curves of estimated OS in ATL patients receiving allogeneic BMT, PBSCT, or unrelated CBT. (B) Kaplan-Meier curves of estimated OS in ATL patients receiving allogeneic BMT or PBSCT with MAC or RIC.

Table 2. Multivariate analysis of factors influencing OS in ATL patients receiving allogeneic HSCT

Variable	No.	HR	95% CI	*P*
Age range at transplantation, y				
15-55	368	1.000		Reference
56-72	210	1.334	(1.035-1.719)	.026
Sex				
Female	267	1.000		Reference
Male	311	1.376	(1.113-1.702)	.003
Disease status at transplantation				
CR	205	1.000		Reference
Non-CR	335	1.940	(1.511-2.490)	< .001
Unknown	38	1.744	(1.114-2.731)	.015
PS				
0	219	1.000		Reference
1	260	1.498	(1.171-1.916)	.001
2-4	74	4.057	(2.957-5.565)	< .001
Unknown	25	1.489	(0.863-2.570)	.153
Relationship between recipient and donor				
HLA-matched related	210	1.000		Reference
HLA-mismatched related	62	1.296	(0.917-1.831)	.142
Unrelated	306	1.276	(1.009-1.613)	.042
Preconditioning regimen				
MAC	278	1.000		Reference
RIC	300	1.087	(0.845-1.398)	.515

from diagnosis to transplantation (0.5-4.9, 4.9-6.9, 6.9-10.1, or 10.1-143.2 months), PS (0, 1, 2-4, or unknown), source of stem cells (BM or PBSCs), relationship between recipient and donor (HLA-matched related, HLA-mismatched related, or unrelated), ATL clinical subtype (chronic/smoldering, acute, lymphoma, or unknown), and preconditioning regimen (MAC or RIC). Five variables, age, sex, disease status, PS, and relationship between recipient and donor, were retained by stepwise Cox regression analysis by minimizing the AIC, as was the preconditioning regimen, which received special emphasis in this study. Of these 6 variables, the following 5 significantly affected OS: older age (56-72 years compared with 15-55 years; hazard ratio [HR], 1.334; 95% CI, 1.035-1.719), male sex (HR, 1.376; 95% CI, 1.113-1.702), not being in CR compared with CR (HR, 1.940; 95% CI, 1.511-2.490), worse PS (1 compared with 0; HR, 1.498; 95% CI, 1.171-1.916, 2-4 compared with 0; HR, 4.057; 95% CI, 2.957-5.565), and transplantation from an unrelated donor compared with HLA-matched related donor (HR 1.276; 95% CI, 1.009-1.613).

Multivariate analysis of factors influencing OS including acute GVHD in ATL patients receiving allogeneic BMT or PBSCT

Of the 586 ATL patients receiving allogeneic HSCT other than unrelated CBT, 2 were excluded because of lack of data on HLA and 57 were excluded because of missing any data on the time from transplantation to onset of acute GVHD or the severity of acute GVHD. Thus, multivariate analysis on 527 ATL patients was performed using the following 7 variables: age, sex, disease status, PS, relationship of the donor to the recipient, preconditioning regimen, and incidence and severity of acute GVHD. Of these, 5 variables significantly affected OS; they were male sex (HR, 1.472; 95% CI, 1.168-1.855), not in CR (HR, 1.943; 95% CI, 1.491-2.532), worse PS (1 compared with 0; HR, 1.534; 95% CI, 1.182-1.991, 2-4 compared with 0; HR, 3.223; 95% CI, 2.256-4.605), transplantation from an unrelated donor compared with that from an HLA-matched related donor (HR, 1.449; 95% CI, 1.115-1.882), and acute GVHD. HRs for death of recipients having grades 1 or 2 and 3 or 4 acute GVHD compared with recipients having no acute GVHD were 0.753 (95% CI, 0.576-0.984), and 1.538 (95% CI, 1.123-2.107), respectively (supplemental Table 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). This result suggesting that an appropriate level of acute GVHD contributed to better OS but that severe GVHD contributed to inferior OS was consistent with our previous report.[12] In contrast, the inclusion of a posttransplant time-varying covariate, acute GVHD, into the present study resulted in a decrease in the number of evaluable patients. In addition, the inclusion of patients who died so early after transplantation that onset of acute GVHD would not yet have occurred provided unacceptable bias leading to the finding that recipients without acute GVHD had worse OS compared with recipients with acute GVHD. Thus, we conducted the present subsequent analyses that aimed to clarify the significance of the preconditioning regimen MAC versus RIC in ATL patients by only including time-fixed covariates that were present pretransplantation.

Interactions of the preconditioning regimen with age, disease status, and PS for OS

Statistical interactions between the preconditioning regimens and age, disease status, or PS at transplantation for OS were tested by adding an interaction term into the multivariate analysis that included the following 6 variables: age, sex, disease status,

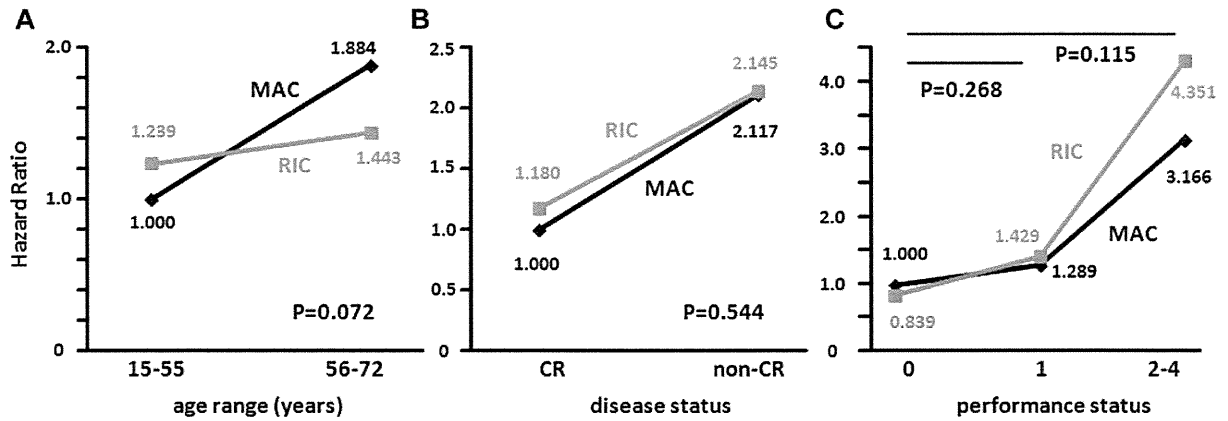

Figure 2. Interactions of the preconditioning regimen with age, disease status, and performance status for OS. Statistical interactions between the preconditioning regimens (MAC or RIC) and age range (15-55 vs 56-72 years; A), disease status (CR vs non-CR; B), and performance status (0 vs 1 or 2-4; C) were analyzed.

Table 3. Multivariate analysis of factors influencing OS in the subgroup of ATL patients receiving transplantation after MAC

Variable	No.	HR	95% CI	P
Age range at transplantation, y				
15-55	246	1.000		Reference
56-72	32	1.667	(1.051-2.643)	.030
Sex				
Female	120	1.000		Reference
Male	158	1.458	(1.053-2.019)	.023
Disease status at transplantation				
CR	95	1.000		Reference
Non-CR	159	2.071	(1.409-3.043)	< .001
Unknown	24	1.536	(0.822-2.870)	.178
PS				
0	102	1.000		Reference
1	120	1.322	(0.909-1.922)	.144
2-4	36	3.073	(1.920-4.919)	< .001
Unknown	20	1.109	(0.565-2.175)	.764
Relationship between recipient and donor				
HLA-matched related	96	1.000		Reference
HLA-mismatched related	21	1.165	(0.618-2.196)	.637
Unrelated	161	1.323	(0.920-1.902)	.131
Type of MAC				
TBI-based	208	1.000		Reference
BU-based	46	0.757	(0.475-1.206)	.242
Mel-based	21	1.388	(0.819-2.353)	.223
Others	3	0.666	(0.158-2.817)	.581

PS, relationship of the donor to the recipient, and preconditioning regimen. Among the 578 patients for whom multivariate analysis for OS was conducted (Table 2), when the HR for death of MAC recipients of a younger age (15-55 years) was determined as 1.000, the HRs of MAC recipients in the older age group (56-72 years) and RIC recipients in the younger and older age groups were 1.884, 1.239, and 1.443, respectively ($P_{interaction} = 0.072$; Figure 2A). When the HR for death of MAC recipients with CR at transplantation was determined as 1.000, HRs of MAC recipients with non-CR and RIC recipients with CR and non-CR were 2.117, 1.180, and 2.145, respectively ($P_{interaction} = 0.544$; Figure 2B). When the HR for death of MAC recipients with PS 0 at transplantation was determined as 1.000, HRs of MAC recipients with PS 1 and RIC recipients with PS 0 and 1 were 1.289, 0.839, and 1.429, respectively ($P_{interaction} = 0.268$), and HRs of MAC and RIC recipients with PS 2 to 4 were 3.166 and 4.351, respectively ($P_{interaction} = 0.115$; Figure 2C).

Multivariate analysis of factors influencing OS in the subgroup of ATL patients who had transplantation after MAC

Of the 280 ATL patients who received MAC, 1 patient was excluded because of missing data on the time from diagnosis to transplantation and one was excluded because of lack of data on HLA. Multivariate analysis was therefore conducted on 278 patients and included the variables of age, sex, disease status, PS, and relationship of the donor to recipient, which were found to have significantly affected OS in the entire subject population (Table 2). Also included was a sixth variable, the type of MAC (TBI, BU, Mel-based, or others). Of these 6 variables, 4 significantly affected OS, namely, older age (HR, 1.667; 95% CI, 1.051-2.643), male sex (HR, 1.458; 95% CI, 1.053-2.019), not in CR (HR, 2.071; 95% CI, 1.409-3.043), and worse PS (2-4 compared with 0; HR, 3.073; 95% CI, 1.920-4.919; Table 3).

Multivariate analysis of factors influencing OS in the subgroup of patients receiving transplantations after RIC

Of the 306 ATL patients receiving RIC, 3 were excluded because of lack of data on the time from diagnosis to transplantation, 2 were excluded because of receiving BMT and PBSCT together, and 1 was excluded because of lack of data on HLA. Thus, multivariate analysis on 300 ATL patients was performed using the following 6 variables: age, sex, disease status, PS, relationship of the donor to the recipient, and type of RIC (Flu + BU, Flu + Mel-based, or others). Of these, 4 significantly affected OS, namely, male sex (HR, 1.475; 95% CI, 1.100-1.978), not in CR (HR, 1.743; 95% CI, 1.249-2.432), worse PS (1 compared with 0; HR, 1.803; 95% CI, 1.293-2.516, 2-4 compared with 0; HR, 6.175; 95% CI, 3.908-9.756), and type of RIC (Flu + Mel compared with Flu + BU based; HR, 0.645; 95% CI, 0.453-0.918; Table 4).

Multivariate analysis of TRM and ATL-related mortality

Among the 586 ATL patients receiving allogeneic BMT or PBSCT, 14 could not be assigned to either the TRM or ATL-related mortality category because detailed information regarding cause of death was missing. The Fine and Gray proportional hazards model was applied to the remaining 572 patients to identify variables affecting TRM and ATL-related mortality, respectively. The variables included age, sex, disease status, PS, and relationship between recipient and donor, which was shown to significant affect OS in the entire patient population (Table 2), and the preconditioning regimen, namely, MAC or RIC. Among these variables, sex and PS were significantly associated with TRM. The HR for TRM of male patients was 1.383 (95% CI, 1.026-1.863). HRs for TRM of recipients with PS 1 and PS 2 to 4 compared with PS 0 were 1.509 (95% CI, 1.075-2.118) and 3.004 (95% CI, 1.915-4.714), respectively. Conversely, disease status, PS, and the preconditioning regimen were significantly associated with ATL-related mortality. HR for ATL-related mortality of recipients not in CR was

Table 4. Multivariate analysis of factors influencing OS in the subgroup of patients receiving transplantation after RIC

Variable	No.	HR	95% CI	P
Age range at transplantation, y				
15-55	122	1.000		Reference
56-72	178	1.127	(0.834-1.523)	.435
Sex				
Female	147	1.000		Reference
Male	153	1.475	(1.100-1.978)	.009
Disease status at transplantation				
CR	110	1.000		Reference
Non-CR	176	1.743	(1.249-2.432)	.001
Unknown	14	1.959	(0.998-3.843)	.051
PS				
0	117	1.000		Reference
1	140	1.803	(1.293-2.516)	< .001
2-4	38	6.175	(3.908-9.756)	< .001
Unknown	5	4.979	(1.849-13.409)	.001
Relationship between recipient and donor				
HLA-matched related	114	1.000		Reference
HLA-mismatched related	41	1.279	(0.836-1.959)	.257
Unrelated	145	1.237	(0.895-1.710)	.198
Type of RIC				
Flu + BU-based	165	1.000		Reference
Flu + Mel-based	86	0.645	(0.453-0.918)	.015
Others	49	0.854	(0.557-1.310)	.470

Table 5. Multivariate analysis of TRM and ATL-related mortalities in patients receiving allogeneic HSCT

	TRM				ATL-related mortality			
Variable	No.	HR	95% CI	P	No.	HR	95% CI	P
Age range at transplantation, y								
15-55	116/362	1.000		Reference	93/362	1.000		Reference
56-72	79/210	1.403	(0.954-2.064)	.085	62/210	0.955	(0.658-1.385)	.810
Sex								
Female	75/262	1.000		Reference	66/262	1.000		Reference
Male	120/310	1.383	(1.026-1.863)	.033	89/310	1.226	(0.886-1.697)	.220
Disease status at transplantation								
CR	58/205	1.000		Reference	32/205	1.000		Reference
Non-CR	121/330	1.238	(0.906-1.691)	0.180	114/330	2.203	(1.469-3.302)	< .001
Unknown	16/37	1.507	(0.873-2.603)	0.140	9/37	1.511	(0.663-3.444)	.330
PS								
0	54/213	1.000		Reference	44/213	1.000		Reference
1	91/260	1.509	(1.075-2.118)	.017	74/260	1.272	(0.872-1.856)	.210
2-4	41/75	3.004	(1.915-4.714)	< .001	30/75	1.679	(1.035-2.723)	.036
Unknown	9/24	1.214	(0.614-2.403)	0.580	7/24	1.965	(0.802-4.818)	.140
Relationship between recipient and donor								
HLA-matched related	62/206	1.000		Reference	60/206	1.000		Reference
HLA-mismatched related	18/62	0.924	(0.532-1.606)	0.780	26/62	1.392	(0.873-2.220)	.160
Unrelated	115/304	1.429	(1.033-1.975)	.031	69/304	0.843	(0.589-1.209)	.350
Preconditioning regimen								
MAC	100/274	1.000		Reference	61/275	1.000		Reference
RIC	95/298	0.786	(0.538-1.148)	0.210	94/304	1.579	(1.080-2.308)	.019

2.203 (1.469-3.302). The HR for ATL-related mortality of recipients with PS 2 to 4 compared with PS 0 was 1.679 (95% CI, 1.035-2.723), and the HR of patients receiving RIC compared with MAC was 1.579 (95% CI, 1.080-2.308; Table 5).

Cumulative incidence of TRM and ATL-related mortality

Among the 572 ATL patients receiving allogeneic BMT or PBSCT, the cumulative incidence of TRM one year after transplantation was 32.7% (95% CI, 27.1-38.4) in MAC recipients and 29.2% (95% CI, 24.0-34.5) in RIC recipients. These figures at 3 years were 37.7% (95% CI, 31.8-43.6) and 33.3% (95% CI, 27.7-38.9), respectively (Figure 3). The cumulative incidence of ATL-related mortality 1 year after transplantation was 18.5% (95% CI, 14.1-23.4) for MAC and 25.0% (95% CI, 20.1-30.1) for RIC recipients and was 22.5% (95% CI, 17.5-27.9) and 33.2% (95% CI, 27.6-38.9), respectively, at 3 years (Figure 3).

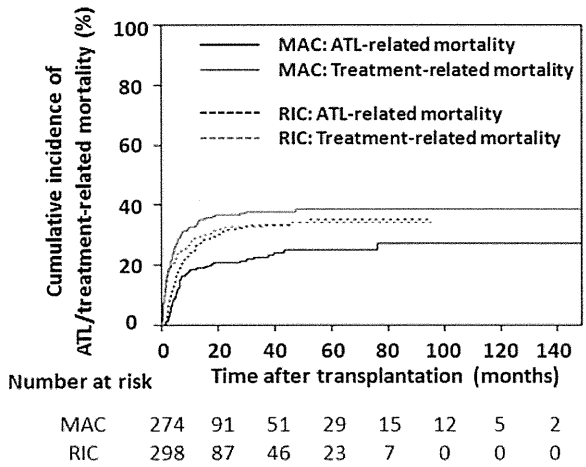

Figure 3. Cumulative incidence of ATL-related and TRMs in patients receiving BMT or PBSCT. Probabilities of ATL-related and TRMs in recipients of MAC or RIC were estimated using cumulative incidence curves to accommodate competing events.

Discussion

To the best of our knowledge, the present study is the largest retrospective study of ATL patients receiving allogeneic HSCT. Results showed that for allogeneic BMT or PBSCT for ATL, RIC was applied more frequently in older patients, as is reasonable and expected. RIC patients more often received PBSCT and had related donors. We surmise this was because RIC was initially proposed in the setting of PBSCT from HLA-matched sibling donors.[30]

The OS plot of ATL patients receiving allogeneic HSCT reached a plateau, leading to long-term survival of a subgroup of ATL patients. Recipients of CBT had a significantly worse prognosis than recipients of BMT or PBSCT, which was consistent with our previous report.[11] Direct comparison of transplantation outcomes between unrelated CBT and the other types of allogeneic HSCT was not possible because the selection of the graft source is an individual process strongly influenced by donor availability and the patient's ATL status. However, even considering such potential biases, the outcome of unrelated CBT seems clearly unsatisfactory. Thus, novel strategies to further improve the outcomes of unrelated CBT are warranted.

Among ATL patients receiving allogeneic BMT or PBSCT, multivariate analysis revealed 5 significant independent variables affecting OS, namely, age, sex, disease status, PS, and relationship between the recipient and donor. Of these factors, younger age, good ATL disease status, and PS at transplantation contributing to better OS were to be expected. The contribution to a better OS of HSCT from HLA-A, -B, and -DR–matched related donors also would be expected. The reason why the female sex was an independent favorable factor is not fully understood but is consistent with results of our previous study.[11] With respect to preconditioning, there was no significant difference in OS between MAC

and RIC recipients. To further clarify the clinical significance of preconditioning in allogeneic BMT or PBSCT for ATL, we analyzed the interactions of preconditioning with age, disease status, and PS. There was a clear trend indicating that RIC contributed to better OS in older patients compared with MAC. In contrast, the associations between MAC and RIC to OS were almost similar even if ATL patients at transplantation were in CR or not. In general, when considering allogeneic HSCT for many other types of leukemia/lymphoma patients who are in non-CR, it seems more usual to apply MAC for those patients because MAC should have the more potent effect in eradicating residual leukemia/lymphoma cells than RIC. However, the present study does not support this strategy at least in HSCT for ATL. The associations between MAC and RIC to OS were almost similar even when the PS at transplantation was 0, 1, or 2 to 4. In general, considering allogeneic HSCT for patients who have a worse PS, it seems to be more usual to apply RIC because RIC should be less toxic for recipients than MAC. However, the present study also does not support this strategy, at least in HSCT for ATL.

In the subgroup analyses stratified by MAC or RIC, older age was an independent unfavorable prognostic factor in MAC recipients, but not in RIC recipients. Female sex, good ATL disease status, and PS significantly contributed to better OS in both groups. Among MAC recipients, there was no significant difference in OS according to the type of MAC, but among RIC recipients, a Flu + Mel–based regimen contributed to better OS compared with a Flu + BU–based regimen. Although RIC regimens that contain alemutuzumab have been widely used in various parts of the world,[31] we had no data available as to whether any of the regimens used included alemtuzumab. Thus, we were not able to clarify the significance of the inclusion of alemtuzumab as a conditioning agent.

Multivariate analysis of variables contributing to mortality demonstrated that there was significantly more ATL-related mortality in RIC recipients. Although not statistically significant, a clear trend showed an association of increased TRM but not ATL-related mortality in older patients. Male sex was significantly associated with increased TRM, which might contribute to the better OS of female recipients. ATL patients not in CR had greater ATL-related mortality, but not TRM. A poor PS was significantly associated with both ATL-related mortality and TRM, but the association was closer with TRM. HSCT from unrelated donors was significantly associated with increased TRM but not with ATL-related mortality.

Cumulative incidence curves of TRM and ATL-related mortalities in MAC and RIC recipients showed characteristic features as illustrated in Figure 3. In comparison with the black lines indicating ATL-related mortality, the red lines showing TRM rise in the early phase after transplantation. Two solid lines for MAC had quite different trajectories, with TRM being greater than ATL-related mortality at any time after transplantation. In contrast, the 2 dotted lines for RIC nearly joined at 24 months after transplantation and were almost identical thereafter. Both lines for RIC were between those for MAC TRM and ATL-related mortality.

Currently, several promising new agents for ATL are being developed.[32-35] These novel treatments should increase the number of ATL patients with a sufficient disease control status and who have maintained a good PS who could become suitable candidates for transplantation. This would require further improvement in allogeneic HSCT for ATL as well as better rescue strategies for patients relapsing after HSCT. Although treatment by AZT/IFN-α[6] and/or alemutuzumab[34,36] are applied for ATL patients in many countries, none of these agents are currently approved in Japan for the treatment of ATL under the national health insurance. Therefore, there are currently no data on their clinical impact on outcome after allogeneic HSCT for ATL. We do expect, however, that the application of AZT/IFN and alemutuzumab would contribute to improved outcomes of HSCT for ATL.

Although this study reports significant novel findings for allogeneic HSCT for ATL patients, it also has inherent limitations common among observational retrospective studies. Eligibility for transplantation as well as choice of transplantation protocol, including the selection of MAC or RIC, was determined by the physicians at each institution. Regarding mortality analysis, it is not easy to determine whether death of an ATL patient after allogeneic HSCT is TRM or ATL-related mortality. This is partially because relapsed ATL patients sometimes achieve partial or complete remission on decreasing or discontinuing immunosuppressive agents, donor lymphocyte infusions, or chemotherapy, which can result in long-term remission and survival.[9,13,18]

In conclusion, allogeneic BMT or PBSCT not only with conventional MAC but also RIC is an effective treatment that results in long-term survival of selected patients with ATL. Posttransplantation outcomes are influenced by the recipient's age, sex, PS, disease status at transplantation, and the relationship between recipient and donor. Although no significant difference in OS between MAC and RIC recipients was observed, there was a clear trend that RIC contributed to better OS in older patients. Regarding results of analysis of mortality, RIC was more significantly associated with ATL-related mortality in comparison with MAC. More definitive conclusions on the role of allogeneic HSCT in the therapeutic algorithm for ATL will need to be drawn from well-designed prospective clinical trials.

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Authorship

Contribution: T.I., M.H., K.K., R.T., and A.U. designed the research, organized the project, and wrote the paper; T.I. and T.N. performed statistical analysis; H.S. and R.S. collected data from JSHCT; Y.M. collected data from JMDP; K.K. collected data from JCBBN; and all authors interpreted data, reviewed, and approved the final manuscript.

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Tax is a potential molecular target for immunotherapy of adult T-cell leukemia/lymphoma

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We expanded CTL specific for Tax (a human T-lymphotropic virus type-1-encoded gene product) *in vitro* from PBMC of several adult T-cell leukemia/lymphoma (ATL) patients, and document its potential significance as a target for ATL immunotherapy. Tax-specific CTL responses against tumor cells were restricted by Tax-expression and the appropriate human leukocyte antigen (HLA) type. Tax-specific CTL recognized HLA/Tax-peptide complexes on autologous ATL cells, even when their Tax expression was so low that it could only be detected by RT-PCR but not by flow cytometry. Recognition resulted in interferon gamma (IFN-γ) production and target cell lysis. This would be the first report that Tax-specific CTL from ATL patients specifically recognized and killed autologous tumor cells that expressed Tax. The Tax-specific CTL responded to as little as 0.01 pM of the corresponding peptide, indicating that their T-cell receptor avidity was much higher than that of any other CTL recognizing viral or other tumor antigens. This is presumably the reason why the Tax-specific CTL recognized and killed autologous ATL cells despite their very low Tax expression. In addition, cell cycle analyses and experiments with primary ATL cell-bearing mice demonstrated that ATL cells present at the site of active cell proliferation, such as in the tumor masses, expressed substantial amounts of Tax, but it was minimally expressed by the tumor cells in a quiescent state, such as in the blood. The present study not only provides a strong rationale for exploiting Tax as a possible target for ATL immunotherapy but also contributes to our understanding of the immunopathogenesis of ATL. (*Cancer Sci* 2012; 103: 1764–1773)

Adult T-cell leukemia/lymphoma (ATL) is a distinct hematologic malignancy caused by human T-lymphotropic virus type 1 (HTLV-1).[1,2] ATL has a long latency period of 50–60 years, so affected individuals have usually been exposed to HTLV-1 early in their lives via agents including infected lymphocytes, mainly from mother's breast milk.[3,4] Only small subpopulations (approximately 5%) of HTLV-1-infected individuals progress to ATL, but there are no clear biomarkers separating those who will develop ATL from those who remain asymptomatic carriers (AC).[2] There are four clinical subtypes of ATL: acute, lymphoma, chronic and smoldering.[5] The two former types have more aggressive clinical courses (aggressive variants), while the latter are less aggressive (indolent variants).

Human T-lymphotropic virus type 1 Tax, a virus-encoded regulatory gene product, is required for the virus to transform cells,[6] and is thought to be indispensable for oncogenesis. Therefore, Tax has been considered as a molecular target for immunotherapy against ATL, and many such investigations have been published.[7–10] However, it has been reported that the level of Tax expression in HTLV-1-infected cells decreases during disease progression, and *Tax* transcripts are detected only in approximately 40% of established ATL cases.[11] Moreover, weak or absent responses to Tax have been observed in ATL patients,[12] leading to controversy as to whether Tax is an appropriate target for immunotherapy of ATL. In the present study, we expanded Tax-specific CTL *in vitro* from PBMC of several ATL patients, and tested their ability to respond to several ATL cell lines, HTLV-1-immortalized lines and to autologous ATL cells. The aim was to clarify the involvement of Tax-specific CTL (Tax-CTL) in the immunopathogenesis of ATL, and to confirm the significance of Tax as a potential immunotherapeutic target in ATL.

Materials and Methods

Primary adult T-cell leukemia/lymphoma cells. Primary ATL cells were separated from PBMC using anti-human CD4 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). All donors provided informed written consent before sampling according to the Declaration of Helsinki, and the present study was approved by the institutional ethics committees of Nagoya City University Graduate School of Medical Sciences.

Cell lines. TL-Su and TL-Om1 were provided by the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). TCL-Kan was kindly provided by Professor Mari Kannagi (Tokyo Medical and Dental University, Tokyo, Japan).[13] HUT102, ATN-1, MT-2 and MT-1 have been previously described.[14,15] MT-4 was purchased from the Health Science Research Resources Bank (Osaka, Japan). HUT102, ATN-1, MT-1 and TL-Om1 are ATL cell lines, and TL-Su, TCL-Kan, ILT-#37, MT-2 and MT-4 are HTLV-I-immortalized lines. K562 is the chronic myelogenous leukemia blast crisis cell line.[16]

Human leukocyte antigen typing. Genotyping of HLA-A, B and C was performed using an HLA-typing Kit (WAKFlow HLA-typing kit, WAKUNAGA Pharmacy, Hiroshima, Japan).

Expansion of human T-lymphotropic virus type 1 Tax-specific CTL. PBMC from ATL patients or HTLV-1 AC were suspended in RPMI-1640 supplemented with 10% autologous plasma and 0.1 μM of the corresponding Tax epitope peptides (LLFGYPVYV or SFHSLHLLF) at a cell concentration of 2.0×10^6/mL. These two synthetic peptides were purchased from Invitrogen (Carlsbad, CA, USA). The cell suspension was cultured at 37°C in 5%CO_2 for 2 days, and then an equal volume of RPMI-1640 supplemented with 100 IU/mL of IL-2

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was added. After subsequent culture for 5 days, an equal volume of ALyS505N (Cell Science & Technology Institute, Sendai, Japan) supplemented with 100 IU/mL of IL-2 was added, and the cells were cultured with appropriate medium (ALyS505N with 100 IU/mL of IL-2) for 7 days. Cytomegalovirus (CMV)-pp65 specific CTL were expanded in the same manner using peptides such as NLVPMVATV or QYDPVAALF (Invitrogen). Viable cell counts were determined using the trypan blue assay.

Antibodies, tetramers and flow cytometry. Phycoerythrin-conjugated HLA-A*02:01/Tax11–19 (LLFGYPVYV), HLA-A*24:02/Tax301–309 (SFHSLHLLF), HLA-A*02:01/pp65 495–503 (NLVPMVATV) and HLA-A*24:02/pp65 341–349 (QYDPVAALF) tetramers, and phycoerythrin-Cyanin5-conjugated anti-CD8 monoclonal antibody (mAb) were purchased from Medical & Biological Laboratories, Nagoya, Japan. Allophycocyanin-conjugated anti-human CD45 mAb (2D1) and PerCP-conjugated anti-CD4 mAb (SK3) were purchased from BD Biosciences (San Jose, CA, USA). Tax expression was assessed by FITC-conjugated anti-Tax mAb Lt-4.[17] FITC-conjugated anti-interferon gamma (IFN-γ) mAb (45.15) was purchased from Medical & Biological Laboratories. Cell cycle assessments were performed by BrdU Flow Kits (BD Biosciences). Cells were analyzed on a FACSCalibur (BD Biosciences) with the aid of FlowJo software (Tree Star, Ashland, OR, USA).

CTL assay. Cytotoxic activity was determined by a standard 4-h chromium^{51} release assay as previously described.[18] All values given are means of triplicate determinations.

Quantitative RT-PCR. *Tax*, *human CD4* and *β-actin* mRNA were amplified as previously described.[19] The primer set for Tax was as follows: sense, 5′-AAGACCACCAACACCA TGGC-3′; and antisense, 5′-CCAAACACGTAGACTGGGTAT CC-3′.

Animals. NOD/Shi-*scid*, IL-2Rγ^{null} (NOG) mice were purchased from the Central Institute for Experimental Animals (Kawasaki, Japan). All of the *in vivo* experiments were approved by the Ethics Committee of the Center for Experimental Animal Science, Nagoya City University Graduate School of Medical Sciences.

Results

Expansion of Tax-specific CTL. Expansion of Tax-CTL was performed by stimulating PBMC from 14 ATL patients and 6 HTLV-1 AC with synthetic peptides. PBMC from patients 1, 2, 3, 6, 8, 9 and 13 were stimulated with Tax11–19, and those from patients 4, 5, 7, 10, 11, 12 and 14 with Tax301–309 (Tables 1 and 2). Patients 1–6 were all in complete remission (CR) at the time of blood sampling. Patient 1 had achieved CR after allogeneic hematopoietic stem cell transplantation (HSCT) 5 years previously, patients 2 and 3 after systemic chemotherapy and anti-CCR4 mAb treatment,[20,21] patient 4 after systemic chemotherapy alone, and patient 5 after allogeneic HSCT 9 months earlier (and was receiving FK506 at the time of sampling). Finally, patient 6 achieved CR after systemic chemotherapy and anti-CCR4 mAb treatment, and was receiving prednisolone at the time of sampling. As shown in Table 1, Tax-CTL could be expanded *in vitro* (fold expansion >10) by stimulation with Tax peptide in 13 of 17 ATL cases. With respect to HTLV-1 AC, we confirmed efficient expansion (fold expansion >10^2) of Tax-CTL from six of six individuals using Tax11–19 or Tax301–309 peptides in the same manner (data not shown), which are consistent with a previous report.[22] Although the degree of expansion of Tax-CTL varied among the ATL patients, there was a trend for higher rates in PBMC from those with indolent variant ATL not on any systemic treatment, or from patients with aggressive ATL in treatment-induced remission, compared to lower or absent expansion in patients initially diagnosed with an aggressive variant. In particular, patient 8 progressed from chronic to acute subtype during the present study. Tax-CTL could be efficiently expanded from this patient during the chronic phase, but no longer after progression to acute subtype. This was despite the finding that the percentage of HLA-A*02:01/Tax11–19 tetramer-positive cells in the PBMC was almost the same as before disease progression (Fig. 1). These observations collectively indicate that insufficient responses to Tax observed in ATL patients, which are also reported by other investigators,[12,23,24] are related to disease progression from indolent to aggressive clinical variants. Subsequently, patient 8 received systemic chemotherapy but failed to achieve CR. He then received allogeneic HSCT with reduced intensity conditioning and entered partial remission. At this time, when he was not receiving immunosuppression after HSCT, his Tax-CTL could again be efficiently expanded from PBMC. This indicates that substantial anti-Tax responses can be restored by appropriate anti-ATL therapies, when the patient is brought from active ATL into remission (Fig. 1). Even though patients were in CR, immunosuppressive agents such as FK506 or prednisolone were likely to have prevented CTL expansions, as observed in patients 5 and 6, consistent with reports that HTLV-1 AC liver transplant recipients developed ATL under immunosuppression.[25,26] In patient 14, the Tax-CTL expansion rate was drastically increased by depletion of CD4+ cells, most of which consisted of the ATL cells themselves. This suggests that Tax-specific immune responses were suppressed by the tumor cells, consistent with our previous report that ATL cells from a subgroup of patients functioned as regulatory T (Treg) cells.[27]

T-cell receptor avidity of the expanded Tax-specific CTL. Specific IFN-γ production following stimulation with serial concentrations (0.01–100 pM) of Tax11–19 or Tax301–309 peptides was used as a readout to measure the T-cell receptor (TCR) avidity of the expanded Tax-CTL. Intracellular IFN-γ was clearly detected specifically even at a peptide concentration of 0.01 pM in both HLA-A*02:01-restricted Tax-CTL from patient 1 (Fig. 2A) and HLA-A*24:02-restricted Tax-CTL from patient 7 (Fig. 2B). We also analyzed the TCR avidity of CMV-pp65-specific CTL expanded from the same patients. Specific IFN-γ production by HLA-A*02:01-restricted pp65-CTL was lower than Tax-CTL at any peptide concentration. Furthermore, no specific IFN-γ production by HLA-A*24:02 pp65-CTL could be detected at all at peptide concentrations of 0.01–1 pM. In general in the literature, peptide concentrations of other viral or tumor antigen epitopes that the corresponding specific CTL recognize and respond to are in the range 1 nM–10 μM, although this varies according to the antigen.[28–32] Collectively, the results presented here indicate that the TCR avidities of these Tax-CTL can be considered to be extremely high.

Expression of human T-lymphotropic virus type 1 Tax in adult T-cell leukemia/lymphoma cells. Given the high TCR avidity of Tax-CTL, we next analyzed whether these CTL could recognize, respond to and kill ATL cells. To this end, Tax expression in ATL cell lines, HTLV-1-immortalized lines, K562 and short-term cultured primary ATL cells was assessed (Fig. 3). Tax expression was detected both by flow cytometry and RT-PCR in TL-Su, TCL-Kan, HUT102, MT-2 and MT-4, but not in K562, MT-1 or TL-Om1 by either technique. No Tax protein was seen in ATN-1 or in short-term cultured primary ATL cells from patients 7, 8 and 14, although *Tax* mRNA was present at levels 1/10–1/100th of those in TL-Su.

Tax-specific CTL responses against autologous adult T-cell leukemia/lymphoma cells. PBMC from patient 7 were stimulated with HLA-A*24:02 restricted Tax301–309 peptide, and the resulting CTL were expanded (Fig. 4A, upper-left panel).

Table 1. Tax-specific CTL expansion in adult T-cell leukemia/lymphoma (ATL) patients

Patient number	Clinical subtype	ATL status at blood sampling	Total cells (number)		Tax tetramer + cells/ lymphocytes (%)		Tax tetramer + cells (number)		Expansion rate†
			Day 0	Day 14	Day 0	Day 14	Day 0	Day 14	
Patient 1	Acute	Complete remission	4.5×10^6	9.5×10^6	0.01	4.51	4.5×10^2	4.28×10^5	951.1
Patient 2	Acute	Complete remission	3.0×10^6	2.8×10^6	<0.01	10.02	$<3.0 \times 10^2$	2.81×10^5	936.7
Patient 3	Chronic	Complete remission	8.6×10^6	1.5×10^7	0.02	9.02	1.72×10^3	1.35×10^6	784.9
Patient 4	Lymphoma	Complete remission	7.5×10^6	1.1×10^7	0.06	10.92	4.5×10^3	1.02×10^6	226.7
Patient 5	Acute	Complete remission	3.0×10^6	1.0×10^7	0.03	0.15	9.0×10^2	1.50×10^4	16.7
Patient 6	Lymphoma	Complete remission	4.3×10^6	3.5×10^6	<0.01	0.62	$<4.3 \times 10^2$	2.17×10^4	>50.5
Patient 7	Chronic	Watchful waiting	2×10^7	1.0×10^8	1.32	12.50	2.64×10^5	1.25×10^7	47.3
Patient 8	Chronic	Watchful waiting	6.5×10^6	9.2×10^6	0.01	7.05	6.5×10^2	6.49×10^5	998.5
Patient 8′‡	Acute	Before treatment	5.26×10^6	5.5×10^6	0.02	0.02	1.05×10^4	1.10×10^4	1.05
Patient 8″‡	Acute	Partial remission	3.5×10^6	6.8×10^6	0.06	26.36	2.1×10^3	1.79×10^6	852.4
Patient 9	Smoldering	Under systemic phototherapy for skin	3.0×10^6	5.8×10^6	0.02	28.78	6.0×10^2	1.67×10^6	2783.3
Patient 10	Lymphoma	Initially diagnosed	7.3×10^6	1.2×10^7	<0.01	0.28	$<7.3 \times 10^2$	3.36×10^4	>46.0
Patient 11	Acute	Initially diagnosed	4.3×10^6	4.1×10^6	<0.01	0.14	$<4.3 \times 10^2$	5.74×10^3	>13.3
Patient 12	Acute	Initially diagnosed	5.2×10^6	ND	ND	ND	ND	ND	ND
Patient 13	Acute	Initially diagnosed	1.0×10^7	ND	ND	ND	ND	ND	ND
Patient 14	Acute	Diagnosed as relapse with acute type phenotype	6.0×10^6	7.0×10^6	0.01	0.03	6.0×10^2	2.10×10^3	3.5
Patient 14 (CD4-subset)§			6.0×10^6	2.6×10^6	0.01	3.79	6.0×10^2	9.90×10^4	165.0

†Cell numbers of Tax tetramer + cells on day 14 was divided by that of day 0. ‡Patient 8 progressed from chronic to acute subtypes, and then he received allogeneic hematopoietic stem cell transplantation. §CD4+ cells were depleted on day 4. ATL, adult T-cell leukemia/lymphoma; CTL, cytotoxic T lymphocytes; HTLV-1, human T-lymphotropic virus type-1; ND, not detected.

In this culture, HLA-A2-restricted Tax11–19 specific CTL were also expanded (Fig. 4A, middle-left panel), even though the Tax11–19 peptide was not used as a stimulator. We surmised that pre-existing Tax-CTL, including these HLA-A2 Tax11–19 CTL, were stimulated by the ATL cells constitutively expressing HLA-A2/Tax11–19 complexes, contained in the cultured PBMC. These expanded T-cells were co-cultured with ATL cell lines, HTLV-1-immortalized lines or autologous ATL cells, and their responses were evaluated by IFN-γ production. HLA-A*24:02/Tax301–309 tetramer-positive fractions of these expanded CD8-positive cells produced IFN-γ when co-cultured with autologous ATL cells or ATN-1 (Fig. 4A), even though the Tax expression was so low as to be undetectable by flow cytometry, and only detectable by RT-PCR (Fig. 3). These tetramer-positive cells also responded to TL-Su and MT-2, but did not respond to the other ATL cell lines, or HTLV-1-immortalized lines tested. This indicates that only target cells having both HLA-A*24:02 and Tax were recognized (Table 2 and Fig. 3). The HLA-A*24:02/Tax301–309 tetramer-negative fractions of these expanded CD8-positive cells also produced IFN-γ when stimulated with autologous ATL cells. This suggests that they recognize unidentified Tax-derived epitopes, or antigens derived from HTLV-1 components other than Tax, or ATL-related tumor antigens not of viral origin. Finally, the HLA-A*02:01/Tax11–19 tetramer-positive fractions within these expanded CD8-positive cells were also found to produce IFN-γ on challenge with autologous ATL cells and TCL-Kan, but not the other ATL cell lines or HTLV-1-immortalized lines. This indicates that HLA-A2 and Tax expression were both required for recognition. HLA-A*02:01/Tax11–19 tetramer-negative cells also produced IFN-γ when stimulated by TCL-Kan. Because both patient 7 and TCL-Kan share HLA-B*46:01 and HLA-C*01:02 (Table 2), the tetramer-negative cells might be recognizing unidentified Tax-derived epitopes, other HTLV-1 antigens or ATL tumor antigens-derived epitopes presented on a different shared MHC allele. These effector cells did not respond to K562 by IFN-γ production, showing that they had no NK activity.

Next, PBMC from patient 8 at chronic stage were investigated in a similar manner, stimulated with Tax11–19 peptide (Fig. 4B, upper-left panel). HLA-A*02:01/Tax11–19 tetramer-positive cells in these expanded CD8-positive cells also produced IFN-γ (Fig. 4B) when stimulated with Tax RT-PCR-positive but flow cytometry-negative autologous ATL cells

Table 2. Human leukocyte antigen (HLA) information

	HLA-A		HLA-B		HLA-C	
TL-Su	*11:01	*24:02	*15:01	*40:02	*03:04	*04:01
TCL-Kan	*02:06	*02:07	*46:01	*56:01	*01:02	*07:02
K562						
HUT102	*30:02	*66:02				
ATN-1	*11:01	*24:02	*54:01	*67:01	*01:02	*07:02
MT-1	*11:01	*26:01	*39:01	*40:02	*03:04	*07:02
MT-2	*24:02	*24:02	*40:02	*51:01	*03:03	*14:02
MT-4	*11:01	*31:01	*39:02	*67:01	*07:02	*07:02
TL-Om1	*02:01	*02:01	*52:01	*52:01	*12:02	*12:02
Patient 1	*02:01	*02:01	*15:01	*40:02	*03:04	*07:02
Patient 3	*02:01	*31:01				
Patient 4	*24:02	*26:01				
Patient 5	*02:06	*24:02				
Patient 6	*02:06	*31:01				
Patient 7	*02:07	*24:02	*46:01	*52:01	*01:02	*12:02
Patient 8	*02:01	*02:06	*35:01	*55:02	*01:02	*03:03
Patient 9	*02:01	*31:01				
Patient 10	*11:01	*24:02				
Patient 11	*11:01	*24:02				
Patient 12	*02:06	*24:02				
Patient 13	*02:03	*31:01				
Patient 14	*24:02	*31:01	*07:02	*40:01	*03:04	*07:02

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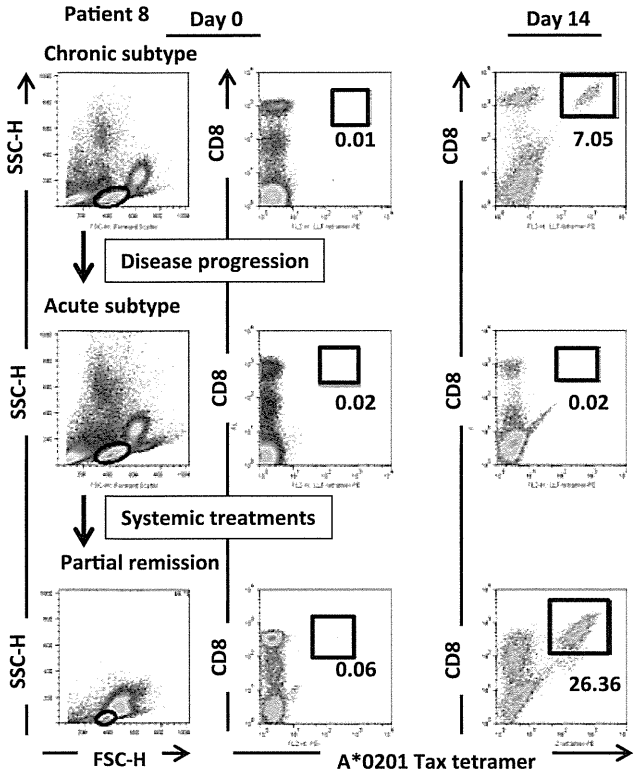

Fig. 1. Expansion of Tax-specific CTL from PBMC of patient 8 at different clinical stages. Flow cytometric analyses of the expanded cells are presented. The lymphocyte population was determined by FSC-H and SSC-H levels (left panels) and the data are plotted to show CD8 and human leukocyte antigen (HLA)-A*02:01/Tax tetramer-positivity (right two panels). Both CD8 and HLA-A*02:01/Tax tetramer-positive cells are gated, and their percentages relative to the entire lymphocyte population are indicated in each panel. Patient 8 progressed from chronic to acute stage disease. His Tax-CTL could be efficiently expanded during the chronic phase (upper panels), but no longer after progression to acute stage (middle panels). Subsequently, he received allogeneic hematopoietic stem cell transplantation, and achieved partial remission. At this time, his Tax-CTL could be efficiently expanded from PBMC once more (lower panels).

(Fig. 3). These tetramer-positive cells responded to TCL-Kan but not to the other ATL cell lines or HTLV-1-immortalized lines. Thus, their recognition was also restricted by the expression of HLA-A2 and Tax (Table 2 and Fig. 3). HLA-A*02:01/Tax11–19 tetramer-negative fractions were also stimulated by autologous ATL cells, again suggesting recognition of unidentified epitopes. HLA-A*02:01/Tax11–19 tetramer-negative cells also produced IFN-γ when stimulated by TCL-Kan. Because patient 8 and TCL-Kan are both HLA-C*01:02-positive (Table 2), these effector cells might be recognizing unidentified epitopes presented on this shared MHC allele. Again, there was no IFN-γ production against K562.

We also repeated these experiments with PBMC from patient 14, and evaluated them in the same manner. In this case as well, the HLA-A*24:02/Tax301–309 tetramer-positive cells responded to autologous ATL cells and ATN-1 (Fig. 4C), again despite the very low level of Tax expression. They also responded to TL-Su, but not the other ATL cell lines or HTLV-1-immortalized lines, showing HLA-A*24:02 and Tax restriction (Table 2 and Fig. 3). Once more, the HLA-A*24:02/Tax301–309 tetramer-negative cells were also stimulated by autologous ATL cells, indicating recognition of unidentified epitopes presented on autologous MHC molecules. HLA-A*24:02/Tax301–309 tetramer-negative cells also produced IFN-γ when stimulated with TL-Su, which shares HLA-C*03:04 with patient 14 (Table 2). Again, no NK activity was detectable.

Lysis of autologous adult T-cell leukemia/lymphoma cells by Tax-specific CTL. Cells from patient 7 expanded by Tax301–309 peptide (Fig. 4A) killed TL-Su, MT-2, ATN-1 and autologous ATL cells in an E/T ratio-dependent manner, but did not lyse MT-1 or HUT102 (Fig. 5, left panel). Lysis depended on the presence of both HLA-A*24:02 and Tax (Table 2 and Fig. 3). Although as mentioned before, the level of Tax expression by these autologous ATL cells and ATN-1 was so low as to be detectable only by RT-PCR and not by flow cytometry, objective lysis of both cells was still observed. The patient 7 Tax-CTL expanded by Tax301–309 peptide stimulation also killed TCL-Kan. HLA-A2-restricted Tax11–19 CTL included in the effector subset presumably contributed to the lyses of TCL-Kan as well as autologous tumor cells (Fig. 4A, middle-left panel). Again, these expanded cells did not possess NK activity. The cells from patient 8 at chronic stage expanded by Tax11–19 peptide (Fig. 4B) killed TCL-Kan and autologous ATL cells, but not TL-Om1 (Fig. 5, middle panel) in an HLA-A2-restricted and Tax-restricted manner (Table 2 and Fig. 3). Again, Tax expression by the autologous ATL cells was extremely low, but the targets were, nonetheless, killed. As with the other patients, there was no NK activity present in the expanded cells.

Finally, cells from patient 14 stimulated by Tax301–309 peptide (Fig. 4C) killed TL-Su and autologous ATL cells, but not MT-1 (Fig. 5, right panel), restricted by HLA-A*24:02 and Tax (Table 2 and Fig. 3), again with no NK activity.

Tax expression in primary adult T-cell leukemia/lymphoma cells induced by short-term culture. It was previously reported that although Tax expression was not detectable in primary ATL cells by flow cytometry in most cases, short-term culture of such cells could induce Tax expression in nearly half of cases.[33] Tax expression and its regulation in primary ATL cells is currently not fully understood. We tested Tax expression of primary ATL cells from patients 7, 8, 13 and 14, as listed in Table 1, and 2 additional patients, 15 and 16 (both chronic type). Tax protein was not present in any primary uncultured ATL cells isolated with anti-human CD4 microbeads from patients' peripheral blood. In all cases, these cells were in a quiescent state, as determined by 7-AAD staining (Fig. 6A). Cells incorporating BrdU (S phase) and those having double DNA content (G2/M phase) first appeared on culture of the primary ATL cells for several days, indicating that they had begun to cycle. At the same time, Tax-expressing cells appeared in three of six cases (patients 7, 8 and 13) (Fig. 6B). These findings indicate that Tax expression was induced in primary ATL cells when they were actively cycling (i.e. cells not in G0 phase). Because most primary ATL cells in the peripheral blood are in a quiescent state (G0 phase), they express little or no Tax.

Tax expression in primary adult T-cell leukemia/lymphoma cell-bearing NOG mice. NOG mice bearing primary ATL cells were established using ATL cells of patients 7, 12 and 13, as previously described.[34] ATL mice from patient 7 presented with large intraperitoneal tumor masses, and tumor cells aggressively infiltrated into liver and spleen, but into the blood only to a lesser extent. Setting the *Tax/human CD4* mRNA level of TL-Su as unity, these values for blood cells, liver, spleen and tumor cell suspensions were 0.00195 ± 0.00065 (standard deviation), 0.023000 ± 0.00312, 0.00626 ± 0.00214 and 0.19533 ± 0.02185, respectively. Because there was little ATL cell infiltration into bone marrow, the *Tax/human CD4* mRNA value of bone marrow cells was under the limit of detection