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Brief report

Relapse of leukemia with loss of mismatched HLA resulting from uniparental disomy after haploidentical hematopoietic stem cell transplantation

Itzel Bustos Villalobos,[1] Yoshiyuki Takahashi,[1] Yoshiki Akatsuka,[2,3] Hideki Muramatsu,[1] Nobuhiro Nishio,[1] Asahito Hama,[1] Hiroshi Yagasaki,[1] Hiroh Saji,[4] Motohiro Kato,[5] Seishi Ogawa,[6] and Seiji Kojima[1]

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We investigated human leukocyte antigen (HLA) expression on leukemic cells derived from patients at diagnosis and relapse after hematopoietic stem cell transplantation (HSCT) using flow cytometry with locus-specific antibodies. Two of 3 patients who relapsed after HLA-haploidentical HSCT demonstrated loss of HLA alleles in leukemic cells at relapse; on the other hand, no loss of HLA alleles was seen in 6 patients who relapsed after HLA-identical HSCT. Single-nucleotide polymorphism array analyses of sorted leukemic cells further revealed the copy number–neutral loss of heterozygosity, namely, acquired uniparental disomy on the short arm of chromosome 6, resulting in the total loss of the mismatched HLA haplotype. These results suggest that the escape from immunosurveillance by the loss of mismatched HLA alleles may be a crucial mechanism of relapse after HLA-haploidentical HSCT. Accordingly, the status of mismatched HLA on relapsed leukemic cells should be checked before donor lymphocyte infusion. (*Blood*. 2010;115(15):3158-3161)

Introduction

Human leukocyte antigen (HLA) molecules expressed on the cell surface are required in presenting antigens to T cells. The HLA class I antigens are vital in the recognition of tumor cells by tumor-specific cytotoxic T cells. The loss of HLA class I molecules on the cell surface membrane may lead to escape from T-cell immunosurveillance and the relapse of leukemia. Previously, loss of HLA class I haplotype has been described in solid tumors.[1-3] However, there are few reports concerning HLA-haplotype loss in leukemia.[4,5]

We examined HLA class I expression in leukemic blasts from patients who relapsed after hematopoietic stem cell transplantation (HSCT) to analyze whether the loss of HLA on leukemic cells was related to the relapse after HLA-identical or haploidentical HSCT.

Methods

Patients and transplantation procedure

We identified 9 children with acute leukemia who relapsed after HSCT. Their leukemic samples were cryopreserved both at the time of the initial diagnosis and of relapse. The patients' characteristics are summarized in supplemental Table 1 (available on the *Blood* website; see the Supplemental Materials link at the top of the online article). Three patients received HSCT from an HLA-haploidentical family donor, and the other 6 patients received HSCT from an HLA-matched donor (4 siblings and 2 unrelated donors).

Written informed consent was given by the parents according to the protocol approved by the ethics committee of Nagoya University Graduate School of Medicine in accordance with the Declaration of Helsinki.

HLA class I expression on leukemic cells

Samples were collected at diagnosis and post-transplantation relapse. HLA expression of leukemic blasts and normal cells was analyzed by flow cytometry as previously reported.[6] Anti-HLA A2-FITC (cloneBB7.2) and anti-HLA A24-FITC (clone17a10) monoclonal antibodies were purchased from Medical & Biological Laboratories; HLA-A11 (IgM), HLA-A30, HLA-31 (IgM), HLA-25, HLA-26 (IgM), HLA-Bw6 (IgG3), and HLA-Bw4 (IgG3) antibodies were purchased from One Lambda. For leukemic cell markers, CD13-PE (IgG1) were purchased from Immunotech and CD34-APC (IgG1) were purchased from BD Biosciences. Samples were analyzed with FACSCalibur cytometer and CellQuest software. The method of genomic HLA typing was previously reported.[7]

Isolation of DNA and single nucleotide polymorphism analysis

The CD13+/CD34+ leukemic blasts were sorted by flow cytometry from bone marrow cells at the time of diagnosis and of relapse. Genomic DNA was extracted from leukemic cells sorted by a fluorescence-activated cell sorter as well as from phytohemagglutinin-stimulated patient-derived T cells and subjected to single nucleotide polymorphism (SNP) array analysis using GeneChip NspI arrays (Affymetrix) according to the manufacturer's protocol. Allele-specific copy number was detected using Copy Number Analyzer for GeneChip software as previously described.[8]

Limiting dilution-based CTLp frequency assay

The frequencies of cytotoxic T-lymphocyte precursor (CTLp) specific for the recipient-mismatched HLA molecules were analyzed using a standard limiting dilution assay.[9]
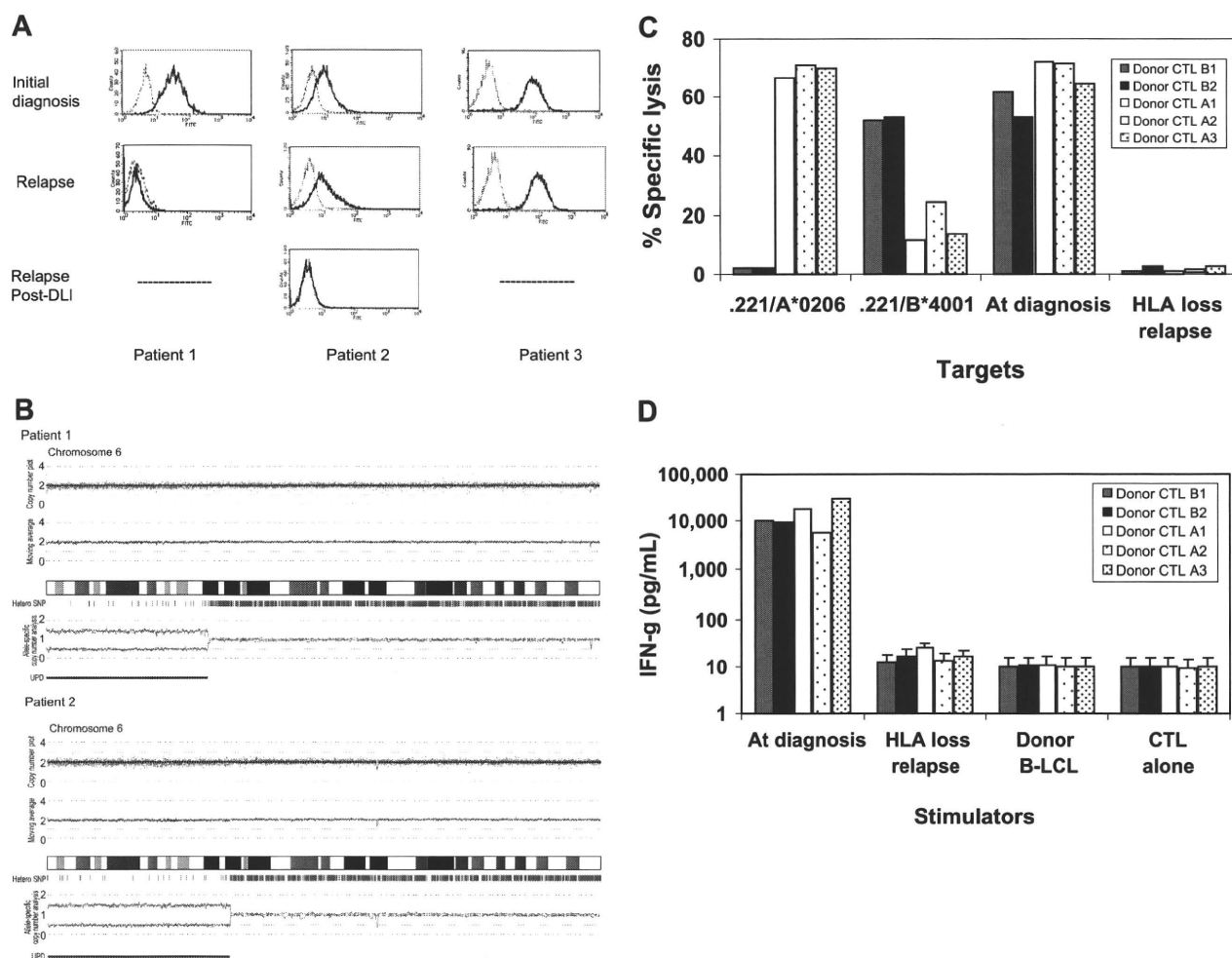
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Figure 1. The loss of mismatched HLA expression on leukemic blasts caused by uniparental disomy on chromosome 6p impaired recognition and killing of donor's alloreactive cytotoxic T lymphocytes. (A) Leukemic blasts at the time of initial diagnosis and at the time of relapse after hematopoietic stem cell transplantation (HSCT) and donor lymphocyte infusion (DLI) were gated by CD34^{+} and CD13^{+}, and then the surface expression of mismatched human leukocyte antigen (HLA) alleles was examined with anti–HLA-A2 antibodies. In 3 patients with acute myelogenous leukemia (AML) who experienced relapse after HLA-haploidentical HSCT, HLA-A2 expression was lost in patient 1 at relapse 15 months after HSCT and lost in patient 2 at second relapse 6 months after DLI. (B) Single nucleotide polymorphism (SNP) array analyses of sorted leukemic cells with the loss of an HLA allele revealed that the short arm of chromosome 6 shows copy number-neutral loss of heterozygosity or acquired uniparental disomy as detected by dissociated allele-specific copy number plots (red and blue lines at the bottom), resulting in the total loss of the mismatched HLA haplotype in both patient 1 and patient 2. The presence of acquired uniparental disomy is also indicated by normal total copy numbers with missing heterozygous SNPs (green bars) in the distal part of the short arm. (C) Recipient alloantigen-specific cytotoxic T-lymphocyte (CTL) clones were generated by a conventional cloning method from cytotoxicity-positive wells obtained in the limiting dilution assays using the donor CD8^{+} cells as responders. Donor CTL clones A1, A2, and A3 were specific for HLA-A*0206. Donor CTL clones B1 and B3 were specific for HLA-B*4001, all of which recognize mismatched HLA alleles between the donor and recipient. Those 5 representative CTL clones were tested for HLA specificity and recognition of leukemic blasts obtained at the time of the initial diagnosis and at the time of HLA loss relapse after DLI by a standard ⁵¹Cr-release assay at the effector/target ratio of 30:1. (D) Their interferon-γ production was also assessed against leukemic blasts collected at the time of diagnosis and at the time of HLA-loss relapse.

Cytotoxic assay of CTL clones against leukemic blasts and a mismatched HLA cDNA-transfected B-lymphoblastoid cell line

The remaining cells of several cytotoxicity-positive wells used for the CTLp assay for the donor were used to obtain allo-HLA–restricted CTLs. CTL clones were isolated by standard limiting dilution and expanded as previously described.[10,11]

The HLA class I–deficient 721.221 B-lymphoblastoid cell line was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2mM L-glutamine, and 1mM sodium pyruvate. Retroviral transduction was conducted as previously described.[12]

The cytotoxicity of CTL clones against target cells was analyzed by conventional chromium 51 (⁵¹Cr) release assay as previously reported.[13]

CTL clones (10⁴ cells/well) were mixed with the indicated stimulator cells (10⁴ cells/well) in 96-well, round-bottom polypropylene plates and spun at 1200g for 3 minutes before overnight incubation in 200 μL of RPMI 1640 medium supplemented with 10% fetal bovine serum. On the next day, 50 μL of supernatant was collected and interferon-γ was measured by enzyme-linked immunosorbent assay with 3,3′,5,5′-tetramethylbenzidine substrate (Sigma-Aldrich).

Results and discussion

Three children with high-risk acute myelogenous leukemia (AML) received haploidentical grafts from their parents but relapsed 8, 14, and 15 months after HSCT. Patient 2 received 3 courses of donor lymphocyte infusion (DLI) for relapsed leukemia after haploidentical HSCT. After the third unmanipulated DLI (10⁷ CD3⁺/kg), she experienced acute grade-III graft-versus-host disease and achieved complete remission. However, she experienced a second relapse 6 months later. To monitor residual disease in those patients, we used flow cytometric analysis with antibodies specific for the mismatched HLA alleles between the donor and patient. Surprisingly, we found total loss of

HLA-A2 expression on $CD13^+/CD34^+$ leukemic cells from bone marrow in 2 of 3 patients who underwent HLA-haploidentical HSCT, whereas microscopic analysis showed relapse (Figure 1A). To test whether HLA class I molecules could be up-regulated, samples were cultured for 48 hours in medium supplemented with tumor necrosis factor-α or interferon-γ and measured again; however, no restoration was observed (data not shown).

Next, to examine the potential loss of genes encoding the undetectable HLA alleles, we sorted $CD13^+/CD34^+$ leukemic blasts and performed DNA genotyping. We found that, in addition to the HLA-A locus, the HLA-B, -C, and -DR loci were not encoded; only the mismatched haplotype was lost in both patients (supplemental Table 2). We then questioned whether this phenomenon would also occur in HLA-matched HSCT settings using anti-HLA class I antibodies. We did not observe any loss of HLA class I expression in any of the patients at the time of relapse (supplemental Figure 1). These results suggest that loss of HLA class I haplotype at the time of posttransplantation relapse is uncommon in HLA-matched HSCT.

To elucidate the mechanism of the loss of the mismatched HLA haplotype, we performed an SNP array analysis of genomic DNA extracted from leukemic blasts at the time of diagnosis and of relapse. Genomic DNA from patient-derived T cells was used as a reference. Leukemic cells at the time of relapse showed copy number-neutral loss of heterozygosity or an acquired uniparental disomy (UPD) of the short arm of chromosome 6 encompassing the HLA locus, whereas no allelic imbalance was identified at the time of diagnosis (Figure 1B). Loss of one allele from one parent and duplication of the remaining allele from the other parent led to UPD.[14]

In patient 2, we examined whether the number of CTLp had changed during the posttransplantation course. Limiting dilution analysis with a split-well ^{51}Cr-release assay was carried out to compare the CTLp frequencies specific for the mismatched antigens between the recipient and donor. Interestingly, the CTLp frequencies were recovered after DLI (Table 1). Restoration of CTLp after 3 DLIs could eradicate such leukemic cells, lasting for 6 months thereafter.

Next, we generated allo-HLA–restricted CTLs from $CD8^+$ cells obtained at day 520 in patient 2 and tested with the 721.221 B-lymphoblastoid cell line transfected with 1 of 3 mismatched HLA alleles (Figure 1C-D).

Despite high transplantation-related mortality resulting from severe graft-versus-host disease and posttransplantation infections, haploidentical HSCT has been widely used with the expectation of a strong graft-versus-leukemia effect.[15] However, our observation provides a possible limitation of this strategy. Indeed, 2 of 3 patients showed genomic loss of the recipient-specific HLA-haplotype, which led to escape from the graft-versus-leukemia effect and relapse of the disease.

Vago et al also reported a similar observation in 5 of 17 (29.4%) patients whose disease relapsed after haploidentical HSCT.[16] Relapsed leukemic cells may possess genomic instability that elicits genetic diversity.[17] Immunologic pressure by alloreaction to major HLA antigens may select leukemic variants of HLA class I loss, which results in the survival and proliferation of these variants.

In haploidentical HSCT, the importance of natural killer (NK)–cell alloreactivity is emphasized to achieve the graft-versus-leukemia effect.[18,19] HLA loss on leukemic blasts may in turn enhance the NK-cell alloreactivity. Our 2 patients with HLA loss had a group 1 homozygous HLA-C locus that is a suppressive killer immunoglobulin-like receptor (KIR) for NK cells and a KIR-matched donor (supplemental Table 2). Because UPD does not

Table 1. The CTLp frequency reactive to the recipient alloantigen in the recipient after transplantation and the donor

Samples	Maximum CD8$^+$ input*	No. of growing wells†	CTLp frequency^{-1} (95% confidence interval)
Donor	33 300	8	8.6×10^5 (1.49×10^6-5.0×10^5)
Day 100	35 500	0	UD
Day 180	17 700	0	UD
Day 300‡	86 000	0	UD
Day 520§	95 000	7	4.3×10^5 (7.2×10^5-2.5×10^5)

Purified CD8$^+$ T cells from the peripheral blood mononuclear cells obtained after transplantation from patient 2 and her donor were cultured at 2- or 3-fold serial dilutions with 33 Gy-irradiated 3×10^4 leukemic blasts cryopreserved at the time of initial diagnosis in 96-well, round-bottom plates in advanced RPMI 1640 medium supplemented with 4% pooled human serum, interleukin-6 (IL-6), and IL-7 (10 ng/mL; both from R&D Systems). The IL-2 (50 U/mL) was added on day 7 with a half medium change. For each dilution, there were at least 12 replicates. On day 14 of culture, a split-well analysis was performed for recipient-specific cytotoxicity against ^{51}Cr-radiolabeled recipient T-cell blasts, donor T-cell blasts, and leukemic blasts harvested at the time of initial diagnosis and at the time of relapse after DLI if indicated. The supernatants were measured in a γ counter after 4-hour incubation. The wells were considered to be positive for cytolytic activity if the total counts per minute released by effector cells was more than 3 SD above the control wells (mean counts per minute released by the target cells incubated with irradiated stimulator cells alone). The CTLp frequency was calculated using L-Calc software (StemCell Technologies). The CTLp frequencies reactive with recipient T-cell blasts in CD8$^+$ T cells obtained around days 100, 180, and 300 (4 months before relapse) were undetectable, whereas the CTLp frequency obtained at day 520 (1 month after the third DLI or 2 weeks after remission confirmed by bone marrow aspirate) was close to the CTLp frequency in the donor CD8$^+$ cells. Complete remission and more than 99% donor chimerism were confirmed on those days

CTLp indicates CTL precursor; and UD, undetermined because no growing wells are present.

*Number of input CD8$^+$ T cells seeded at the highest number per well.

†Number of wells out of 12 wells that received the highest CD8$^+$ cells and showed detectable growth.

‡Corresponds to 4 months before relapse.

§Corresponds to 1 month after the third DLI or 2 weeks after complete remission was confirmed by bone marrow aspirate.

change the total copy number of the gene, donor NK cells should have been suppressed even after UPD occurred in these patients. Interestingly, the remaining patient who experienced relapse without HLA loss after HLA-haploidentical HSCT had a KIR-mismatched donor, so alloreactive NK cells were possibly enhanced to kill leukemic blasts with HLA loss.

Although one limitation of our study is an insufficient number of cases, our results combined with those in a recent report[16] suggest that leukemic cells occasionally escape from immunosurveillance through the loss of the mismatched HLA haplotype by the mechanism of UPD after haploidentical HSCT. DLI for relapsed AML is less effective than that for chronic myelogenous leukemia after HLA-matched HSCT.[20] However, DLI is effective even for the relapse of AML after haploidentical HSCT.[21] Evaluation of loss or down-regulation of HLA on relapsed leukemic blasts after HLA-haploidentical HSCT should be considered because DLI would probably be ineffective in patients whose leukemic cells lose HLA class I antigen.

Acknowledgments

Authorship

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Down syndrome and GATA1 mutations in transient abnormal myeloproliferative disorder: mutation classes correlate with progression to myeloid leukemia

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Down syndrome and *GATA1* mutations in transient abnormal myeloproliferative disorder: mutation classes correlate with progression to myeloid leukemia

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Twenty percent to 30% of transient abnormal myelopoiesis (TAM) observed in newborns with Down syndrome (DS) develop myeloid leukemia of DS (ML-DS). Most cases of TAM carry somatic *GATA1* mutations resulting in the exclusive expression of a truncated protein (GATA1s). However, there are no reports on the expression levels of GATA1s in TAM blasts, and the risk factors for the progression to ML-DS are unidentified. To test whether the spectrum of transcripts derived from the mutant *GATA1* genes affects the expression levels, we classified the mutations according to the types of transcripts, and investigated the modalities of expression by in vitro transfection experiments using *GATA1* expression constructs harboring mutations. We show here that the mutations affected the amount of mutant protein. Based on our estimates of GATA1s protein expression, the mutations were classified into GATA1s high and low groups. Phenotypic analyses of 66 TAM patients with *GATA1* mutations revealed that GATA1s low mutations were significantly associated with a risk of progression to ML-DS (*P* < .001) and lower white blood cell counts (*P* = .004). Our study indicates that quantitative differences in mutant protein levels have significant effects on the phenotype of TAM and warrants further investigation in a prospective study. (*Blood*. 2010;116(22):4631-4638)

Introduction

In children with Down syndrome (DS), the risk of developing acute megakaryocytic leukemia (AMKL) is estimated at 500 times higher than in children without DS. Interestingly, neonates with DS are at a high risk of developing a hematologic disorder referred to as transient abnormal myelopoiesis (TAM). It has been estimated that 5% to 10% of infants with DS exhibit the disorder, and in most cases, it resolves spontaneously within 3 months. However, approximately 20% of the severe cases are still subject to fatal complications and 20% to 30% of patients who escape from early death develop AMKL referred to as myeloid leukemia of DS (ML-DS) within 4 years.[1-4]

Recent studies found that high white blood cell (WBC) count, failure of spontaneous remission, early gestational age (EGA) and liver fibrosis or liver dysfunction are significantly associated with early death.[5-7] Most of the same covariates were found in all of the reports. However, the risk factors for the progression to ML-DS remain elusive.

Blast cells in most patients with TAM and ML-DS have mutations in exon 2 of the gene coding the transcription factor GATA1,[8-14] which is essential for normal development of erythroid and megakaryocytic cells.[15-18] The mutations lead to exclusive expression of a truncated GATA1 protein (referred to as GATA1s)

translated from the second methionine on exon 3. These findings strongly suggest that the qualitative deficit of GATA1 contributes to the genesis of TAM and ML-DS. The analysis of megakaryocyte-specific knockdown of *GATA1* in vivo has revealed a critical role for this factor in megakaryocytic development. Reduced expression (or complete absence) of GATA1 in megakaryocytes leads to increased proliferation and deficient maturation as well as a reduced number of circulating platelets.[19,20] Mice harboring a heterozygous *GATA1* knockdown allele frequently develop erythroblastic leukemia.[21] These observations indicate that the expression levels of GATA1 are crucial for the proper development of erythroid and megakaryocytic cells and compromised GATA1 expression is a causal factor in leukemia.[22] Nevertheless, the impact of a quantitative deficit of the factor on the pathogenesis of TAM and ML-DS has not been examined.

In this study, we classified the *GATA1* mutations observed in TAM patients according to the types of transcripts, and investigated the modalities of gene expression by in vitro transfection assays using *GATA1* expression constructs. We report here that the spectrum of the transcripts derived from the mutant genes affects protein expression and the risk of progression from TAM to ML-DS.

Methods

Patients

This study was approved by the Ethics Committee of Hirosaki University Graduate School of Medicine, and all clinical samples were obtained with informed consent from the parents of all patients with TAM, in accordance with the Declaration of Helsinki. The following clinical data were collected: sex, gestational age, birth weight, time of diagnosis, symptom at diagnosis, and clinical presentation. The following laboratory data were obtained: a complete blood cell count at diagnosis including WBC and the percentage of blasts in the peripheral blood, coagulation parameters, liver enzymes (alanine aminotransferase and aspartate aminotransferase), and total bilirubin. The procedure for the detection of *GATA1* mutations was described previously.[13] Genomic DNA was directly extracted from peripheral blood or bone marrow with the QIAamp blood mini kit (QIAGEN). Total RNA was extracted from white blood cells prepared by removal of erythrocytes by hypotonic buffer treatment of peripheral blood. Clinical features, outcomes, and characteristics of *GATA1* mutations are indicated in Table 1.

Construction of GATA1 expression vectors

To construct *GATA1* minigene expression vectors, fragments of the normal human *GATA1* gene from a part of intron 1 to the stop codon located on exon 6 were amplified by polymerase chain reaction (PCR; Prime STAR HS: Takara Bio) and subcloned to mammalian expression vector pcDNA3.1 (+)/Neo (Invitrogen). To introduce mutations identical to those observed in TAM patients into the expression vector, the regions containing mutations were amplified by PCR from patient samples and inserted into the expression plasmid. To construct expression vectors carrying cDNA, we performed PCR using cDNA derived from baby hamster kidney 21 (BHK-21) cells transfected with *GATA1* minigene vectors. The PCR products were subcloned to pcDNA3.1(+)/Neo. Details of the sequence of each expression construct are described in Table 2.

Transfection

BHK-21, a baby hamster kidney fibroblast cell line, was cultured with Dulbecco modified Eagle medium supplemented 10% fetal bovine serum. *GATA1* expression vectors were transfected into BHK-21 cells using FuGENE HD transfection reagent (Roche Diagnostics) according to the manufacturer's methods. After 24 hours, protein and total RNA were extracted.

Western blot analysis

Lysates of transfected BHK-21 cells were transferred to Hybond-P (GE Healthcare) and processed for reaction with anti-GATA1 antibody M-20 (Santa Cruz Biotechnology) or anti-neomycin phosphotransferase II (NeoR) antibody (Millipore) as described previously.[23]

Northern blot analysis

Two micrograms of total RNA were transferred to Hybond-N+ (GE Healthcare) and hybridized with *GATA1* or *NeoR* DNA probe. Hybridization and detection were performed with the Gene Images AlkPhos Direct Labeling and Detection System (GE Healthcare) according to the manufacturer's instructions.

RT-PCR

To detect alternatively spliced transcripts derived from *GATA1* minigene constructs or from patients' peripheral blood mononuclear cells (obtained by Ficoll-Hypaque fractionation), we performed reverse transcription (RT)–PCR using primers T7: 5′ AATACGACTCACTATAG 3′ and GATA1 AS1, and GATA1 S1 and GATA1 AS1, respectively.[13] Densitometric analyses were performed by the Quantity-One software (Version 4.5.2; Bio-Rad Laboratories).

Statistical analysis

The cumulative incidence of the progression to ML-DS was analyzed with the Gray test. Differences in the distribution of individual parameters among patient subsets were analyzed using the Pearson χ^2 test or Fisher exact test for categorized variables and the Mann-Whitney U test for continuous variables. The univariate Cox proportional hazards model was used to obtain the estimates and the 95% confidence interval of the relative risk for prognostic factors.

Results

Patient characteristics and outcomes

From 2003 to 2008, we screened *GATA1* mutations in clinical samples obtained from 78 patients with TAM upon request from referring hospitals. Acquired *GATA1* mutations were detected in a total of 72 (92.3%) patients among them. Of the 72 patients, 6 harbored multiple *GATA1* mutant clones and were excluded from this study because we could not determine a dominant clone in these patients. Those 6 have not progressed to ML-DS. For the remaining 66 patients (32 male and 34 female), the clinical characteristics and laboratory data at diagnosis are described in Table 1 and summarized in Table 3. Early death within the first 6 months of life occurred in 16 patients (24.2%). The covariates correlated with early death were as follows: EGA, low birth weight, high WBC count at diagnosis, high percentage of peripheral blast cells, complication of effusions, and bleeding diatheses. These prognostic factors were identified in previous studies.[5-7] Eleven (16.7%) cases subsequently developed ML-DS. The median age at diagnosis of ML-DS was 396 days (range 221-747 days). Univariate analysis revealed no covariates correlated with progression to ML-DS except the low total bilirubin level at diagnosis ($P = .023$).

GATA1 mutations affect expression levels of GATA1s protein

We first asked whether the spectrum of transcripts derived from the mutant *GATA1* genes affected the expression levels of the translation products. The transcripts coding GATA1s protein were categorized into 3 groups as follows: loss of the first methionine, splicing errors, and premature termination codon (PTC). Furthermore, the PTC group was divided into 2 subcategories by the location of introduced PTC. In this report, we refer to the mutation that causes PTC before the second methionine at codon 84 as PTC type 1, and after codon 84 as PTC type 2. We constructed cDNA expression vectors for each class of mutations observed in TAM patients, and transfected these constructs into BHK-21 cells (Figure 1). The details of the *GATA1* mutations are described in Table 2. Western blot analysis revealed that GATA1s proteins were most abundantly expressed in mutants with splicing errors. The transcripts from mutants that had lost the first methionine were also efficiently translated. In contrast, in the cells expressing PTC type 1 or type 2 constructs, GATA1s expression levels were uniformly low. Note that the translation efficiency of the PTC type 2 transcripts was lowest among them.

To test the possibility that mutations in *GATA1* have an effect on the quantity of the transcripts, we next prepared human *GATA1* minigene expression vectors, and assessed the expression levels. Consistent with the results using cDNA expression vectors, Western blot analysis showed that the expression levels of GATA1s were lower in cells expressing PTC type 2 mutations, whereas the expression levels of the proteins from PTC type 1 mutations were not uniformly low (Figure 2Ai). Northern blot analysis revealed that the lowest expression levels of *GATA1* mRNAs were observed

Table 1. Clinical features and mutation characteristics in TAM patients with *GATA1* mutations

Patient No.	Sex	WBC, ×10⁹/L	Outcome	*GATA1* mutation*	Consequence of mutation	Mutation type
1[13,24]	F	63.9	CR	207 C>G	Tyr69stop	PTC 1-3′
2[13]	F	89.0	Early death	199 G>T	Glu67stop	PTC 1-3′
3[13]	F	NA	NA	174 ins 19 bp CAGCCACCGCTGCAGCTGC	Frame shift at codon58, stop at codon 73	PTC 1-3′
4[13]	F	128.8	CR	IVS1 to IVS2 del 1415 bp	Splice mutant	Splicing error
5[13]	F	NA	NA	49 C>T	Gln17stop	PTC 1-5′
6[13]	F	248.6	NA	Loss of 2nd exon	Splice mutant	Splicing error
7[13]	F	31.2	CR	Loss of 2nd exon	Splice mutant	Splicing error
8[13]	M	199.6	CR	−11 to +33 del 44 bp	No translation from Met1	Loss of 1st Met
9[13]	M	44.9	Early death	45 ins C	Frame shift at codon15, stop at codon 39	PTC 1-5′
10[13]	M	50.9	CR	37 G>T	Glu13stop	PTC 1-5′
11[13]	F	103.0	Early death	90-91 del AG	Frame shift at codon 30, stop at codon 38	PTC 1-5′
12[13]	F	14.6	Evolved to ML-DS	116 del A	Frame shift at codon 39, stop at codon 136	PTC 2
13[13]	M	423.0	CR	185 ins 22 bp GCTGCAGCTGCGGCACTGGCCT	Frame shift at codon 62, stop at codon 74	PTC 1-3′
14[13]	M	201.2	CR	189 C>A	Tyr63stop	PTC 1-3′
15[13]	M	NA	NA	1 A>G	No translation from Met1	Loss of 1st Met
16[13]	F	28.3	CR	189 C>A	Tyr63stop	PTC 1-3′
17[13]	M	203.0	Evolved to ML-DS	38-39 del AG	Frame shift at codon 13, stop at codon 38	PTC 1-5′
18[13]	M	31.3	CR	189 C>A	Tyr63stop	PTC 1-3′
19[13]	M	NA	NA	90-91 del AG	Frame shift at codon 30, stop at codon 38	PTC 1-5′
20[13]	F	114.0	Early death	187 ins T	Frame shift at codon 63, stop at codon 67	PTC 1-3′
21[25]	F	26.0	Evolved to ML-DS	194 ins 20 bp GGCACTGGCCTACTACAGGG	Frame shift at codon 65, stop at codon 143	PTC 2
22[25]	F	25.0	Evolved to ML-DS	194 ins 20 bp GGCACTGGCCTACTACAGGG	Frame shift at codon 65, stop at codon 143	PTC 2
23	F	49.9	CR	3 G>T	No translation from Met1	Loss of 1st Met
24	F	46.2	NA	IVS1 3′ boundary AG>AA	Splice mutant	Splicing error
25	F	10.5	CR	194 ins 19 bp GCACTGGCCTACTACAGGG	Frame shift at codon 65, stop at codon 73	PTC 1-3′
26[24]	F	244.0	Evolved to ML-DS	1 A>G	No translation from Met1	Loss of 1st Met
27	F	38.3	CR	Loss of 2nd Exon	Splice mutant	Splicing error
28[24]	F	34.6	CR	IVS1 to exon2 del 148 bp	Splice mutant	Splicing error
29	M	25.9	Evolved to ML-DS	160 ins TC	Frame shift at codon 54, stop at codon 137	PTC 2
30	F	52.3	Evolved to ML-DS	187 ins CCTAC	Frame shift at codon 63, stop at codon 138	PTC 2
31[24]	F	221.0	CR	183-193 del 11 bp CTACTACAGGG	Frame shift at codon 62, stop at codon 63	PTC 1-3′
32	M	149.7	CR	2 T>G	No translation from Met1	Loss of 1st Met
33[24]	M	132.3	Evolved to ML-DS	101-108 del 8 bp TCCCCTCT	Frame shift at codon 34, stop at codon 36	PTC 1-5′
34[24]	F	220.0	Early death	90-91 del AG	Frame shift at codon 30, stop at codon 38	PTC 1-5′
35[24]	M	166.0	Early death	IVS2 5′ boundary GT>CT	Splice mutant	Splicing error
36[24]	M	57.6	Early death	193-199 GACGCTG>TAGTAGT	Asp65stop	PTC 1-3′
37[24]	M	247.6	Early death	Exon2 to IVS2 del 218 bp	Splice mutant	Splicing error
38[24]	M	93.3	Early death	IVS1 3′ boundary AG>AA	Splice mutant	Splicing error
39[24]	M	290.8	Early death	186 ins 12 bp GGCACTGGCCTA	Tyr62stop	PTC 1-3′
40	F	7.8	CR	2 T>C	No translation from Met1	Loss of 1st Met
41[24]	M	136.6	Early death	IVS2 5′ boundary GT>GC	Splice mutant	Splicing error
42	M	33.1	Early death	187 ins 8 bp TGGCCTAC	Frame shift at codon 63, stop at codon 139	PTC 2
43	M	9.0	CR	22 ins G	Frame shift at codon 8, stop at codon 39	PTC 1-5′
44	M	24.1	Evolved to ML-DS	149 ins 20 bp AGCAGCTTCCTCCACTGCCC	Frame shift at codon 50, stop at codon 143	PTC 2
45[24]	F	53.3	CR	173 C>TGCTGCAGTGTAGTA	Frame shift at codon 58, stop at codon 141	PTC 2
46	F	119.0	CR	1 A>C	No translation from Met1	Loss of 1st Met
47	M	33.0	CR	189 C>A	Tyr63stop	PTC 1-3′
48	M	178.2	Early death	188 ins 22 bp GCAGCTGCGGCACTGGCCTACT	Frame shift at codon 63, stop at codon 74	PTC 1-3′
49	F	73.6	CR	3 G>A	No translation from Met1	Loss of 1st Met
50	F	12.9	CR	158 ins 7 bp AGCACAG	Frame shift at codon 53, stop at codon 69	PTC 1-5′
51	M	13.0	CR	154-161 del 8 bp ACAGCCAC	Frame shift at codon 52, stop at codon 64	PTC 1-5′
52	M	105.5	Early death	4 G>T	Glu2stop	PTC 1-5′
53	F	98.3	CR	4 G>T	Glu2stop	PTC 1-5′
54	F	356.9	CR	219 A>C	Splice mutant	Splicing error
55	F	25.8	Evolved to ML-DS	157 ins CA	Frame shift at codon 53, stop at codon 137	PTC 2
56	M	97.4	Evolved to ML-DS	185-188 del 4 bp ACTA	Frame shift at codon 62, stop at codon 135	PTC 2
57	F	97.3	Early death	3 G>A	No translation from Met1	Loss of 1st Met
58	M	NA	CR	3 G>A	No translation from Met1	Loss of 1st Met
59	M	20.2	CR	150 ins 5 bp TGGCT	Frame shift at codon 50, stop at codon 52	PTC 1-5′
60	M	133.4	CR	174 ins 19 bp CAAAGCAGCTGCAGCGGTG	Frame shift at codon 58, stop at codon 73	PTC 1-3′
61	M	NA	CR	220 G>T	Splice mutant	Splicing error
62	M	120.2	CR	220 G>A	Splice mutant	Splicing error
63	F	39.0	CR	97-139 del 43 bp	Frame shift at codon 33, stop at codon 122	PTC 2
64	F	NA	NA	156 ins C	Frame shift at codon 52, stop at codon 67	PTC 1-5′
65	F	32.4	CR	174 ins 7 bp CTGCAGC	Frame shift at codon 58, stop at codon 69	PTC 1-3′
66	M	69.4	Early death	174-177 GGCA>TGCGGTGG	Frame shift at codon 58, stop at codon 68	PTC 1-3′

We previously reported the *GATA1* mutations of the indicated patients.

F indicates female; M, male; CR, complete remission; NA, not available; and IVS, intervening sequence.

*For cDNA nucleotide numbering, nucleotide number 1 corresponds to the A of the ATG translation initiation codon in the reference sequence.

Table 2. GATA1 expression vectors used in this study

Name	Patient no.	*GATA1* mutation*	Last normal GATA1 amino acid	PTC	Mutation type
WG	–	–	Ser413	–	Normal
SP1	24, 38	intron1 3′ boundary AG>AA	Ser413	–	Splicing error
SP2	41	intron2 5′ boundary GT>GC	Ser413	–	Splicing error
L	46	1 A>C	(Met1 is replaced by Val1)	–	Loss of 1st Met
P1-1	11, 19, 34	90, 91 del AG	Gly31	38	PTC 1-5′
P1-2	14, 16, 18, 47	189 C>A	Tyr62	63	PTC 1-3′
P1-3	25	194 ins 19 bp	Arg64	73	PTC 1-3′
P1-4	17	38, 39 del AG	Ser12	38	PTC 1-5′
P1-5	33	101-108 del 8 bp	Phe33	36	PTC 1-5′
P1-6	50	158 ins 7 bp	Tyr52	69	PTC 1-5′
P1-7	3	174 ins 19 bp	Ala58	73	PTC 1-3′
P1-8	48	188 ins 22 bp	Try62	74	PTC 1-3′
P2-1	21, 22	194 ins 20 bp	Arg64	143	PTC 2
P2-2	44	149 ins 20 bp	Ala49	143	PTC 2
P2-3	29	160 ins TC	Ala53	137	PTC 2

– indicates not applicable.

*For cDNA nucleotide numbering, nucleotide number 1 corresponds to the A of the ATG translation initiation codon in the reference sequence.

in cells transfected with PTC type 2 constructs, whereas the mRNA levels in mutants that had lost the first methionine and PTC type 1 mutants were almost comparable to those of control minigene constructs harboring wild type *GATA1* gene (Figure 2Aiii). Thus, abundant proteins were produced from *GATA1* mRNAs in mutants with splicing errors and those that lost the first methionine. Conversely, relatively low levels of protein were produced by PTC type 2 mutants because of inefficient translation and reduced levels of message (Figure 2Ai,iii). However, in the case of PTC type 1 mutations, especially P1-1 and P1-4, we could find no correlation between the amount of transcripts or translation efficiency and the expression levels of GATA1s proteins (Figure 2Ai,iii).

GATA1s expression levels largely depend on the amount of the alternative splicing form

To investigate the precise relationship between PTC type 1 mutations and GATA1s protein levels, we examined more type 1 mutations

using the minigene constructs. Western blot analysis showed relatively higher expression of the proteins in samples expressing P1-5, P1-7, P1-8, P1-2, and P1-3 than the other constructs (Figure 2Bi). Each mutation in the mutant minigene construct is described in Table 2. Interestingly, all samples that expressed higher levels of GATA1s protein exhibited intense signals at lower molecular weights than the dominant GATA1 signal (Figure 2Biii). Because the size of the lower molecular weight band was identical to that observed in the splicing error mutant (Figure 2Biii), we speculated that the signal might be derived from a transcript lacking exon 2 (Δexon 2) by alternative splicing. To examine that possibility, we attempted Northern blot analysis using the *GATA1* exon 2 fragment as a probe, and as expected, only the longer transcript was detected (Figure 2Biv). To confirm the correlation between the amount of Δexon 2 transcript and GATA1s protein, we performed a quantitative assessment by densitometric analysis. The results showed a strong correlation between Δexon 2 transcript and GATA1s protein

Table 3. Findings at diagnosis and during the course of TAM were significantly associated with early death and the progression to leukemia (univariate analysis)

Variable	Total (n = 66)	Early death (n = 16)	P	Progressed to ML-DS (n = 11)	P
Sex					
Male, n (%)	32 (48.5)	11 (68.8)		5 (45.5)	
Female, n (%)	34 (51.5)	5 (31.3)	.088	6 (54.5)	.947
Median gestational age, wk (range)	37.35 (30.0-40.6)	34.6 (30.0-38.4)		38.1 (32.6-40.6)	
Term versus preterm					
Term (≥ 37 weeks), n (%)	27 (58.7)	4 (30.8)		5 (71.4)	
Preterm (< 37 weeks), n (%)	19 (41.3)	9 (69.2)	.021	2 (28.6)	.465
Median birth weight, kg (range)	2.5 (1.4-3.5)	2.2 (1.6-2.7)		2.5 (1.6-3.5)	
Not LBW versus LBW					
Not LBW (≥ 2.5 kg), n (%)	24 (52.2)	3 (23.1)		3 (42.9)	
LBW (< 2.5 kg), n (%)	22 (47.8)	10 (76.9)	.025	4 (57.1)	.184
Median WBC, ×10⁹/L (range)	69.4 (7.8-423.0)	104.3 (33.1-290.8)		26 (14.6-244.0)	
WBC < 70 × 10⁹/L vs WBC > 70 × 10⁹/L					
WBC < 70 × 10⁹/L, n (%)	30 (50.8)	4 (25.0)		7 (63.6)	
WBC > 70 × 10⁹/L, n (%)	29 (49.2)	12 (75.0)	.020	4 (36.4)	.755
Median peripheral blasts, % (range)	56.0 (4.0-94.0)	78.0 (8.0-93.0)	.031	49.5 (6.0-66.0)	.752
Median AST, IU/L (range)	61 (16-4341)	79 (41-3866)	.620	51 (16-153)	.553
Median ALT, IU/L (range)	39 (4-653)	41 (7-473)	.455	12 (4-96)	.615
Median T-Bil mg/dL (range)	6.3 (0.6-46.0)	6.06 (2.4-16.5)	.922	3.01 (1.82-6.50)	.023
Effusions, n (%)	16 of 44 (36.4)	8 of 11 (72.7)	.007	1 of 7 (14.3)	.912
Bleeding diatheses, n (%)	13 of 45 (28.9)	8 of 12 (66.7)	.001	1 of 7 (14.3)	.123

Some clinical data were not available. We defined the number of patients for whom clinical data was available as (n).

LBW indicates low birth weight; AST, aspartate transaminase; ALT, alanine transaminase; and T-Bil, total bilirubin.
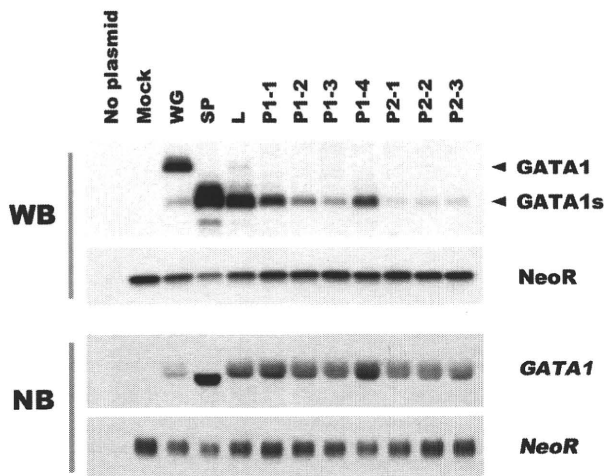
Figure 1. Effects of mutant transcripts of *GATA1* on the expression level of the truncated protein. The *GATA1* mutations observed in TAM patients are classified according to the types of transcripts. The translational efficiency of each transcript was assessed by Western blot analysis in BHK-21 cells transfected with *GATA1* cDNA expression vectors (top part of the panel) and Northern blot analysis (bottom part of the panel), respectively. WG indicates wild type GATA1; SP, splicing error mutation (Δexon 2); L, loss of first methionine mutation; P1, PTC type 1 mutation; P2, PTC type 2 mutation. The details of the *GATA1* mutations are summarized in Table 1. NeoR indicates Neomycin phosphotransferase II.

levels ($r = 0.892$, $P = .003$), but not with the long transcript containing exon2 nor total *GATA1* mRNA (supplemental Figure 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). Next, we performed RT-PCR using primers recognizing both transcripts, and calculated the ratio of Δexon 2 to the long transcript (Figure 2Bvi-vii). The intensive short transcript was detected in all samples with higher expression of GATA1s (P1-5, P1-7, P1-8, P1-2, and P1-3; Figure 2Bvii). Interestingly, most of these mutations were clustered in the 3′ region of exon 2 (Table 2, Figure 2Bvii). These results suggest that the location of the mutation predicts the efficiency of alternative splicing and GATA1s expression levels.

To examine whether differential splicing efficiency could also be observed in TAM blasts with PTC type 1 mutations, RT-PCR analysis was performed using patients' clinical samples. Intense transcription of the short form was observed in the samples from the patients who had *GATA1* mutations located on the 3′ side of exon 2 (+169 to +218 in mRNA from the ATG translation initiation codon; Figure 3A-B). We refer to them as PTC type 1-3′ and the mutations located on the 5′ side of exon 2 as PTC type 1-5′.

Correlation of the phenotype and *GATA1* mutations in TAM patients

Based on these results, *GATA1* mutations were classified into 2 groups: a high GATA1s expression group (GATA1s high group) including the loss of first methionine type, the splicing error type, and PTC type 1-3′, and a low GATA1s expression group (GATA1s low group) including PTC type 1-5′ and PTC type 2. We classified TAM patients into these 2 groups in accordance with the GATA1s expression levels estimated from the mutations and compared their clinical data. High counts of WBC and blast cells were significantly associated with the GATA1s high group ($P = .004$ and $P = .008$, respectively; Table 4). Although high WBC count was correlated with early death, there were no significant differences in the cumulative incidence of early death between the 2 groups (Figure 4). Importantly, TAM patients in the GATA1s low group had a

significantly higher risk for the development of leukemia ($P < .001$; Figure 4). Of 11 TAM patients who progressed to ML-DS, 10 belonged to the GATA1s low group. Notably, 8 patients among them had PTC type 2 mutations (Tables 1, 5).
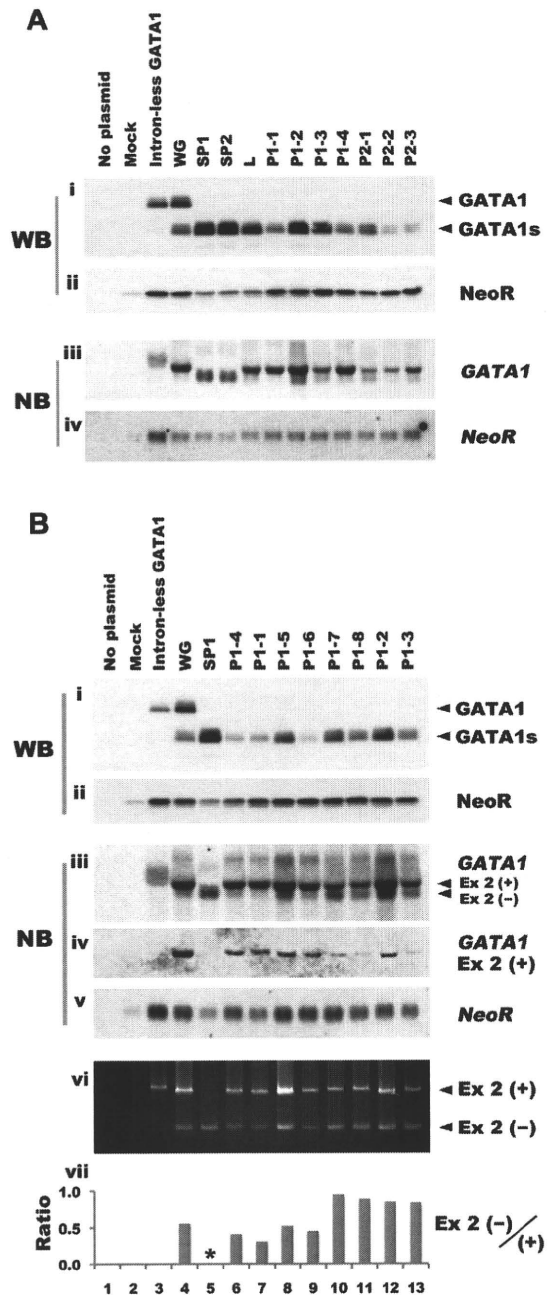

Figure 2. GATA1 mutations affect the expression level of the truncated protein. (A) The expression levels of GATA1s protein and mRNA were assessed in BHK-21 cells transfected with human *GATA1* minigene expression vectors carrying mutations observed in TAM patients. Western blot analysis was performed with anti-GATA1 (i) or anti-NeoR antibody (ii). Northern blot analysis was carried out with *GATA1* exon 3-6 fragment (iii) or *NeoR* cDNA (iv) as probe. (B) The expression levels of GATA1s protein and mRNA in BHK-21 cells transfected with human *GATA1* minigene expression vectors with PTC type 1 mutation. Levels were assessed by Western blot analysis with anti-GATA1 antibody (i), anti-NeoR antibody (ii). Northern blot analysis was performed with GATA1 exon 3-6 (iii), exon 2 (iv), or NeoR cDNA (v). To detect the transcripts derived from the human *GATA1* minigene expression construct, RT-PCR analysis was carried out using primers described in "RT-PCR" (vi). Ex 2(+) and Ex 2(−) indicate PCR products or transcripts with or without exon 2, respectively. Ratio of Ex 2(−)/(+) was calculated from the results of a densitometric analysis of the RT-PCR. The asterisk denotes unavailable data (vii).
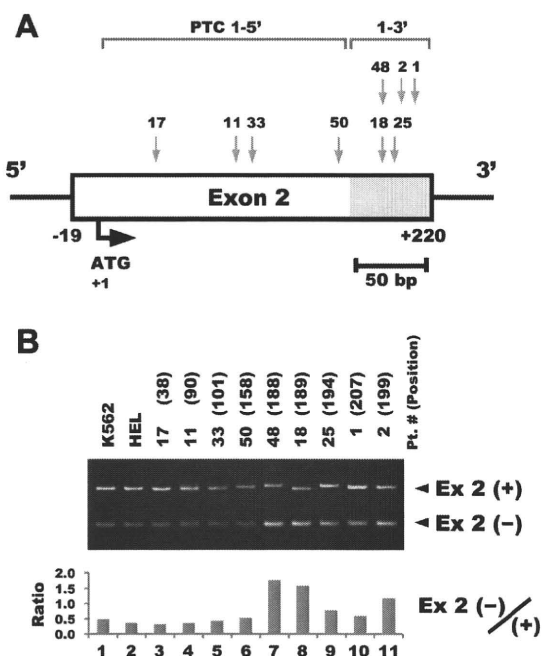
Figure 3. The location of the PTC type 1 mutation affects the efficiency of alternative splicing in TAM blast cells. (A) The location of the *GATA1* mutation in each TAM patient. Details of the mutation in each sample are described in Table 1. (B) RT-PCR analysis of *GATA1* in TAM blast cells harboring PTC type 1 mutations. RT-PCR was performed using primers recognizing both the long transcript including exon 2 and Δexon 2 (top). All of the patient samples consisted of mononuclear cells from peripheral blood. The numbers in parentheses indicate the number of nucleotides in mRNA from the translation initiation codon. Ex 2(+) and Ex 2(−) indicate PCR products with or without exon 2, respectively (middle). Ratio of Ex 2(−)/(+) was calculated from the results of a densitometric analysis of the RT-PCR (bottom). Note that the intense bands of the short form were observed in the samples from the patients who have *GATA1* mutations located on the 3′ side of exon 2 (lanes 7-11).

To validate this observation, we examined the proportion of mutation types in 40 ML-DS patients observed in the same period of time as this surveillance. The results showed a significantly higher incidence of GATA1s low type mutations in ML-DS than in TAM ($P = .039$; Table 5). These results further support the present findings that quantitative differences in the mutant protein have a significant effect on the risk of progression to ML-DS.

Table 4. Correlations between patient covariates and GATA1 expression levels

	GATA1s expression group		
	High (n = 40)	Low (n = 26)	P
Sex: male/female, n	19/21	13/13	.843*
Gestational age, wk	37.3 (30.0-40.0)	37.9 (32.6-40.6)	.487
Birth weight, kg	2.5 (1.6-3.3)	2.5 (1.4-3.5)	.698
WBC, ×10⁹/L	105.65 (7.8-423.0)	39.0 (9.0-220.0)	.004
Number of blasts, ×10⁹/L	72.1 (0.42-301.6)	13.4 (0.45-189.2)	.008
AST, IU/L	68.5 (23-501)	46.5 (16-4341)	.113
ALT, IU/L	41.0 (5-407)	12.5 (4-653)	.075
T-Bil mg/dL	6.7 (0.6-15.3)	4.65 (1.82-46.0)	.270
Effusions, n (%)	11 of 27 (40.7)	5 of 17 (29.4)	.447†
Bleeding diatheses, n (%)	8 of 29 (27.6)	5 of 16 (31.3)	.528†

Values are given as the median (range). *P* values estimated by Mann-Whitney *U* test.

*Pearson χ² test.
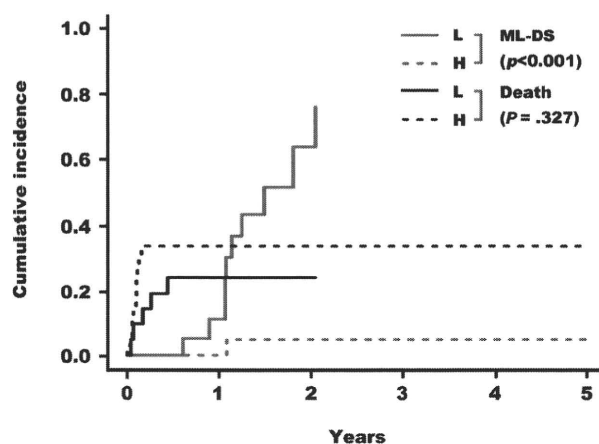†Fisher exact test.

Figure 4. Cumulative incidence of early death and of ML-DS in children with TAM. Based on the estimated GATA1s expression levels, patients were classified in 2 groups: GATA1s high and low groups. TAM patients in the GATA1s low group had a significantly higher risk for the development of leukemia (*P*(gray) < .001).

Discussion

In TAM, *GATA1* mutations lead to the expression of proteins lacking the N-terminal transactivation domain. In addition to this qualitative change, we showed here that the mutations affect the expression level of the truncated protein. The mutations were classified into 2 groups according to the estimated GATA1s expression level. Comparison of the clinical features between the 2 groups revealed that GATA1s low mutations were significantly associated with a high risk of progression to ML-DS and lower counts of both WBC and blast cells. These results suggest that quantitative differences in protein expression caused by *GATA1* mutations have significant effects on the phenotype of TAM.

GATA1s was shown previously to be produced from wild-type *GATA1* through 2 mechanisms: use of the alternative translation initiation site at codon 84 of the full-length transcript and alternative splicing of exon 2.[12,26] However, the translation efficiencies of GATA1s from the full-length of mRNA and short transcripts have not been investigated. Our results clearly showed that the Δexon 2 transcript produced GATA1s much more abundantly than did the full-length transcript. The translation efficiencies of GATA1s from full-length transcripts containing PTC were also lower than the alternative spliced form. These results support our contention that GATA1s expression levels largely depend on the amount of the Δexon 2 transcript. Thus, one cannot predict the expression level of GATA1s protein from the total amount of the transcript.

The differences in the quantities of GATA1s proteins expressed by PTC type 1-5′ and -3′ mutations revealed the importance of the location of the mutation for splicing efficiency and protein expression. The splicing efficiency is regulated by *cis*-elements located in exons and introns (referred to as exonic and intronic splicing enhancers or silencers), and transacting factors recognizing these elements.[27,28] The PTC type 1-3′ mutations induced efficient skipping of exon 2 (Figures 2Bvi-vii, 3A-B). These mutations might affect exonic splicing enhancers or silencers located in exon 2. To predict the splicing pattern from the mutations more accurately, the elucidation of *cis*-elements and transacting splicing factors, which regulate the splicing of exon 2 of *GATA1*, will be very important.

Table 5. Summary of outcomes and *GATA1* mutation types in TAM patients

Mutation type	Outcome of TAM				TAM Total (n = 66)		ML-DS Total (n = 40)	
	CR	Early death	Evolved to ML-DS	NA				
High group								
Loss of 1st Met, n (%)	7	1	1	1	10 (15.2)		3 (7.5)	
Splicing error, n (%)	7	4	0	2	13 (19.7)	40 (15.2)	6 (15.0)	16 (40.0)
PTC 1-3′, n (%)	10	6	0	1	17 (25.8)		7 (17.5)	
Low group								
SPTC 1-5′, n (%)	6	4	2	3	15 (22.7)	26 (39.4)	14 (35.0)	24 (60.0)
PTC 2, n (%)	2	1	8	0	11 (16.7)		10 (25.0)	

The nonsense mediated RNA decay pathway (NMD), a cellular mechanism for detection of PTC and prevention of translation from aberrant transcripts,[29,30] might regulate the expression of GATA1s protein derived from PTC type 2 mutations, which contained PTCs after the second methionine at codon 84. We consistently detected low amounts of transcripts of *GATA1* in samples expressing PTC type 2 mutations, whereas the expression levels of *GATA1* mRNA from PTC type 1 mutations were comparable with that from wild-type *GATA1* (Figure 2Aiii). These results suggest that the location of PTC relative to alternative translation initiation sites is important for effective NMD surveillance.

Available evidence indicates that acute leukemia arises from cooperation between one class of mutations that interferes with differentiation (class II mutations) and another class that confers a proliferative advantage to cells (class I mutations).[31] Recent reports showed that introducing high levels of exogenous GATA1 lacking the N-terminus did not reduce the aberrant growth of GATA1-null megakaryocytes, but instead induced differentiation.[32,33] This observation suggested that abundant GATA1s protein functions like a class I mutation in TAM blasts. In contrast, reducing GATA1 expression leads to differentiation arrest and aberrant growth of megakaryocytic cells.[19,20] The present data suggest that GATA1s is expressed at very low levels in TAM blasts with GATA1s low mutations. These levels may not be sufficient to provoke normal maturation. Together, these findings suggest that the low expression of GATA1s might function like class II mutations in TAM blasts. Additional class I mutations or epigenetic alterations might be more effective in the development of leukemia in blast cells expressing GATA1s at low levels.

In the present study, we identified a subgroup of TAM patients with a higher risk of developing ML-DS. Of 66 children, 11 (16.7%) with TAM subsequently developed ML-DS and 10 of them belonged to the GATA1s low group harboring the PTC type 2 or PTC type 1-5′ mutations. Surprisingly, 8 of 11 patients (73%) with the PTC type 2 mutations developed ML-DS (Tables 1, 5), whereas 2 of 15 patients (13.3%) with PTC type 1-5′ mutations developed leukemia. The estimated expression levels of GATA1s from PTC type 2 mutations were lower than those from PTC type 1-5′ mutations (Figures 1, 2Ai). These results suggest that the type 2 mutations may be a more significant risk factor for developing ML-DS (supplemental Figure 2). However, our classification of *GATA1* mutations mainly rested on extrapolation from in vitro transfection experiments (Figures 1-2) and RT-PCR analyses of a small number of patient samples (Figure 3). The stability of the transcripts and the splicing efficiency of the second exon of *GATA1* will be regulated through complex mechanisms. To confirm our findings, precise mapping of the mutations that affect the expres-

sion levels of GATA1s and a prospective study with a large series of TAM patients are necessary.

Finally, we proposed the hypothesis that the quantitative differences in GATA1s protein expression caused by mutations have a significant effect on the phenotype of TAM. The observations described here provide valuable information about the roles of *GATA1* mutations on multistep leukemogenesis in DS patients. Moreover, the results might have implications for management of leukemia observed in DS infants and children. Because the blast cells in both TAM and subsequent ML-DS appear highly sensitive to cytarabine,[34-39] the preleukemic clone could be treated with low-dose cytarabine without severe side effects, and elimination of the preleukemic clone might prevent progression to leukemia.

Authorship

Contribution: R.K. and T. Toki designed, organized, and performed research, analyzed data, and wrote the paper; K.T. designed research and collected and analyzed clinical data; G.X. and R.W. performed mutation screening; A.S., H.K., K. Kawakami, M.E., D.H., K. Kogawa, S.A., Y.I., S.I., T. Taga, Y.K., and Y.H. provided clinical samples and data; A.H. and S.K. performed mutation screening and provided clinical samples and data; and E.I. designed and organized research, analyzed data, and wrote the paper.

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Autoimmune lymphoproliferative syndrome-like disease with somatic KRAS mutation

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Autoimmune Lymphoproliferative Syndrome Like Disease With Somatic *KRAS* Mutation

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Abstract

Autoimmune lymphoproliferative syndrome (ALPS) is classically defined as a disease with defective FAS-mediated apoptosis (Type I–III). Germline *NRAS* mutation was recently identified in Type IV ALPS. We report two cases with ALPS like disease with somatic *KRAS* mutation. Both of the cases were characterized by prominent autoimmune cytopenia and lymphoadenopathy/splenomegaly. These patients did not satisfy the diagnostic criteria for ALPS or juvenile myelomonocytic leukemia (JMML), and are likely to be defined as a new disease entity of RAS associated ALPS like disease (RALD).

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Introduction

ALPS is a disease characterized by dysfunction of the FAS-mediated apoptotic pathway[1,2], currently categorized as Type Ia, germline *TNFRSF6/FAS* mutation; Type Ib, germline *FAS ligand* mutation; Type Is, somatic *TNFRSF6/FAS* mutation; and Type II, germline *Caspase 10* mutation. Patients exhibit lymphadenopathy, hepatosplenomegaly, and autoimmune diseases such as immune cytopenia and hyper-γ-globulinemia. An additional subclassification has been proposed that includes Types III and IV, whereby Type III has been defined as that with no known mutation but with a defect in FAS-mediated apoptosis, and Type IV as one showing germline *NRAS* mutation[3]. Type IV is considered exceptional because the FAS-dependent apoptosis pathway is not involved in the pathogenesis, and this subclass is characterized by a resistance to IL-2 depletion-dependent apoptosis. Recent updated criteria and classification of ALPS suggested type IV ALPS as a RAS associated leukoproliferative disease [4]. JMML is a chronic leukemia in children. Patients show lymphadenopathy, hepatosplenomegaly, leukocytosis associated with monocytosis, anemia, thrombocytopenia, and occasional autoimmune phenotypes. About 80% of patients with JMML have been shown to have a genetic abnormality in their leukemia cells including mutations of *NF1*, *RAS* family[5], *CBL*, or *PTPN11*. The hallmarks of the laboratory findings of JMML include spontaneous colony formation in bone marrow (BM) or peripheral blood (PB) mononuclear cells (MNC) and hypersensitivity to granulocyte-macrophage colony-stimulating factor (GM-CSF) of CD34 positive BM-MNC[6].

Germline RAS pathway mutations cause Costello (*HRAS*), Noonan (*PTPN11*, *KRAS*, and *SOS1*), and cardio-facio-cutaneous (CFC) syndromes (*KRAS*, *BRAF*, *MEK1*, and *MEK2*). Patients with Costello and Noonan syndromes have an increased propensity to develop solid and hematopoietic tumors, respectively[7],

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among these tumors the incidence of JMML in patients with germline mutation of *NF1* or *PTPN11* is well known.

We present two cases with autoimmune cytopenia and remarkable lymphadenopathy and hepatosplenomegaly, both of which were identified as having a somatic KRAS G13D mutation without any clinical features of germline *RAS* mutation such as CFC or Noonan syndrome.

Patients and Methods

All studies were approved by the ethical board of Tokyo Medical and Dental University.

Case 1

A 9-month-old boy had enormous bilateral cervical lymphadenopathy and hepatosplenomegaly (Supplemental data 1 Fig. 1a, b). Blood test revealed presence of hemolytic anemia and autoimmune thrombocytopenia. hyper-γ-globulinemia with various auto-antibodies was also noted. ALPS and JMML were nominated as the diseases to be differentially diagnosed. Detailed clinical history and laboratory data are provided as Supplemental data 1. The patient did not satisfy the criteria for the diagnosis of ALPS or JMML as discussed in results and discussion section.

Case 2

A 5-month-old girl had a fever, massive hepatosplenomegaly (Supplemental data 1 Fig. 1d). She was initially diagnosed with Evans syndrome based on the presence of hemolytic anemia and autoimmune thrombocytopenia with hyper-γ-globulinemia and auto-antibodies. Spontaneous colony formation assay and GM-CSF hypersensitivity of BM-MNC showed positivity. Then, tentative diagnosis of JMML was given, even though she showed no massive

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monocytosis or increased HbF. Detailed clinical history and laboratory data is provided as Supplemental data 1.

Detailed methods for experiments are described in Supplemental data 2

Results and Discussion

Case 1 showed a high likelihood of being a case of ALPS according to the symptoms and clinical data presented (Supplemental data 1, Table 1) except for number of Double-negative T (DNT) cells which was only 1.4% of TCRαβ cells (Fig. 1a). JMML was also nominated as a disease to be differentiated, because remarkable hepatosplenomegaly with thrombocythemia and moderate monocytosis was noted. However, no hypersensitivity to GM-CSF as determined by colony formation assay for BM-MNC (data not shown) or phospho STAT5 staining (data not shown) was observed. DNA sequence for JMML associated genes such as *NRAS, KRAS, HRAS, PTPN11* and *CBL* was determined, and KRAS G13D mutation was identified (Fig. 1b). The mutation was seen exclusively in the hematopoietic cell lineage and no mutation was seen in the oral mucosa or nail-derived DNA. Granulocytes, monocytes, T cells, and B cells were all positive for KRAS G13D mutation (data not shown). The proportion of mutated cells in each hematopoietic lineage was quantitated by mutation allele specific quantitative PCR methods, which revealed mutated allele was almost equally present in granulocytes, T cells and B cells (Fig 1c). CD34-positive hematopoietic stem cells (HSC) was also positive for KRAS G13D mutation, and 60% of CFU-GM colonies developed from isolated CD34 cells carried KRAS G13D mutation (data not shown). These observations suggest that the mutation occurred at the HSC level, and HSCs consists of wild type and mutant HSCs.

NRAS mutated Type IV ALPS was previously characterized by apoptosis

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