different transcripts in leukemic blasts, namely, 1-8, 1-6-8, 1-5-6-8, 1-4-5-6-8, and 1-5-6-7-8 [5]. In our study, all samples of 29 pediatric AML patients with normal karyotype had the *BAALC* isoform that consisted of exon 1, 6, and 8 sequences (1-6-8). However, three samples (10.3%) also had the *BAALC* isoform that contained in addition the exon 5 sequence (1-5-6-8) (Fig. 3a, b). One of the three patients with the 1-5-6-8 isoform also had high *BAALC* expression. With regard to prognosis, all three patients with the 1-5-6-8 *BAALC* isoform have died (Table 2). Two relapsed after complete remission and the third died after intracranial hemorrhage during induction therapy. Of the 29 normal karyotype patients who were subjected to *BAALC* isoform and expression analyses, *FLT3*-ITD were found in eight (27.6%), *FLT3*-D835Mt in two (6.9%), *NRAS* mutations in two (6.9%), and *KRAS* mutations in four (13.8%), but no *NPM1* gene mutations were found [4]. There are no differences of *FLT3*-ITD, *MLL*-PTD and *CEBPA* in high and low *BAALC* expression group (data not shown). Of the three 1-5-6-8 *BAALC* isoform-bearing patients, one had the *FLT3*-ITD mutation and another patient had a *RAS* mutation. A previous study did not detect a difference in outcome between cytogenetically normal adult AML patients with and without *NRAS* mutations [34]. Thus, it seems that the possession of the 1-5-6-8 *BAALC* isoform by pediatric AML patients with normal karyotype may be associated with a candidate for some adverse prognostic factor. Studies with greater patient numbers should be performed to confirm this. Recent reports have suggested that the isoform patterns of other genes (i.e., *AML1-ETO9a* [21], *WT1* [22], *PML/RARa* [23], *Ikaros* [24], and *FHIT* [25]) are of prognostic significance, which supports the significance of investigating the *BAALC* isoform patterns.

CEBPA mutations were detected in four of the 49 AML patients with normal karyotype (8.2%). Two of these belonged to the M1 subset and the remaining two belonged to the M2 subset. One patient had biallelic mutations in both the N-terminal part and bZIP domain of *CEBPA*. To date, two categories of *CEBPA* mutations have been reported: out-of-frame ins/del mutations that often occur in the N-terminal region, and in-frame ins/del mutations that often occur in the C-terminal region [12, 13]. The mutations in both the N-terminal part and bZIP domain have been described in adult AML, but the reported frequencies vary considerably, ranging between 11 and 47% [35]. In a study of pediatric AML patients, of whom six had a normal karyotype, four of the six (67%) had one or more *CEBPA* mutations [36], but the clinical outcomes associated with these mutations are unclear. Notably, in the absence of poor prognostic factors, adult patients with *CEBPA* mutations have been shown to have favorable clinical outcomes [33, 37]. In our study, none of the patients exhibiting a *CEBPA* mutation also had the *FLT3*-ITD mutation and all maintained complete remission for at least 5 years. The statistical significance was not indicated for insufficient sample numbers in AML 99 protocol between normal karyotype patients with CEBPA mutation (+) ($n = 4$) and mutation (−) ($n = 45$) with regard to their OS (100 vs. 55.4%, $P = 0.14$) or EFS (100 vs. 48.9%, $P = 0.09$) (supplementary Fig. 1). But differing from previous report about pediatric AML patients with *CEBPA* mutations, the presentation of clinical information about them may be evaluated. Thus, in the absence of other adverse factors, *CEBPA* mutations may also be suspected to favorable prognostic factors for pediatric AML with normal karyotype.

In summary, we report here for the first time that the presence of the 1-5-6-8 *BAALC* isoform may be associated with a poor prognosis for pediatric AML patients with normal karyotype. In contrast, *CEBPA* mutations are suspected to a good prognosis.

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CASE REPORT

Ring/marker chromosome derived from chromosome 7 in childhood acute megakaryoblastic leukemia with monosomy 7

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Abstract In some cases of childhood acute megakaryoblastic leukemia (AMKL), G-band analysis reveals supernumerary ring/marker chromosomes along with monosomy 7. However, their origin and relevance are poorly understood. We experienced three patients with AMKL, one of whom had Down's syndrome, whose blasts at the first visit exhibited both monosomy 7 and a ring/marker chromosome. For one case, precise molecular-cytogenetic techniques revealed that the ring chromosome was derived from a chromosome 7. It was strongly suggested that the ring chromosome was derived from a chromosome 7 in another case. The ring or one of the 2 marker chromosomes was derived from a chromosome 7 in the other case. All patients responded well to initial induction therapy. While it is not clear whether the ring/marker chromosome 7 affects the long-term prognosis of acute myeloid leukemia with monosomy 7, it may be of prognostic relevance to distinguish pure monosomy 7 from monosomy 7 with a ring/marker chromosome 7. For this purpose, conventional G-banding could be complemented with additional techniques such as spectral karyotyping or fluorescence in situ hybridization, which characterize the aberration in more detail. These methods may be useful for determining the optimal treatment and for elucidating the etiology of AMKL itself.

Keywords Monosomy 7 · Spectral karyotyping · Fluorescence in situ hybridization

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1 Introduction

Acute myeloid leukemia (AML) is a disease of the myeloid compartment of the hematopoietic system and is characterized by the accumulation of undifferentiated blast cells in the peripheral blood and bone marrow. The prognosis of childhood AML has improved significantly over the past decades [1]. Acute megakaryoblastic leukemia (AMKL) is a biologically heterogeneous form of AML that occurs in 10–20% of all cases of AML and has a bimodal age distribution with peaks in early childhood and adulthood [2].

Specific molecular and cytogenetic abnormalities in AML, including AMKL, define distinct pathological features and clinical behavior, in particular the response to therapy and survival. In some AML cases, some or all of chromosome 7 are deleted. AML with monosomy 7 cases is generally considered to have a poor prognosis [3]. This may relate to the possibility that genes on chromosome 7 may play critical roles in controlling the growth and division of cells.

Childhood AMKL can be divided into two groups, namely, Down syndrome (DS)-AMKL and primary AMKL that occurs in the absence of DS (known as non-DS-AMKL). Athale et al. [4] have found that while the two AMKL groups do not differ in terms of microscopic BM findings and the expression of surface antigens, the treatment outcome for patients with non-DS-AMKL is very poor. In contrast, Hama et al. [5] showed that the blasts of

DS-AMKL patients are less mature than those of non-DS-AMKL patients in terms of morphology and immunophenotyping but that the two groups had excellent outcomes. This discrepancy may be resolved by the recent study by Bourquin et al. [6] who showed that DS- and non-DS-AMKL patients have distinct gene expression profiles and that non-DS-AMKL patients can be subdivided into two molecular phenotypes. The meaning of monosomy 7 may be different between DS-AMKL and non-DS-AMKL. Kudo et al. [7] showed that in DS-AMKL, the presence of monosomy 7 is a greater risk factor of adverse outcome. By contrast, the prognostic significance of monosomy 7 in non-DS-AMKL is poorly understood.

In this paper, we describe three cases of childhood AMKL with a ring/marker chromosome 7 in addition to monosomy 7. We discuss the possibility that spectral karyotyping (SKY) and fluorescence in situ hybridization (FISH) analyses performed at the time of diagnosis could be useful for determining the optimal treatment regimen (Table 1).

2 Case reports

2.1 Case #1: DS-AMKL

A boy was diagnosed with constitutional trisomy 21 at birth and underwent surgery for a congenital heart anomaly (atrial and ventricular septal defect). In the neonatal period, he had a history of mild thrombocytopenia, but a transient abnormal myelopoiesis was not evident. At 3 years old, he was admitted with a fever, petechiae, and slowly progressing thrombocytopenia. He had dysmorphic features such as hypertelorism and epicanthal folds. Laboratory tests showed a white blood count of 3300/μl with 1% blasts, Hb of 10.3 g/dl, and platelet counts of 55000/μl. The blood film showed mild anisocytosis, poikilocytosis, and occasional hypogranulated platelets. Upon marrow aspiration, the patient exhibited 'dry tap' and a drop of aspirate revealed that the myeloid and erythroid lineages lacked dysplastic features. A bone marrow biopsy revealed mature megakaryoblast congestion and marked fibrosis. He was diagnosed with AMKL and achieved complete remission (CR) after the first induction therapy, which involved the use of the current JPLSG AML-D05 protocol (AraC 100 mg/m^2 × 7 + VP16 150 mg/m^2 × 3 + THP-ADR 25 mg/m^2 × 2). The patient's blasts were negative for the *FLT3*-internal tandem duplication (ITD). Direct cytogenetic analysis of the bone marrow metaphases revealed three of the 19 cells that were analyzed had 48,XY,−7,add(11)(q23),+21,+21,+r1. SKY was not possible because of insufficient material. Therefore, we employed the FISH technique using a chromosome 7 probe (D7Z1) and found that 0 of 100 cells showed a single D7Z1 signal. Though we could not definitely determine whether FISH probe was on the ring chromosome, add(11)(q23), or

Table 1 Summary of three cases

	Case #1	Case #2	Case #3
Age	3 years 4 months	1 year 0 month	2 years 3 months
Down syndrome	(+)	(−)	(−)
Dry tap	(+)	(+)	(−)
Karyotype of BM cells	48,XY,−**7**,add(11)(q23),+21c,+21,+**r1** [3] 47,XY,+21 [16]	47,XY,−**7**,+21,+**r1** [11] 46,XY [9]	46,X,−X,−2,−**7**,add(17)(q25),del(20)(q11.2),+**r1**,+**mar1**,+**mar2** [20]
Constitutional karyotype	47,XY,+21c [20]	46,XY [20] (BM) 46,XY [100] (buccal mucosa)	46,XX [20]
Surface marker on blast	CD13, CD33, HLA-DR, CD7, CD41a, CD61	CD13, CD33, HLA-DR, CD7, CD41a, CD61	CD13, CD33, HLA-DR, CD7, CD41a, CD61
Trilineage dysplasia	(−)	(−)	(+)
Single signal of chromosome 7 by FISH	0/100 cells	2/100 cells	5/100 cells
SKY	Not done	Refer to Fig. 1	Not done
Induction therapy	JPLSG AML-D05	JPLSG AML-D05	JPLSG AML-05
Grade 3/4 toxicity	Hematopoietic	Hematopoietic	Hematopoietic
CR after first induction	Yes	Yes	Yes
CR duration	13 months	10 months	7 months

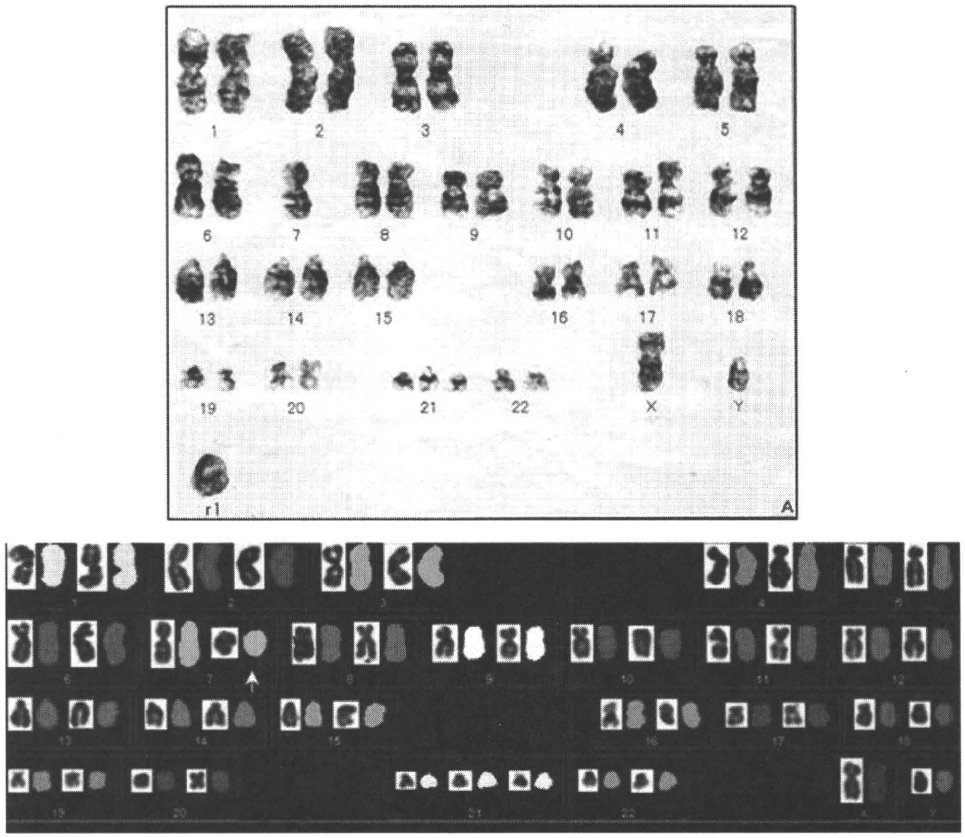

Fig. 1 The results of G-banding and SKY (case #2)

other chromosomes which appeared normal in interphase nuclei, it was strongly suggested that the ring chromosome was derived from a chromosome 7. The CR status of the patient continues to persist for 13 months.

2.2 Case #2: non-DS-AMKL-1

A 1-year-old male who had a past history of hydronephrosis and afebrile convulsion was admitted for further routine laboratory tests to search for an abnormality. Laboratory tests revealed a white blood count of 10900/μl with 4% blasts, Hb of 7.7 g/dl, and platelet counts of 20000/μl. The peripheral blood smear and a drop of bone marrow aspirate had the same features as case #1. Similarly, a bone marrow biopsy revealed mature megakaryoblast congestion and marked fibrosis. The patient was diagnosed with AMKL. His blasts were negative for *FLT3*-ITD. He had no dysmorphic features such as hypertelorism and epicanthal folds. But since 11 of the 20 bone marrow cells that were examined initially by cytogenetics showed 47,XY,−7,+21,+r1, and 9 of 20 cells exhibited 46,XY, it was not possible to completely exclude the possibility of mosaic Down syndrome and so he was treated by the JPLSG AML-D05 protocol. Since buccal cytogenetics then revealed two signals from chromosome 21 by FISH, the patient's treatment regimen was changed to the Japanese AML99 protocol [8] with a moderate dosage. The response to the initial induction therapy was good and the patient continues to have a CR status for 10 months. SKY and FISH analyses revealed that the ring was from a chromosome 7 (Fig. 1).

2.3 Case #3: non-DS-AMKL-2

A 2-year-old female was admitted for further work-up for purpura. The laboratory tests showed a white blood count of 37500/μl with 73% blasts, Hb of 8.3 g/dl, and a platelet count of 13000/μl. A peripheral blood smear and a drop of bone marrow aspirate had the same features as cases #1 and #2 with multilineage dysplasia in the background. The patient was diagnosed with AMKL. Initial bone marrow cytogenetics revealed that all of the 20 cells examined had 46,X,−X,−2,−7,add(17)(q25),del(20)(q11.2),+r1,+mar1, +mar2. However, subsequent FISH technique using a chromosome 7 probe showed that only 5% cells were single positive, which suggests the existence of extra chromosome 7 derivatives. The patient's blasts were negative for *FLT3*-ITD. She was treated by the JPLSG AML-05 protocol (AraC 200 mg/m^2 × 7 + VP16 150 mg/m^2 × 5 + MIT 5 mg/m^2 × 5). Because (1) the response for initial induction therapy was good and (2) her AMKL was considered to be

different from AMKL with pure monosomy 7, subsequent chemotherapy without stem cell transplantation was performed. Her CR status continues for 7 months.

3 Discussion

In the present paper, we described three cases of childhood AMKL with monosomy 7 where G-banding analysis revealed a supernumerary ring/marker chromosome and SKY or FISH analysis showed that the ring/marker was composed of material from chromosome 7. The aim of this paper was to emphasize that there exists a subset of patients with monosomy 7 who also bear a ring/marker chromosome 7. We also wish to highlight the possibility that the prognosis of these patients might differ from that of patients who have monosomy 7 but lack a ring/marker chromosome, in which case the former patients should be treated differently. About 7% of AML cases bear ring chromosomes [9], but their origin has been analyzed very infrequently; this was particularly true before the advent of FISH analysis. Monosomy 7 has been found to predict a significantly poorer outcome for AML patients compared to AML patients with normal cytogenetics [2]. Indeed, it has been used as an indicator to stratify patients participating in clinical trials [3, 10]. However, when Hasle et al. [11] recently retrospectively analyzed the data of 258 children with myeloid malignancies from several major AML study groups overseas, they found that del(7q) ± other AML patients had superior survival compared to patients with monosomy 7 ± other (51 vs. 30%, $P < .01$). It was concluded that patients with monosomy 7 and those with del(7q) should not be considered as the same cytogenetic subgroup. Thus, it may be, similar to the situation with the del(7q) patients, that the prognosis of cases with a ring/marker chromosome 7 in addition to monosomy 7 might differ from that of patients with pure monosomy 7. These observations suggest that AMKL patients with monosomy 7 who have a ring/marker chromosome should be routinely tested by FISH or SKY to determine the origin of the latter chromosome. Notably, while Frohling et al. [12] proposed that FISH should be used as a complementary method to detect more subtle abnormalities, such as inv(16) and t(11q23), and for suspected variant t(8;21), they made no comment regarding the utility of this method to detect ring/marker chromosomes.

The fact that AMKL patients with both monosomy 7 and a ring/marker chromosome 7 might have a different prognosis than pure monosomy 7 patients complicates the interpretations of several other studies. Several examples of chromosome 7 abnormalities associated with DS and leukemia were reported [13, 14]. In a Japanese study of DS-AML (Down 99) [7], multivariate analysis revealed that monosomy 7, which was observed in six patients, was a risk factor for an adverse outcome. However, four of the six patients had in addition a ring/marker chromosome. Kobayashi et al. [14] showed a case with chemorefractory DS-AMKL showing the absence of one normal chromosome 7 and an additional ring chromosome 7 detected by SKY analysis. In a Japanese study of non-DS-AML (AML99) [8], there were some cases with monosomy 7, all of whom received stem cell transplantation. However, it was not clear whether these cases had a ring chromosome. AML with monosomy 7 is a relative indication for stem cell transplantation in first CR if a suitable donor is available, but it may be that more careful observation of cases like ours could change the indication for stem cell transplantation.

Ring chromosomes have been observed in a wide variety of human neoplasias. In some tumor subentities, ring chromosomes occur so frequently and are such typical features that they can even serve as cytogenetic hallmarks for differential diagnosis [15]. In AML, the usefulness of the ring/marker chromosome as a prognostic factor remains controversial. The generally held view that ring chromosomes in leukemias are a poor prognostic factor must be considered with great caution because ring chromosomes often occur in the context of highly complex aberrant karyotypes [9]. Indeed, there are exceptional cases of leukemias where the presence of ring chromosomes is associated with a better prognosis [9].

Two of our three cases had an additional chromosome 21. Unlike constitutional trisomy 21, the relevance of acquired trisomy 21 is unclear. Acquired trisomy 21 has been assumed to be a cytogenetic feature of childhood non-DS-AMKL since various studies have shown it is present in 22, 33, and 43% of patients with non-DS-AMKL whereas other childhood de novo AMLs have a significantly lower incidence (1.5–6%) [3, 16]. Thus, it may play a major role in AMKL leukemogenesis. Future studies that determine the relevance of this observation are warranted.

In conclusion, we described three cases of childhood AMKL with a ring/marker chromosome 7, two of which had an acquired chromosome 21. When monosomy 7 is detected, it may be useful to employ techniques such as SKY or FISH in addition to conventional G-banding to characterize this aberration in more detail. The resulting information may be indispensable, at least in some cases, for determining the optimal treatment regimen. It may also allow the etiology of AML itself to be elucidated.

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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.

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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, $\times 10^9$/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, $\times 10^9$/L (range)	69.4 (7.8-423.0)	104.3 (33.1-290.8)		26 (14.6-244.0)	
WBC < 70 × 10^9/L vs WBC > 70 × 10^9/L					
WBC < 70 × 10^9/L, n (%)	30 (50.8)	4 (25.0)		7 (63.6)	
WBC > 70 × 10^9/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.

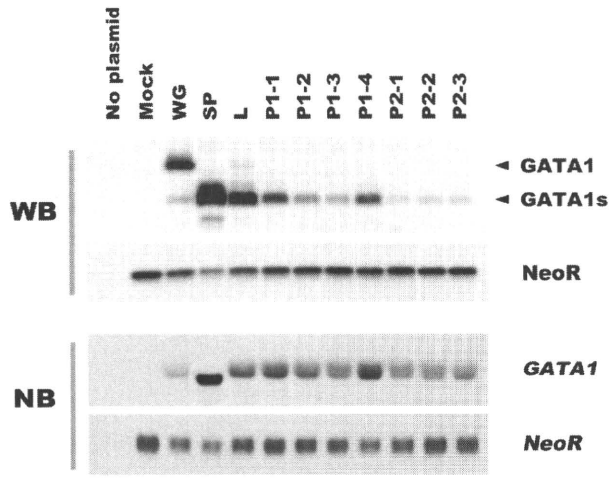

Figure 1. Effects of mutant transcripts of *GATA1* on the expression level of the truncated protein. The *GATA1* mutations observed in TAM patients are classified according to the types of transcripts. The translational efficiency of each transcript was assessed by Western blot analysis in BHK-21 cells 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).

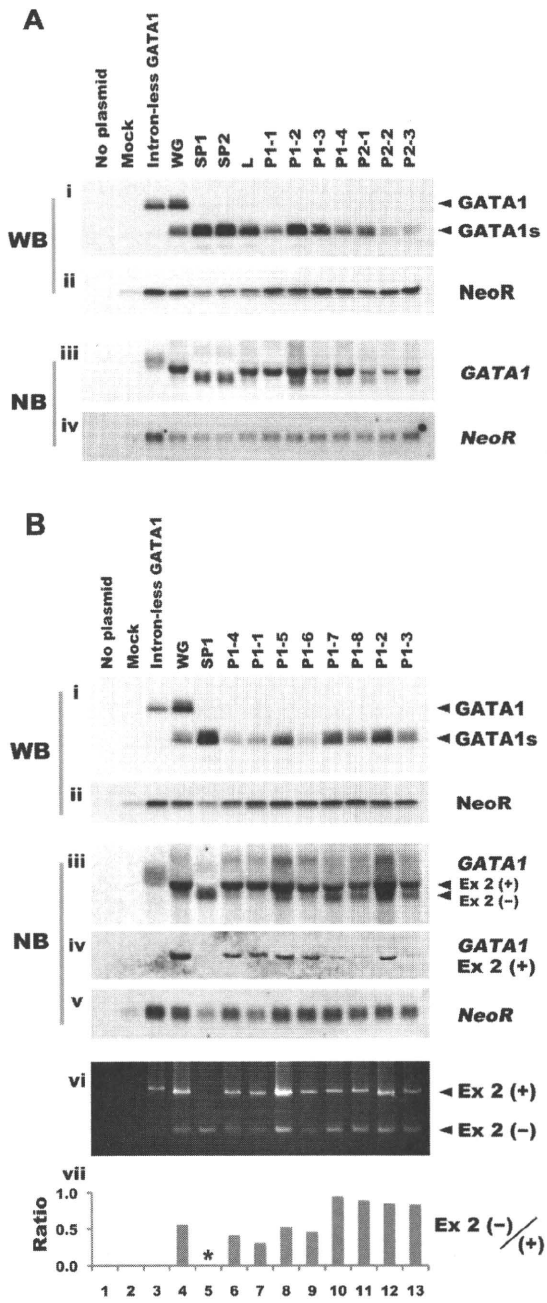

Figure 2. GATA1 mutations affect the expression level of the truncated protein. (A) The expression levels of GATA1s protein and mRNA were assessed in BHK-21 cells transfected with human *GATA1* minigene expression vectors carrying mutations observed in TAM patients. Western blot analysis was performed 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).

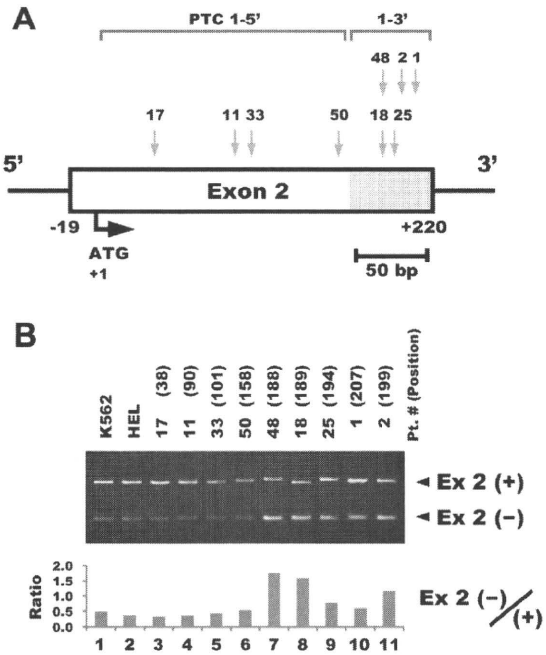

Figure 3. The location of the PTC type 1 mutation affects the efficiency of alternative splicing in TAM blast cells. (A) The location of the *GATA1* mutation in each TAM patient. Details of the mutation in each sample are described in Table 1. (B) RT-PCR analysis of *GATA1* in TAM blast cells harboring 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, $\times 10^9$/L	105.65 (7.8-423.0)	39.0 (9.0-220.0)	.004
Number of blasts, $\times 10^9$/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 χ^2 test.

†Fisher exact test.

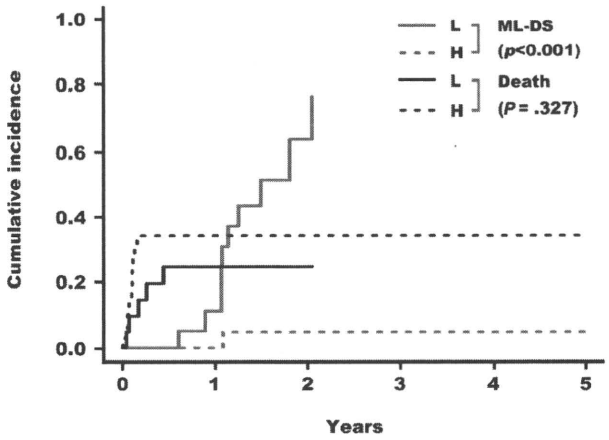

Figure 4. Cumulative incidence of early death and of ML-DS in children with TAM. Based on the estimated GATA1s expression levels, patients were classified in 2 groups: GATA1s high and low groups. TAM patients in the GATA1s low group had a significantly higher risk for the development of leukemia (*P*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

	Outcome of TAM				TAM		ML-DS	
Mutation type	CR	Early death	Evolved to ML-DS	NA	Total (n = 66)		Total (n = 40)	
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 expression 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.

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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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Establishment of a xenograft model of human myelodysplastic syndromes

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The online version of this article has a Supplementary Appendix.

ABSTRACT

Background

To understand how myelodysplastic syndrome cells evolve from normal stem cells and gain competitive advantages over normal hematopoiesis, we established a murine xenograft model harboring bone marrow cells from patients with myelodysplastic syndromes or acute myeloid leukemia with myelodysplasia-related changes.

Design and Methods

Bone marrow $CD34^+$ cells obtained from patients were injected, with or without human mesenchymal stem cells, into the bone marrow of non-obese diabetic/severe combined immunodeficient/$IL2R\gamma^{null}$ hosts. Engraftment and differentiation of cells derived from the patients were investigated by flow cytometry and immunohistochemical analysis.

Results

Co-injection of patients' cells and human mesenchymal stem cells led to successful engraftment of patient-derived cells that maintained the immunophenotypes and genomic abnormalities of the original patients. Myelodysplastic syndrome-originated clones differentiated into mature neutrophils, megakaryocytes, and erythroblasts. Two of the samples derived from patients with acute myeloid leukemia with myelodysplasia-related changes were able to sustain neoplastic growth into the next generation while these cells had limited differentiation ability in the murine host. The hematopoiesis of mice engrafted with patients' cells was significantly suppressed even when human cells accounted for less than 1% of total marrow mononuclear cells. Histological studies revealed invasion of the endosteal surface by patient-derived $CD34^+$ cells and disruption of extracellular matrix architecture, which probably caused inhibition of murine hematopoiesis.

Conclusions

We established murine models of human myelodysplastic syndromes using cells obtained from patients: the presence of neoplastic cells was associated with the suppression of normal host hematopoiesis. The efficiency of engraftment was related to the presence of an abnormality in chromosome 7.

Key words: xenograft, MDS, NOG mouse, niche, MSC.

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Introduction

Myelodysplastic syndromes (MDS) are a heterogeneous group of clonal hematopoietic disorders originating from primitive hematopoietic cells and some of the least studied hematopoietic malignancies due largely to difficulties in creating an *in vivo* model suitable for studying the biology of MDS since these syndromes cause variable degrees of morphological dysplasia in non-lymphoid lineages and accompanying hematopoietic failure.[1,2] The prognosis of MDS patients is generally poor with an approximately 25% risk of the disease evolving into acute myeloid leukemia (AML).[3] A wide variety of cytogenetic abnormalities is recognized in nearly half of MDS patients.[4,5] Although a multi-step process of disease development has been proposed,[6-9] the current understanding of the molecular pathogenesis of this disease is limited and, consequently, the precise mechanisms of how MDS cells evolve from normal hematopoietic cells remain unclear.

Mouse models of human diseases have been proven to be useful tools for elucidating the biology of various diseases and for evaluating the efficacy of evolving therapy.[10] The successful establishment of murine xenograft models for human AML has yielded empirical evidence for the existence of so-called 'cancer stem cells', a minor subpopulation of cells responsible for maintenance of neoplastic proliferation.[11-13] In addition, recent studies demonstrated that chemotherapy-resistant leukemic stem cells reside in the endosteal region of bone marrow.[14,15] These findings helped to clarify how acute leukemia cells are maintained and propagated *in vivo*; however, little is known about the behavior of MDS cells in the bone marrow microenvironment partly because of the difficulties in obtaining a suitable *in vivo* model for this disease. The reason for the selective outgrowth of MDS clones and the concurrent decrease in normal hematopoietic stem cells in patients does, therefore, remain elusive.

To establish a murine model of human MDS, which would undoubtedly be of benefit in the study of the pathology and biology of MDS, we transplanted bone marrow $CD34^+$ cells from patients with MDS and acute myeloid leukemia with myelodysplasia-related changes (AML-MRC) and human mesenchymal stem cells (MSC) as auxiliary cells in murine bone marrow using an established intramedullary co-transplantation method.

Design and Methods

Patients and preparation of human cells

The experimental protocol of this study was approved by the Institutional Review Board of Tokai University, School of Medicine, and all human samples were handled accordingly. Bone marrow samples were obtained from six patients with MDS, eight patients with AML-MRC, and four healthy individuals after obtaining written informed consent. The clinical characteristics and immunophenotypes of the patients are summarized in *Online Supplementary Tables S1 and S2*, respectively. $CD34^+$ cells were selected using the CD34 Progenitor Cell Isolation Kit (Miltenyi Biotec, Sunnyvale, CA, USA) according to the manufacturer's instructions as described previously.[16] The purity of the selected bone marrow CD34+ cells was always more than 95%. Human MSC were purchased from Lonza Walkersville Inc. (Walkersville, MD, USA) and cultured according to the directions supplied by the company. In some experiments, MSC were established from the $CD34^-$ fraction of patients' cells. The ability of the cells to differentiate into adipocytes, chondrocytes and osteoblasts was assessed and confirmed *in vitro* as described previously[17] before the cells were used for this study (*data not shown*).

Antibodies

The following monoclonal antibodies were used for flow cytometry: anti-CD7 (4H9), -CD11b (D12), -CD13 (L138), -CD14 (MϕP9), -CD19 (SJ25C1), -CD36 (CB38(NL07)), -CD38 (HB7), -CD56 (MY31), -CD61 (VI-PL2), -CD64 (10.1), and -HLA-DR (L243, all from BD Biosciences, San Jose, CA, USA); anti-CD33 (WM53), -CD34 (581), -CD41b (P2), -CD45 (J.33), and CD117 (95C3, all from Coulter/Immunotech, Marseille, France); and MPO (MPO-7, DACO, Denmark).

The following antibodies were used for tissue immunostaining: anti-CD15 (80H5, 1:150, Coulter/Immunotech); anti-CD31 (1:100, TECNE Corporation, Minneapolis, MN, USA); anti-CD34 (My10, 1:20), -CD45 (2D1, 1:200), and -CD38 (HIT2, 1:100, all from BD Biosciences); anti-glycophorin A (JC159, 1:400) and -CD61(Y2/51, 1:1000, both from DACO); anti-fibronectin (1:400, Sigma, St Louis, MO, USA); and anti-PCNA (1:200, abcam, Cambridge, UK).

Experimental animals, lentiviral gene transduction, and cell transplantation

Non-obese diabetic/severe combined immunodeficient/IL2Rγ^{null} (NOG) mice were maintained in sterile microisolator cages in the animal facility of Tokai University School of Medicine. The mice were irradiated with 250 cGy from an X-ray irradiator (HW-300, Hitex, Osaka, Japan) 24 h prior to intramedullary transplantation of cells. All procedures were approved by the Animal Care Committee of Tokai University. The MSC were transduced with the *GFP* gene as described previously.[17]

Analysis of human cells

The mice were killed humanely 8 to 16 weeks after transplantation, and the entire bone marrow contents of the injected tibiae were collected in phosphate-buffered saline containing 0.5% bovine serum albumin and 0.5 M EDTA. The total number of bone marrow mononuclear cells was counted for each bone of individual experimental animals. The number of non-human bone marrow cells was obtained by calculation. Aliquots of cells were used to examine the percentages of cells expressing human cell surface antigens. A four-color flow cytometric analysis was conducted using FACSCaliber. Quadrants were set to include at least 97% of the isotype-negative cells. The proportion of each lineage was calculated from 10,000 events acquired using the CELLQuest software package. The remaining cells were saved for secondary transplantation, cytospin preparation for morphological examination, chromosomal analysis and fluorescence *in situ* analysis (FISH). Chromosomal analysis was conducted using a conventional method in the clinical laboratory of the University Hospital, while the FISH analysis was performed at SRL Inc. (Tokyo, Japan). The preparation of the bone marrow for histological studies, immunofluorescent staining and enzyme immunohistochemistry were performed as described previously.[18] Images of stained slides were captured using an LSM510 META confocal microscope with a 63X/1.2 numeric aperture c-Apochromat objective lens (Carl Zeiss, Jena, Germany) and an Olympus Ax80 microscope with a 20X/0.70 numeric aperture UplanApo lens equipped with a DP71 digital camera (Olympus, Japan). Images were transferred to Adobe Photoshop CS4 (Adobe Systems, San Jose, CA, USA)

Histological analysis of bone

For serial transplantation experiments, the percentage of cells in the endosteal region (within 5 cells' distance) was obtained by

counting the cells in the entire field of bone specimens under the light field microscope. More than five slides were examined for each transplant.

Statistics

Data are presented as the mean ± standard deviation. The two-sided *P* value was determined by testing the null hypothesis that the two population medians are equal. *P* values less than 0.05 were considered to be statistically significant.

Results

Engraftment of myelodysplastic syndrome-originated human hematopoietic cells in murine bone marrow

We previously reported that intramedullary injection of cord blood hematopoietic stem cells along with human MSC improved engraftment of human cells in the murine microenvironment.[18] We, therefore, transplanted bone marrow CD34$^+$ cells, which included hematopoietic stem cells and primitive progenitors, obtained from six patients with MDS, eight patients with AML-MRC and four healthy individuals, into the bone marrow of NOG mice with or without human MSC. Flow cytometric analysis detected the presence of human CD45$^+$ cells, at varying frequencies, in the bone marrow of 8/8, 12/23, and 4/4 recipient mice injected with bone marrow CD34$^+$ cells from the MDS patients, the AML-MRC patients, and the healthy individuals, respectively (Table 1). As expected, transplantation of MSC alone did not result in hematopoietic engraftment (*data not shown*). Further lineage analysis revealed a CD33$^+$ myeloid dominant differentiation, 60% or more, in three of six MDS cases (3/8 mice engrafted) and three of eight AML-MRC cases (9/12 mice engrafted), suggesting the engraftment of MDS-originated cells (Table 1; patients 2, 5, 6, 11, 13, and 14, and Figure 1A). Human cells recovered from transplanted animals were positive for cell surface markers found on the original patients' cells (*Online Supplementary Table S2*), such as CD13 (74.90%) and CD56 (32.08%) for patient 11, CD7 (72.13%) and CD41b (60.12%) for patient 13, and CD13 (81.92%, 37.86% of which co-expressed CD34) and CD117 (31.41%) for patient 14.

To confirm that this was indeed engraftment of MDS-originated cells, we performed cytogenetic and morphological analyses on human cells recovered from the mice engrafted with patients' bone marrow cells. FISH analysis confirmed cytogenetic abnormalities of original bone marrow in the human cells isolated from mice engrafted with cells from patients 6, 11, and 13 (Figure 1B and *Online Supplementary Table S3*) in 100% of the cells analyzed. In addition, patient-specific chromosomal abnormalities (monosomy 7 for patient 13 and isochromosome 17 for patient 14) were detected in 100% of cells analyzed (Figure 1C). Morphological observations of cytospin samples and bone marrow histology of mice engrafted with bone marrow cells from patients 2 and 11 showed dyspla-

Table 1. Engraftment of bone marrow CD34$^+$ cells obtained from MDS and AML-MRC patients.

Patient N.	N. of injected cells, x10^4*	Auxiliary cells	# of mice engrafted/ injected	N. of cells recovered, x10^6 /tibia	Percentage of human cells	Percentages of CD34$^+$ cells in human cells†	Percentages of lineage cells in human cells† CD33$^+$	CD19$^+$
1	23.7	allo	1/1	6.8	67.65	10.45	10.8	82.3
2	33.9	allo	1/1	4.0	88.92	39.71	67.37	21.17
3	32.4	allo	1/1	3.0	3.93	9.26	4.4	85.72
4	28.2	allo	1/1	2.18	0.48	9.94	0	95.47
5	40	auto	1/1	3.4	1.95	83.59	96.02	0
		dermal fibroblasts	1/1	2.7	0.83	27.71	51.81	49.13
		-	1/1	4.8	0.36	50.0	57.14	23.81
6	14	allo	1/1	1.6	0.15	8.43	88.0	0
7	236	allo	1/1	5.5	3.18	5.77	2.57	91.32
8	39	allo	0/1	4.8	0	NT	NT	NT
9	100	allo	1/1	6.3	0.14	10.67	7.25	79.81
10	500	allo	1/1	12.0	12.42	9.58	3.15	83.99
11	100	auto	1/1	4.8	0.32	20.59	95.52	3.96
		allo	1/1	0.67	3.28	8.03	86.22	0.71
	138	auto	2/2	3.4, 5.2	7.72, 1.14	28.19, 12.3	80.76,91.54	0.0, 8.0
		CD34$^-$ fraction	0/2	5.8, 6.2	0	NT	NT	NT
		-	1/1	4.8	0.49	13.46	60.0	29.09
12	250-1000	allo, auto	0/6	4.4-9.8	0	NT	NT	NT
	1000	-	0/2	6.9, 7.8	0	NT	NT	NT
13	500	allo	1/1	2.7	10.27	35.71	94.78	1.01
14	400	allo	1/1	0.45	89.29	36.10	80.69	0.73
		auto	2/2	0.63, 0.42	71.06, 83.4	35.74, 28.61	73.68, 90.20	0.24, 0.22
Normal individuals	5, 10, 7.8, 10	allo, auto	4/4	7.0, 3.8, 5.7, 14.7	32.7,2.9,45.5,12.8	8.7,16.2,6.7,8.5	5.9,18.6,7.7,15.1	76.9,71.4,72.9,67.1

*Auto indicates autologous MSC; allo, allogeneic MSC; -, not applicable; NT, not tested. *Indicated numbers of bone marrow CD34$^+$ cells were transplanted with or without 5~15x10^5 auxiliary cells. †Shown here are percentages of cell surface marker expressing cells in the human CD45$^+$ gate.*

sia typically associated with MDS, such as bi-nucleated myelocytes and megakaryocytes with separated nuclei (Figures 1D and 2A, and *data not shown*). In samples prepared with cells recovered from mice engrafted with bone marrow cells from patient 2, myelocytes with variable degrees of normal differentiation were easily seen, but there were also sporadic cells with dysplasia which were not seen in samples from animals engrafted with normal human bone marrow cells. Large blastic cells were prominent in cytospin samples of bone marrow cells prepared from mice engrafted with cells from patients 13 and 14 (Figure 1D and *data not shown*). Considering these findings collectively, mice injected with bone marrow cells from patients 2, 5, 6, 11, 13, and 14 were engrafted with MDS-originated cells. Five of these patients harbored one or more genetic abnormalities, most of which were abnormalities in chromosome 7 (*Online Supplementary Table S1*). In contrast, human cell engraftment of mice transplanted with cells obtained from normal individuals and patients 1, 3, 4, 7, 9, and 10 consisted mainly of B-lineage cells, typical of normal human cell differentiation in the NOG mice environment.[19-21] Analyses of cytospin samples prepared from human cells recovered from the bone marrow of these mice confirmed a B-cell dominant differentiation (Figure 1D). In addition, no clonal markers specific to patients' phenotype were detected by FISH analysis (*Online Supplementary Table S3*, patients 9 and 10). The human engraftment in mice injected with cells from these patients was, therefore, considered to come from a minor population of normal hematopoietic stem cells co-existing in the patients' bone marrow $CD34^+$ cells.

Co-transplantation of bone marrow $CD34^+$ cells along with human MSC facilitated the engraftment of MDS-originated cells (Table 1; patients 5 and 11). In mice transplanted with bone marrow $CD34^+$ cells and MSC, more than 80% of human cells expressed CD33 while less than 4% expressed CD19 (5/5), in contrast to the mice transplanted with bone marrow $CD34^+$ cells alone (23.81% and 29.09% $CD19^+$ cells) or in combination with dermal fibroblasts (49.13% $CD19^+$ cells) in which B-cell proliferation was more notable (3/3), thus suggesting that normal human cells were also engrafted. The co-injection of the $CD34^-$ fraction of bone marrow cells did not yield any human cell engraftment (2/2). These results indicate the unique property of MSC of facilitating the engraftment of MDS-originated cells.

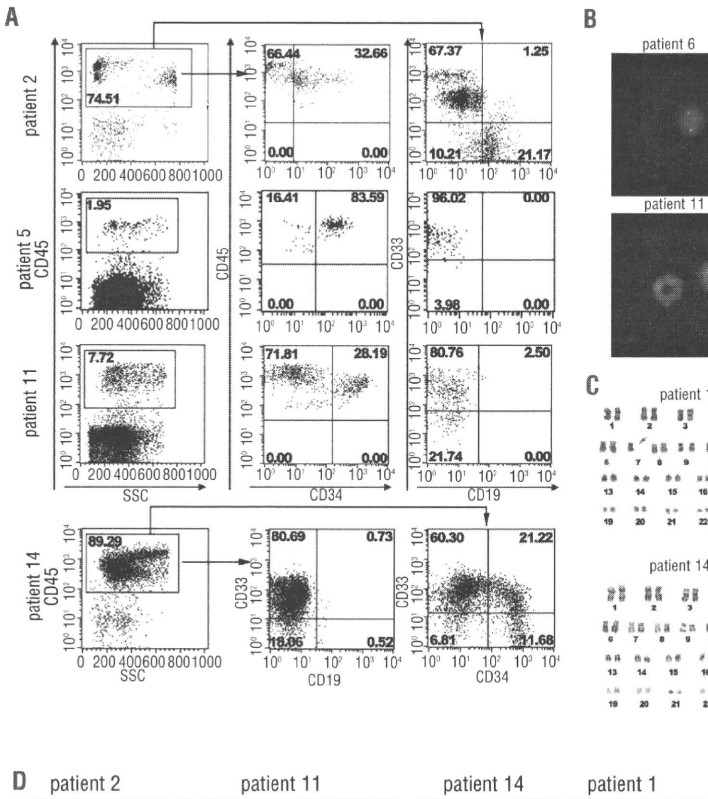

Figure 1. Engraftment of human MDS-originated hematopoietic cells in the bone marrow (BM) of NOG mice. (A) Representative flow cytometric profiles of BM cells recovered from mice engrafted with patients' BM cells. The majority of human CD45-expressing cells were positive for a myeloid marker CD33 in patients 5, 11, and 14, while some $CD19^+$ cells were present in BM cells recovered from the mouse engrafted with cells from patient 2. For patient 14, approximately one quarter of $CD33^+$ cells co-expressed CD34. The percentages of cells in the respective regions are shown. (B) FISH detection of a partial deletion of chromosome 7 and monosomy 7. Human cells recovered from the mice engrafted with BM cells from patient 6 and patient 11 were subjected to FISH analysis using D7Z1 (green signal for centromere of chromosome 7) plus D7S486 (red signal for 7q31 region) probes for patient 6 and D7Z1 (yellow signal) probe for patient 11. In a lower panel, a murine granulocyte with a ring-shaped nucleus which did not hybridize with the human probe is located adjacent to the human cell hybridized with D721. All cells analyzed (10 cells for patient 6 and 100 cells for patient 11) demonstrated the same outcome. (C) Chromosomal analysis of cells recovered from the mice transplanted with MDS-originated cells obtained from the BM of patient 13 and patient 14 demonstrated the maintenance of the original abnormal karyotype, namely isochromosome 17 and monosomy 7 (arrows), respectively. Eight cells were analyzed for patient 13 and 20 cells for patient 14. (D) Wright-Giemsa-stained cytospin preparations made of CD45-sorted human cells. In the cytospin samples for patient 2, various stages of myeloid lineage cells and an eosinophil are shown. An insert shows a myelocyte with pseudo-Pelger anomaly. For patient 11, an arrow indicates a bi-nucleated myelocyte. Inserts show differentiated neutrophils. The majority of cells found in a cytospin preparation of BM cells obtained from the mice engrafted with cells from patient 14 demonstrated fine chromatin formation and conspicuous nucleoli. Cytospin samples of a normal cell-engrafted mouse (patient 1) were composed of lymphocytes.