ated with multiple physical abnormalities, especially cleft lip and/or palate.

Cmejla *et al.* reported that 87.5% of *RPL5*-mutated patients were born small-for-date, whereas only 42.9% of *RPS19*-mutated patients were born small-for-date.[18] However, in our series, the small-for-date phenotype was seen in seven patients, and all of them were *RPL5*-non-mutated patients. Our data suggest that *RPL5* mutations in Japanese DBA patients have no relevance to the small-for-date phenotype, which may be a unique characteristic of Japanese DBA.

According to recent studies, the frequency of malformation, particularly thumb anomalies, in *RPS19*-mutated patients, was relatively low compared to that in *RPL5*- or *RPL11*-mutated patients.[22-24,29] In Italian DBA patients, the risk of malformation was 7-fold higher in *RPL5*-mutated patients than in *RPS19*-mutated patients.[29] In contrast, all of the Japanese DBA patients with *RPS19* mutations had one or more malformations. The frequency of thumb anomalies was significantly higher in patients with *RPS19* mutations, as well as in patients with *RPL5* mutations, compared to in the other groups of patients.

Although steroid therapy is one of the established treatments for DBA, the mechanism of action is unknown and reliable prediction of response to initial steroid therapy is not available.[1,3] *RPS19* mutation status has not been predictive of response in any series.[3] In our cohort, responsiveness to first steroid therapy in Japanese DBA patients was as good as that reported in western populations.[1,3] In this study, no significant differences in response to initial steroid therapy were found between *RPS19*-mutated and *RPS19*-non-mutated groups, or between the groups with *RPS19* mutations and other ribosomal protein gene mutations.

In summary, we found that heterozygous mutations in *RPS19*, *RPL5*, *RPL11* or *RPS17* were present in 27% of Japanese DBA patients. No mutations were detected in *RPS14*, *RPS24* or *RPL35A*. We observed a slightly lower frequency of mutations in ribosomal protein genes in our cohort of Japanese DBA patients than the frequencies reported previously from western countries, although the data from both populations are based on relatively low numbers of patients and values showing significant differences between populations are lacking. Our data suggest an association between *RPL5* mutation and malformations, especially cleft palate, and between *RPS19* mutation and malformations, particularly thumb anomalies. This study also suggests that no association exists between *RPL5* mutations and the small-for-date phenotype or between *RPS19* mutations and non-responsiveness to initial steroid therapy in Japanese DBA patients.

Authorship and Disclosures

EI was the principal investigator and takes primary responsibility for the paper. YK, TT, ST, GX, RNW, KT, and SO performed the laboratory work for this study. SO, TH, AH, SK, DH, YK, RY, KK, RK, TI, TH, MHP, and KS enrolled the patients. EI and YK wrote the paper.

The authors reported no potential conflicts of interest.

List of hospitals and people who cooperated in collecting clinical samples from the DBA patients

Iwate prefectural Chubu Hospital (N. Onodera); Iwata City Hospital (M. Shirai); Osaka City General Hospital (J. Hara) ; Kagoshima City Hospital (K. Kawakami); Kagoshima University (Y. Okamoto); Kyoto University (K. Watanabe); Kyoto Prefectural Yosanoumi Hospital (H. Ogawa); Saitama Children's Medical Center (K. Koh); Shiga Medical Center for Children (T. Kitoh); Shizuoka Children's Hospital (K. Sakaguchi); Tokyo University (K. Ida); National Hospital Organization Saitama Hospital (I. Kamimaki); Dokkyo University (H. Kurosawa); Nakadori General Hospital (A. Watanabe); East Medical Center Moriyama Municipal Hospital, City of Nagoya (M. Yazaki); Nara Medical University (Y. Takeshita); Japanese Red Cross Narita Hospital (S. Igarashi); Hiroshima Red Cross Hospital & Atomic-bomb Survivors Hospital (N. Fujita); Fukushima Medical University (A. Kikuta); Yamagata University (T. Mitsui); Wakayama Medical University (M. Yoshiyama).

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Brief report

Autoimmune lymphoproliferative syndrome–like disease with somatic *KRAS* mutation

Masatoshi Takagi,[1] Kunihiro Shinoda,[2] Jinhua Piao,[1] Noriko Mitsuiki,[1] Mari Takagi,[2] Kazuyuki Matsuda,[3] Hideki Muramatsu,[4] Sayoko Doisaki,[4] Masayuki Nagasawa,[1] Tomohiro Morio,[1] Yoshihito Kasahara,[5] Kenichi Koike,[6] Seiji Kojima,[4] Akira Takao,[2] and Shuki Mizutani[1]

[1]Department of Pediatrics and Developmental Biology, Graduate School of Medicine, Tokyo Medical and Dental University, Tokyo, Japan; [2]Department of Pediatrics, Gifu Municipal Hospital, Gifu, Japan; [3]Department of Laboratory Medicine, Shinshu University School of Medicine, Nagano, Japan; [4]Department of Pediatrics, Nagoya University Graduate School of Medicine, Aichi, Japan; [5]Department of Laboratory Sciences, Kanazawa University School of Health Sciences, Ishikawa, Japan; and [6]Department of Pediatrics, Shinshu University School of Medicine, Nagano, Japan

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 2 cases with ALPS-like disease with somatic *KRAS* mutation. Both cases were characterized by prominent autoimmune cytopenia and lymphoadenopathy/splenomegaly. These patients did not satisfy the diagnostic criteria for ALPS or juvenile myelomonocytic leukemia and are probably defined as a new disease entity of RAS-associated ALPS-like disease (RALD). (*Blood*. 2011;117(10):2887-2890)

Introduction

Autoimmune lymphoproliferative syndrome (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 interleukin-2 (IL-2) depletion-dependent apoptosis. Recent updated criteria and classification of ALPS suggested type IV ALPS as a RAS-associated leukoproliferative disease.[4]

Juvenile myelomonocytic leukemia (JMML) is a chronic leukemia in children. Patients show lymphadenopathy, hepatosplenomegaly, leukocytosis associated with monocytosis, anemia, thrombocytopenia, and occasional autoimmune phenotypes. Approximately 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 mononuclear cells (MNCs) and hypersensitivity to granulocyte-macrophage colony-stimulating factor (GM-CSF) of $CD34^+$ BM-MNCs.[6]

Germline RAS pathway mutations cause Costello (*HRAS*), Noonan (*PTPN11*, *KRAS*, and *SOS1*), and cardio-facio-cutaneous syndromes (*KRAS*, *BRAF*, *MEK1*, and *MEK2*). Patients with Costello and Noonan syndromes have an increased propensity to develop solid and hematopoietic tumors, respectively[7]; among these tumors, the incidence of JMML in patients with germline mutation of *NF1* or *PTPN11* is well known.

We present 2 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 cardio-facio-cutaneous or Noonan syndrome.

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 Figure 1A-B, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). Blood test revealed the presence of hemolytic anemia and autoimmune thrombocytopenia. Hyper-γ-globulinemia with various autoantibodies 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. The patient did not satisfy the criteria for the diagnosis of ALPS or JMML as discussed in "Results and discussion."

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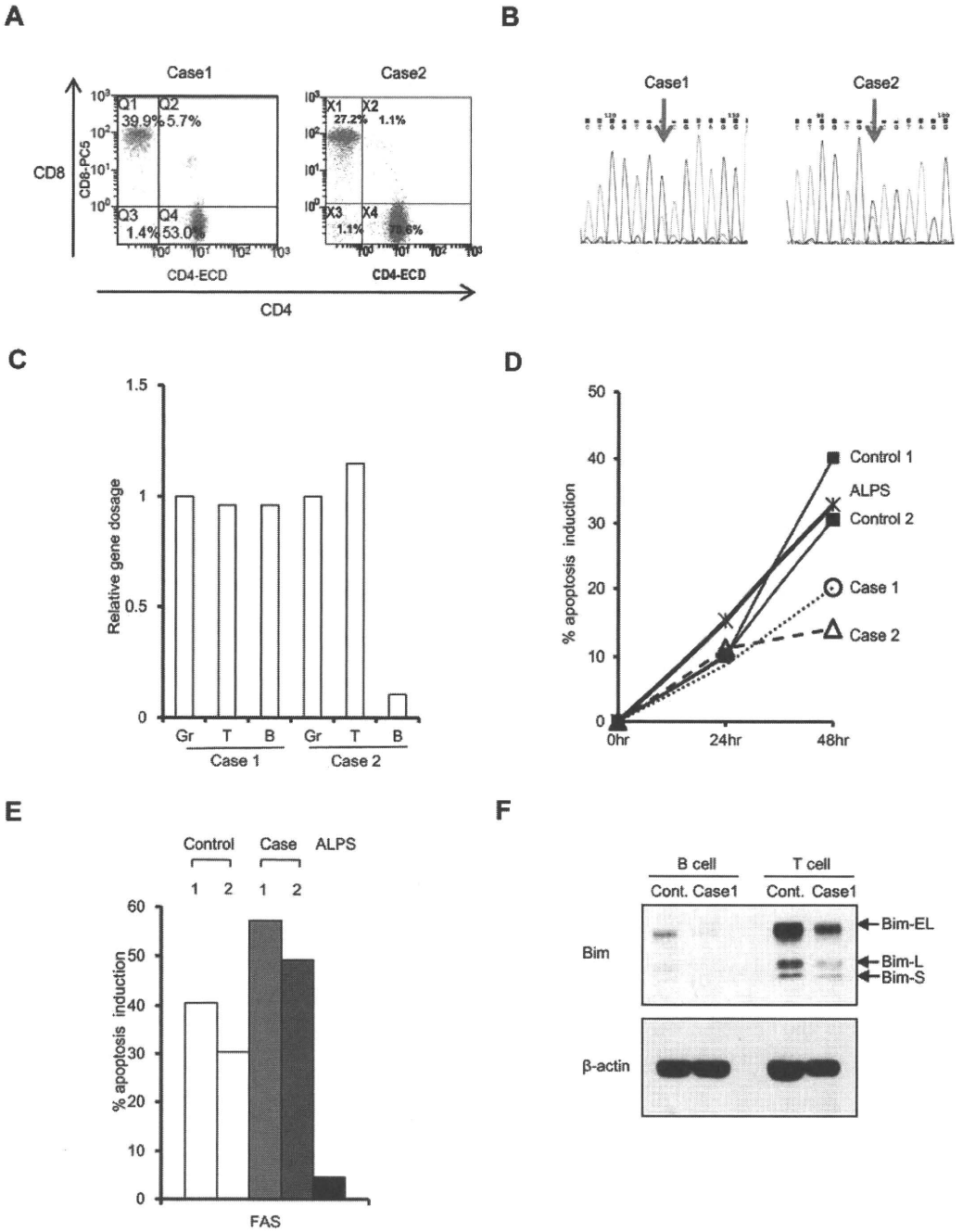

Figure 1. Molecular cell biologic assay of RALD. (A) Flow cytometric analysis of double-negative T cells. CD8 and CD4 double staining was performed in T-cell receptor-αβ-expressing cells. (B) Electropherogram showing KRAS G13D mutation in BM-MNCs in case 1 (left panel) and case 2 (right panel). (C) Gene dosage of mutated allele in granulocytes (Gr), T cells (T), and B cells (B). Relative gene dosage was estimated by a mutant allele-specific polymerase chain reaction method in cases 1 and 2 using albumin gene as internal control. (D) Apoptosis assay using activated T cells. Apoptosis percentage was measured by flow cytometry with annexin V staining 24 and 48 hours after IL-2 depletion. (E) Apoptosis percentage was measured 24 hours after addition of anti-FAS CH11 antibody (final 100 ng/mL). (F) Western blotting analysis of Bim expression.

Case 2

A 5-month-old girl had a fever and massive hepatosplenomegaly (supplemental Figure 1D). She was initially diagnosed with Evans syndrome based on the presence of hemolytic anemia and autoimmune thrombocytopenia with hyper-γ-globulinemia and autoantibodies. Spontaneous colony formation assay and GM-CSF hypersensitivity of BM-MNCs showed positivity. Then, tentative diagnosis of JMML was given, even though she showed no massive monocytosis or increased fetal hemoglobin. Detailed clinical history and laboratory data are provided in supplemental data.

Detailed methods for experiments are described in supplemental data.

Results and discussion

Case 1 showed a high likelihood of being a case of ALPS according to the symptoms and clinical data presented (supplemental Table 1), except for number of double-negative T cells, which was only 1.4% of T-cell receptor-αβ cells (Figure 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-MNCs (data not shown) or phosphor-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 (Figure 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 polymerase chain reaction methods, which revealed that mutated allele was almost equally present in granulocytes, T cells, and B cells (Figure 1C). $CD34^+$ hematopoietic stem cells (HSCs) were also positive for KRAS G13D mutation, and 60% of colony-forming units-granulocyte macrophage (CFU-GM) developed from isolated CD34 cells carried the KRAS G13D mutation (data not shown). These observations suggest that the mutation occurred at the HSCs level, and HSC consists of wild-type and mutant HSCs.

NRAS-mutated type IV ALPS was previously characterized by apoptosis resistance of T cells in IL-2 depletion.[3] Then, activated T cells were subjected to an apoptosis assay by FAS stimulation or IL-2 depletion. Remarkable resistance to IL-2 depletion, but not to FAS-dependent apoptosis (Figure 1D-E), was seen. This was in contrast to T cells from FAS-mutated ALPS type 1a, which showed remarkable resistance to FAS-dependent apoptosis and normal apoptosis induction by IL-2 withdrawal (Figure 1D-E). Western blotting analysis of activated T cells or Epstein-Barr virus-transformed B cells showed reduced expression of Bim (Figure 1F).

In case 2, autoimmune phenotype and hepatosplenomegaly were remarkable, as shown in Supplemental data. The patient was initially diagnosed as Evans syndrome based on the presence of hemolytic anemia and autoimmune thrombocytopenia. Double-negative T cells were 1.1% of T-cell receptor-$\alpha\beta$ cells in the peripheral blood, which did not meet with the criteria of ALPS. Although spontaneous colony formation was shown in peripheral blood- and BM-MNCs, and GM-CSF hypersensitivity was demonstrated in BM-MNCs derived $CD34^+$ cell (supplemental Table 2), she showed no massive monocytosis or increased fetal hemoglobin. Thus, the diagnosis was less likely to be ALPS or JMML. DNA sequencing of JMML-related genes, such as *NRAS, KRAS, HRAS, PTPN11*, and *CBL*, identified somatic, but not germline, KRAS G13D mutation (Figure 1B). KRAS G13D mutation was detected in granulocytes and T cells. Mutation was not identified in B cells by conventional DNA sequencing (data not shown). Mutant allele-specific quantitative polymerase chain reaction revealed that mutated allele was almost equally present in granulocytes and T cells, but barely in B cells (Figure 1C). Activated T cells showed resistance to IL-2 depletion but not to FAS-dependent apoptosis (Figure 1D-E).

Both of our cases were characterized by strong autoimmunity, immune cytopenia, and lymphadenopathy or hepatosplenomegaly with partial similarity with ALPS or JMML. However, they did not meet with the well-defined diagnostic criteria of ALPS[2] or JMML.[6] It is interesting that case 2 presented GM-CSF hypersensitivity, which is one of the hallmarks of JMML. Given the strict clinical and laboratory criteria of JMML and ALPS, our 2 cases should be defined as a new disease entity, such as RAS-associated ALPS-like disease (RALD). Recently defined NRAS-mutated ALPS type IV may also be included in a similar disease entity.

There are several cases of JMML reported simultaneously having clinical and laboratory findings compatible with autoimmune disease.[8,9] Autoimmune syndromes are occasionally seen in patients with myelodysplastic syndromes, including chronic myelomonocytic leukemia.[10] These previous findings may suggest a close relationship of autoimmune disease and JMML. Because KRAS G13D has been identified in JMML,[11-13] it is tempting to speculate that KRAS G13D mutation is involved in JMML as well as RALD. In JMML, erythroid cells reportedly carry mutant RAS, whereas B- and T-cell involvement was variable.[13] In both of our cases, myeloid cells and T cells carried mutant RAS, whereas B cells were affected variably. These findings would support a hypothesis that the clinical and hematologic features are related to the differentiation stages of HSCs where RAS mutation is acquired. JMML-like myelomonocytic proliferation may predict an involvement of RAS mutation in myeloid stem/precursor cell level, whereas ALPS-like phenotype may predict that of stem/precursor cells of lymphoid lineage, especially of T cells. Under the light of subtle differences between the 2 cases presented, their hematologic and clinical features may reflect the characteristics of the stem cell level where *KRAS* mutation is acquired. Involvement of the precursors with higher propensity toward lymphoid lineage may lead to autoimmune phenotypes, whereas involvement of those with propensity toward the myeloid lineage may lead to GM-CSF hypersensitivity while still sharing some overlapping autoimmune characteristics.

One may argue from the other viewpoints with regard to the clinicopathologic features of these disorders. First, transformation in fetal HSCs might be obligatory for the development of JMML[14] and, in HSCs later in life, may not have the same consequences. Second, certain KRAS mutations may be more potent than others. Codon 13 mutations are generally less deleterious biochemically than codon 12 substitutions, and patients with JMML with codon 13 mutations have been reported to show spontaneous hematologic improvement.[12,15] Thus, further studies are needed to reveal in-depth clinicopathologic characteristics in this type of lymphomyeloproliferative disorder.

KRAS mutation may initiate the oncogenic pathway as one of the first genetic hits but is insufficient to cause frank malignancy by itself.[16,17] Considering recent findings that additional mutations of the genes involved in DNA repair, cell cycle arrest, and apoptosis are required for full malignant transformation, one can argue that RALD patients will also develop malignancies during the course of the disease. Occasional association of myeloid blast crisis in JMML and that of lymphoid malignancies in ALPS will support this notion. Thus, the 2 patients are now being followed up carefully. It was recently revealed that half of the patients diagnosed with Evans syndrome, an autoimmune disease presenting with hemolytic anemia and thrombocytopenia, met the criteria for ALPS diagnosis.[18,19] In this study, FAS-mediated apoptosis analysis was used for the screening. Considering the cases we presented, it will be intriguing to reevaluate Evans syndrome by IL-2 depletion-dependent apoptosis assay focusing on the overlapping autoimmunity with RALD.

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Authorship

Contribution: Masatoshi Takagi and S.M. designed entire experiments and wrote the manuscript; K.S., N.M., and Mari Takagi treated patients and designed clinical laboratory test; J.P. performed experiments described in Figure 1B-F; K.M., H.M., and S.D. performed colony and mutational analysis; and M.N., T.M., K.K., S.K., Y.K., and A.T. supervised clinical and immunologic experiments or coordinated clinical information.

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Correspondence: Masatoshi Takagi, Department of Pediatrics and Developmental Biology, Graduate School of Medicine, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo, 113-8519, Japan; e-mail: m.takagi.ped@tmd.ac.jp; and Shuki Mizutani, Department of Pediatrics and Developmental Biology, Graduate School of Medicine, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo, 113-8519, Japan; e-mail: smizutani.ped@tmd.ac.jp.

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

*Rika Kanezaki,[1] *Tsutomu Toki,[1] Kiminori Terui,[1] Gang Xu,[1] RuNan Wang,[1] Akira Shimada,[2] Asahito Hama,[3] Hirokazu Kanegane,[4] Kiyoshi Kawakami,[5] Mikiya Endo,[6] Daisuke Hasegawa,[7] Kazuhiro Kogawa,[8] Souichi Adachi,[9] Yasuhiko Ikeda,[10] Shotaro Iwamoto,[11] Takashi Taga,[12] Yoshiyuki Kosaka,[13] Seiji Kojima,[3] Yasuhide Hayashi,[2] and Etsuro Ito[1]

[1]Department of Pediatrics, Hirosaki University Graduate School of Medicine, Hirosaki, Japan; [2]Department of Hematology/Oncology, Gunma Children's Medical Center, Gunma, Japan; [3]Department of Pediatrics, Nagoya University Graduate School of Medicine, Nagoya, Japan; [4]Department of Pediatrics, Graduate School of Medicine, University of Toyama, Toyama, Japan; [5]Department of Pediatrics, Kagoshima City Hospital, Kagoshima, Japan; [6]Department of Pediatrics, Iwate Medical University, Morioka, Japan; [7]Department of Pediatrics, St Luke's International Hospital, Tokyo, Japan; [8]Department of Pediatrics, National Defense Medical College, Tokorozawa, Japan; [9]Department of Pediatrics, Kyoto University Graduate School of Medicine, Kyoto, Japan; [10]Department of Pediatrics, Aomori City Hospital, Aomori, Japan; [11]Department of Pediatrics, Mie University Graduate School of Medicine, Tsu, Japan; [12]Department of Pediatrics, Shiga University of Medical Science, Ohtsu, Japan; and [13]Department of Hematology and Oncology, Hyogo Children Hospital, Kobe, Japan

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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*R.K. and T.T. contributed equally to this work.

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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.

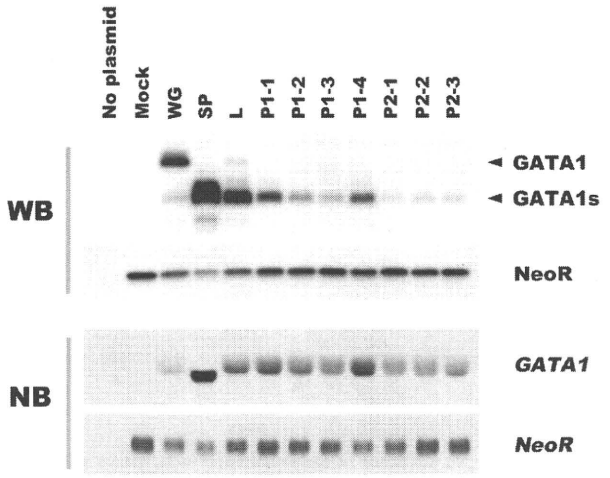

Figure 1. Effects of mutant transcripts of *GATA1* on the expression level of the truncated protein. The *GATA1* mutations observed in TAM patients are classified according to the types of transcripts. The translational efficiency of each transcript was assessed by Western blot analysis in BHK-21 cells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

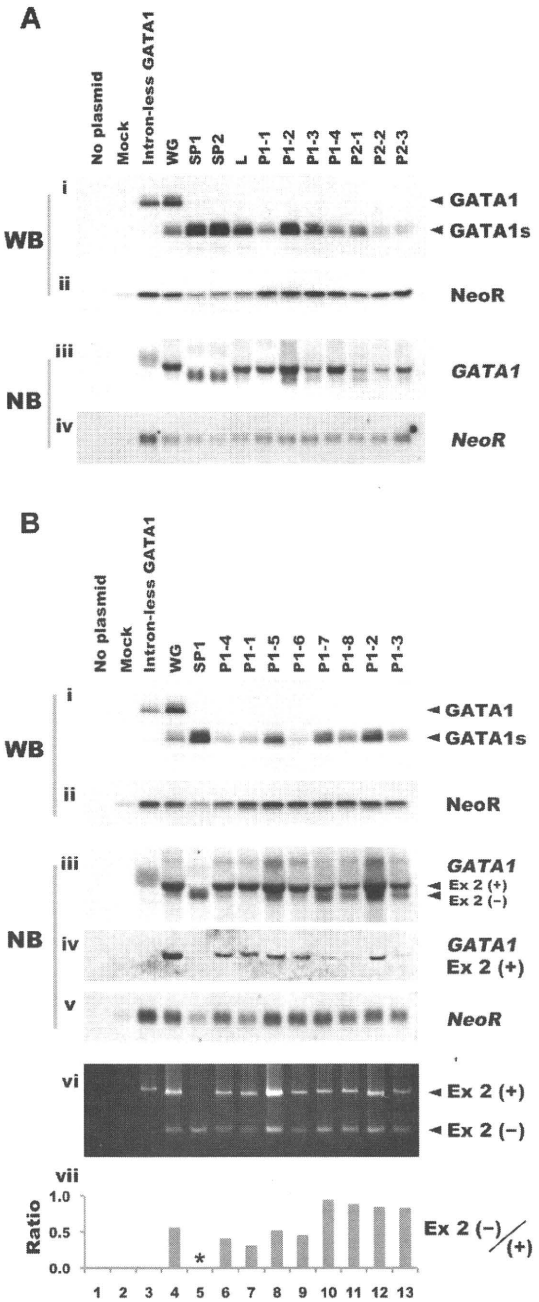

Figure 2. GATA1 mutations affect the expression level of the truncated protein. (A) The expression levels of GATA1s protein and mRNA were assessed in BHK-21 cells transfected with human *GATA1* minigene expression vectors carrying mutations observed in TAM patients. Western blot analysis was performed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

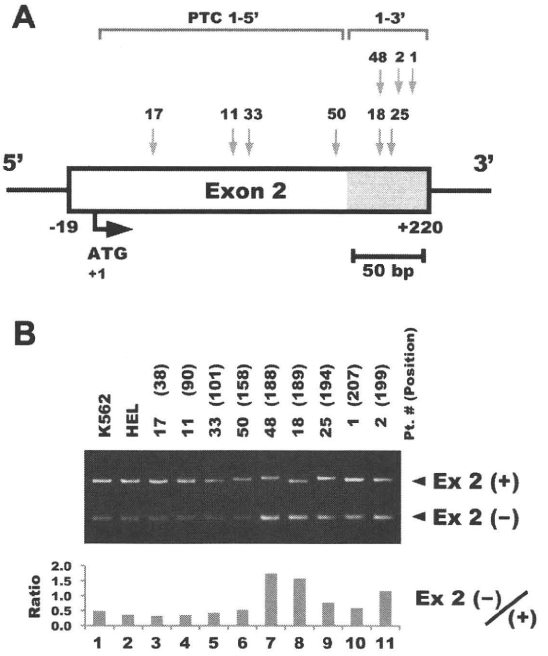

Figure 3. The location of the PTC type 1 mutation affects the efficiency of alternative splicing in TAM blast cells. (A) The location of the *GATA1* mutation in each TAM patient. Details of the mutation in each sample are described in Table 1. (B) RT-PCR analysis of *GATA1* in TAM blast cells harboring 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.

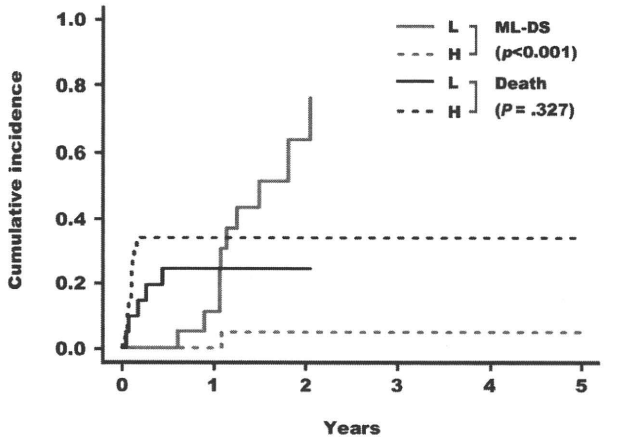

Figure 4. Cumulative incidence of early death and of ML-DS in children with TAM. Based on the estimated GATA1s expression levels, patients were classified in 2 groups: GATA1s high and low groups. TAM patients in the GATA1s low group had a significantly higher risk for the development of leukemia (P (gray)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

Mutation type	Outcome of TAM				TAM		ML-DS	
	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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Correspondence: Etsuro Ito, Department of Pediatrics, Hirosaki University Graduate School of Medicine, 5 Zaifu-cho, Hirosaki, Aomori, 036-8563, Japan; e-mail: eturou@cc.hirosaki-u.ac.jp.

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Recent progress in dyskeratosis congenita

Nobuhiro Nishio · Seiji Kojima

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Abstract Dyskeratosis congenita (DC) is an inherited disease associated with nail dystrophy, abnormal skin pigmentation, oral leukoplakia, bone marrow failure and a predisposition to cancer. DC is a disease of defective telomere maintenance and patients with DC have very short telomeres. To date, mutations in six genes of telomerase and telomere components have been identified in patients with DC. Recently, mutations in telomerase and telomere components were also identified in patients with aplastic anemia, pulmonary fibrosis, and liver diseases who did not have mucocutaneous manifestations. These findings imply that defective telomere maintenance may cause not only classical DC but also a broad spectrum of diseases previously thought to be idiopathic, and have led to a new concept of diseases, termed "syndromes of telomere shortening". An understanding of the role of telomeres in these diseases is indispensable for diagnosis, genetic counseling and clinical management.

Keywords Dyskeratosis congenita · Telomere · Telomerase · Bone marrow failure

1 Introduction

Elizabeth Blackburn, Carol Greider, and Jack Szostak were awarded the 2009 Nobel Prize in Physiology or Medicine for their work describing telomeres and telomerase [1, 2]. Telomeres are DNA–protein structures that protect chromosome ends, which consist of a TTAGGG repeat bound by a cap protein, shelterin. Telomeres cannot be replicated by standard polymerase but only by a specialized transcriptase, called telomerase.

Dyskeratosis congenita (DC) is a rare inherited multisystem bone marrow failure syndrome characterized mainly by mucocutaneous abnormalities including nail dystrophy, mucosal leukoplakia, and abnormal skin pigmentation, along with a predisposition to cancer. Patients with DC have very short germ-line telomeres compared with normal individuals due to a defect of telomere maintenance. DC has been receiving increased attention because "telomere maintenance" is closely associated with life events, including aging and cancer predisposition. Recently, mutations in telomerase and telomere components were also identified in patients with aplastic anemia (AA), pulmonary fibrosis, and liver diseases who did not have mucocutaneous manifestations [3–13]. These findings implicate that defective telomere maintenance causes not only classical DC but also a broad spectrum of diseases previously thought to be idiopathic, and have lead to a new concept of diseases, termed "syndromes of telomere shortening".

In this review, we will discuss recent progress in the understanding of the pathophysiology of DC and other telomere diseases, as well as treatment for these diseases including stem cell transplantation.

2 Dyskeratosis congenita

The incidence of classic DC is approximately 1/1,000,000 individuals [14]. Classic DC presents with a triad of mucocutaneous abnormalities in around 80–90% of patients; abnormal skin pigmentation, nail dystrophy and oral leukoplakia [15]. Skin pigmentation and nail changes

N. Nishio · S. Kojima (✉)
Department of Pediatrics, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Shouwa-ku, Nagoya 466-8550, Japan
e-mail: kojimas@med.nagoya-u.ac.jp

usually appear in childhood followed by oral leukoplakia and bone marrow failure, which develop by the age of 20 years. Other clinical manifestations, including non-mucocutaneous abnormalities, have also been reported. Non-mucocutaneous features such as bone marrow failure and pulmonary fibrosis occasionally precede mucocutaneous abnormalities, making it difficult to diagnose patients with DC based on clinical features alone. The diagnostic criteria for DC proposed by Vulliamy [16] include one or more of the three classic mucocutaneous features combined with hypoplastic bone marrow and at least two other somatic features known to occur in DC. The main causes of death in patients with DC are bone marrow failure/immunodeficiency (60–70%), pulmonary complications (10–15%), and malignancy (10%) [17, 18].

Until now, mutations in six genes involved in telomere maintenance have been identified in patients with DC. Figure 1 shows the schema of telomerase and shelterin complex. *DKC1* gene, encoding dyskerin, is the first gene identified in X-linked DC patients [19]. Dyskerin has a close association with the RNA component of telomerase (TERC), and mutations in dyskerin cause a reduction in accumulation of TERC and reduced telomere length [20]. In addition to its role in the biogenesis of telomerase RNA, dyskerin is involved in ribosomal RNA biogenesis. Dyskerin catalyzes uridine to pseudouridine, which is a critical step for ribosomal RNA maturation and function. These findings imply that both telomere and ribosomal defects may occur in patients with *DKC1* mutations. Subsequently, heterozygous *TERC* mutations were found in autosomal dominant DC patients [21]. Mutation screening demonstrated mutations of other components of telomerase complex including telomerase reverse transcriptase (*TERT*) [22, 23], *NOP10* [24], and *NHP2* [25] in patients with rare autosomal recessive DC. Mutations of *TERT* were also reported in the autosomal dominant family [8]. More recently, heterozygous mutations of *TINF2* encoding TIN2, main component of shelterin which protects telomeres, have been identified in ~11% of DC patients [5, 26].

3 Gene mutations of telomere maintenance in aplastic anemia and other bone marrow failure syndromes

Patients with DC have disease diversity in terms of age at onset, symptoms, and severity; this diversity occurs even among the patients with the same gene mutation. Bone marrow failure sometimes precedes mucocutaneous manifestations in patients with DC, and a substantial proportion of patients with AA have shorter telomeres compared with normal individuals [27, 28]. These observations prompted screening for gene mutations responsible for telomere maintenance in patients with AA and other bone marrow failure syndromes. This screening identified mutations in *TERC* and *TERT* in 3% of patients with AA [7, 9] (Table 1). We also identified *TERT* mutations in 2 of 96 Japanese children with AA, but no patient had a *TERC* mutation [6]. Patients with *TERC* or *TERT* mutations have very short telomeres in blood cells. Recently, Du et al. [4] found that 6 (5.5%) of 109 pediatric patients with severe AA had mutations of *TINF2*. We also screened for mutations of *TINF2*, but none of 96 pediatric patients with AA showed mutations of this gene (unpublished data).

Among three methods of measuring telomere length, including southern blot, real-time polymerase chain reaction, and flow cytometry and fluorescence in situ

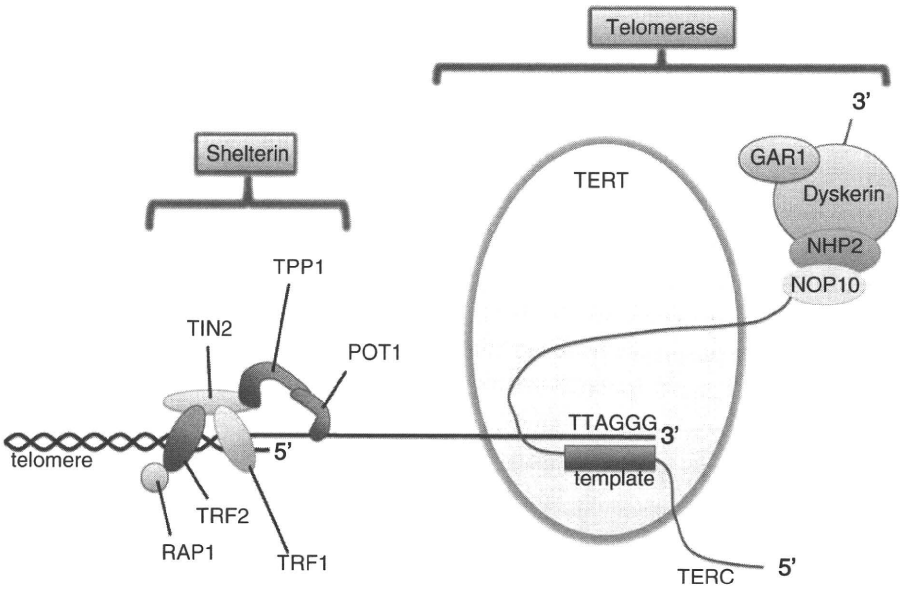

Fig. 1 Schema of telomerase and shelterin complex. Telomerase complex consists of the enzyme telomerase transcriptase (TERT), RNA component (TERC), and dyskerin protein complex (dyskerin, NOP10, NHP2, and GAR1). TERT adds new telomeres (TTAGGG repeats) onto the chromosome end by using the template provided by TERC. The shelterin complex consists of six proteins (TRF1, TRF2, RAP1, POT1, TPP1, and TIN2) and protects telomeres and regulates telomerase

Table 1 Mutations of genes associated with telomere maintenance identified in patients with aplastic anemia

References	Gene	Number of mutated and screened patients
Vulliamy et al. [10]	*TERC*	2/17 (12%)
Vulliamy et al. [8]	*TERT*	2/80 (2.5%)
Yamaguchi et al. [9]	*TERC*	2/150 (1.3%)
Yamaguchi et al. [7]	*TERT*	7/200 (3.5%)
Savage et al. [50]	*TERF1*	1/47 (2.1%)
	TERF2	1/47 (2.1%)
Liang et al. [6]	*TERT*	2/96 (2.1%)
Walne et al. [51]	*TINF2*	2/111 (1.8%)
Du et al. [3]	*TERT*	4/199 (2.0%)
Du et al. [4]	*TINF2*	6/109 (5.5%)

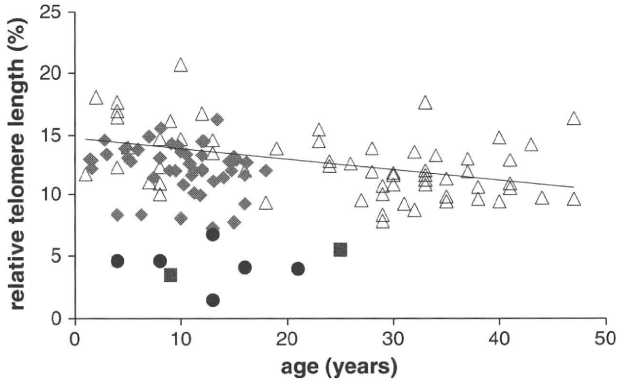

Fig. 2 Relative telomere length in peripheral blood lymphocytes from patients with dyskeratosis congenita (*filled circles*), patients with aplastic anemia harboring *TERT* mutations (*filled squares*), patients with idiopathic aplastic anemia (*filled argyles*) and normal individuals (*open triangles*). Telomere lengths were measured by flow cytometry-fluorescent in situ hybridization (flow-FISH). Relative telomere length was calculated as the ratio between the telomere signal of each sample and the telomere signal of the control cell line (cell line 1301). These data are from the Department of pediatrics, Nagoya University Graduate School of Medicine

hybridization (flow-FISH), flow-FISH is the most appropriate for "prospective" screening [29, 30]. As shown in Fig. 2, patients with DC and AA with the *TERT* mutation demonstrated very short telomeres as compared with idiopathic AA patients and normal individuals. Given the finding that a small subset of patients with apparently idiopathic AA carry telomere gene mutations and recognizing these patients is critical to treatment decisions, it is desirable to screen telomere gene mutations routinely in patients with AA before starting treatment. However, because screening of gene mutations is laborious and time-consuming, we have adopted screening of telomere length in blood cells instead of gene mutations.

It should be noted that short telomeres are not specific for patients with DC but are also seen in patients with bone marrow failure syndromes. Although short telomeres are also found in patients with other congenital bone marrow failure syndromes, such as Shwachman–Diamond syndrome and Fanconi anemia, telomere length in patients with DC is the shortest compared with other bone marrow failure syndromes. In fact, telomere length in most patients with DC is below the first percentile of telomere length found in healthy controls [31].

Family members of patients with DC should receive genetic counseling to rule out if they are silent carriers. In particular, genetic counseling is necessary during the proband search for a donor for hematopoietic stem cell transplantation. Sometimes, telomere length analysis in families with DC demonstrates that mutated carriers with clinical signs of bone marrow failure have the short telomeres. However, telomere length cannot predict the presence or absence of a mutation in family members with bone marrow failure. There are rare cases that show normal telomere length even though they harbor the same mutation as the proband, suggesting that mutation alone does not sufficiently shorten the telomeres [3].

4 Telomere diseases other than bone marrow failure syndromes

Clinical manifestations in patients with DC include not only bone marrow failure, but also other organ failures. Progressive pulmonary fibrosis develops in around 10–15% of patients with DC [17, 18], and is the second most common cause of death. Respiratory failure is also a common fatal complication after hematopoietic stem cell transplantation. Idiopathic pulmonary fibrosis (IPF) is an adult-onset, progressive scarring of the lung of unknown etiology that ultimately leads to respiratory failure. From 2 to 20% of patients with IPF have a family history of the disease that is inherited as an autosomal dominant trait with variable penetrance [12, 32]. Because some individuals in a pedigree of DC had the IPF phenotype, Armanios et al. [12] hypothesized that *TERC* or *TERT* may be candidate genes for familial IPF. They screened 73 probands of IPF and found 6 (8%) had heterozygous mutations in *TERT* or *TERC*. Tsakiri et al. [11] also independently found three missense mutations and one deletion of *TERT* genes in 44 probands of familial IPF and an additional single mutation in 44 sporadic cases of IPF. These mutant telomerase resulted in short telomeres. However, these patients did not show any classic mucocutaneous manifestations of DC.

Liver diseases have been also described as one of the clinical presentations in patients with DC. Some patients with DC develop severe liver complications after hematopoietic stem cell transplantation even if they have a normal liver function at the time of transplant [33]. In parallel with

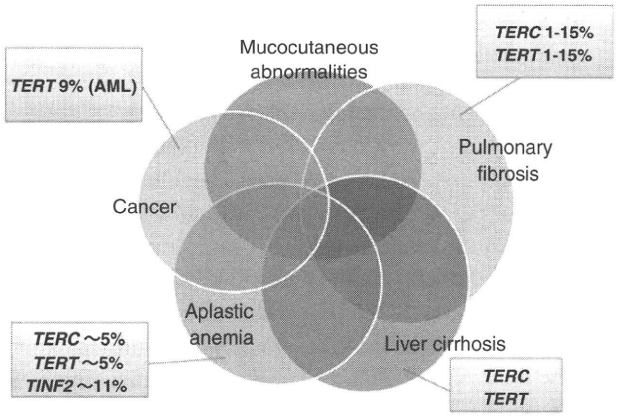

Fig. 3 Schema of phenotypic variations and identified gene mutations in defective telomere maintenance

reports of familial IPF, Calado et al. [13] reported that many relatives of patients with AA and a telomerase mutation had liver diseases, including pathologic fibrosis with inflammation and nodular regenerative hyperplasia. These patients did not present symptoms in childhood or display the characteristic physical abnormalities of DC, but had very short telomeres. These authors proposed that these disorders be collectively considered as "syndromes of telomere shortening". Figure 3 shows the schema of phenotypic variations and identified gene mutations in defective telomere maintenance.

5 Telomere shortening, chromosome instability and cancer predisposition

Patients with DC are prone to hematological malignancies and other solid tumors [17]. The defect of telomere maintenance and telomere attrition leads to chromosomal instability such as loss or gain of chromosomes and end-to-end fusion in in vitro studies and mouse models [34, 35]. Alter et al. recently reported that the expected cancer risk is 11-fold higher in patients with DC compared with the general population. The most frequent solid tumors were head and neck squamous cell carcinomas followed by skin and anorectal cancer [36].

Even outside DC, telomere attrition appears to cause chromosomal instability and cancer predisposition. Calado et al. [37] recently reported that patients with AA with shorter telomeres at diagnosis had a sixfold higher probability of developing clonal malignant disease following immunosuppressive therapy than patients with longer telomeres. They also showed that cultured bone marrow cells of patients with short telomeres in the presence of cytokines and high-dose granulocyte-colony stimulating factor (G-CSF) demonstrated increased telomere-free chromosomal ends and aneuploidy and translocations, including Robertsonian translocations.

Because patients with DC have been thought to be prone to myeloid malignancy, a screening for *TERT* and *TERC* mutations in patients with acute myeloid leukemia (AML) was conducted by the NIH group [38]. The authors found constitutional *TERT* mutations in 9% of patients with AML and a strong association of *TERT* mutations with the risk of cytogenetic abnormalities including trisomy 8 and inversion 16. None of the AML patients with *TERT* mutations had physical abnormalities that led to a suspicion of DC.

In addition, short telomeres have been linked to tumorigenesis of several solid tumors, including esophageal cancer, colorectal cancer, gastric cancer [39], and lung cancer [40]. Recent genome-wide studies demonstrated a higher frequency of *TERT* gene polymorphism in these patients than in normal individuals [41, 42].

6 Treatment of bone marrow failure

Bone marrow failure and immune deficiency are the most common causes of death in up to 60–70% of patients with DC. Androgen (e.g. oxymetholone) has been used to improve cytopenia in patients with DC since the 1960s. However, the mechanism of action of androgen has not been well understood until recently. Calado et al. [43] showed that in vitro exposure of normal peripheral blood cells to androgen produce higher *TERT* mRNA levels, and cells from patients who had heterozygous mutation of telomerase restored their low baseline telomerase activity to normal levels. As telomere shortening is closely associated with malignant disease, androgen therapy might prevent or postpone the development of various types of cancers. Erythropoietin and/or G-CSF combined with androgen has occasionally provided transient hematopoietic recovery to poor responders to androgen alone [44]. However, this combination should be used with caution because severe splenic peliosis and fatal rupture have been reported in two patients with DC who received simultaneous administration of androgen and G-CSF [45].

Allogeneic hematopoietic stem cell transplantation is the only curative treatment for bone marrow failure in patients with DC. However, the outcome in previous reports has been disappointing because of unacceptable transplant-related toxicities, including severe pulmonary/liver complications, especially in transplants from an alternative donor [36, 46]. To avoid these complications, non-myeloablative conditioning regimens have been recently used in several cases. Dietz et al. [47] reported encouraging results of six patients with DC who received a fludarabine-based non-myeloablative regimen. Four patients are alive, three of whom were recipients of unrelated grafts. Non-myeloablative transplants are expected to provide improvement in short-term survival. However,

longer-term follow-up is necessary because the late effects of conditioning agents and allogeneic immune responses within the recipient's organs, such as the lung and liver, remain to be clarified.

7 Future direction

Since the review article concerning DC was published by Walne et al. [14] in 2005 in this journal, many advances have occurred in the understanding of DC; however, many unsolved issues remain. Six causative genes have been identified, but mutations of these genes have been found in only half of patients with DC. Telomere-related gene mutations have been identified in patients with not only DC but also in patients with idiopathic AA, pulmonary fibrosis, and liver disease. These findings indicate that telomere-related diseases have a broad spectrum and may represent a new disease entity. A recent study demonstrated that exogenous expression of *TERC* alone can increase telomere activity and create growth potential and longevity in both *TERC* mutant and *DKC1* mutant cells [48]. More recently, Agarwal et al. [49] established induced pluripotent stem cells derived from a patient with DC and showed that the reprogrammed DC cells overcome a critical limitation in TERC levels to restore telomere maintenance and self-renewal. These findings indicate that drugs or gene therapy that can upregulate TERC activity have attractive therapeutic potential in patients with DC. Multicenter prospective studies are warranted to establish appropriate conditioning regimens aimed at reducing transplant-related mortality. We should improve not only short-term outcomes, such as hematological recovery, but also long-term effects on vital organs, especially the lungs and liver, following stem cell transplantation.

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