

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<sup>+</sup> 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.<sup>3</sup> 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<sup>+</sup> 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<sup>2</sup> or JMML.<sup>6</sup> 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.<sup>8,9</sup> Autoimmune syndromes are occasionally seen in patients with myelodysplastic syndromes, including chronic myelomonocytic leukemia.<sup>10</sup> These previous findings may suggest a close relationship of autoimmune disease and JMML. Because *KRAS* G13D has been identified in JMML,<sup>11-13</sup> 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.<sup>13</sup> 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<sup>14</sup> 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.<sup>12,15</sup> 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.<sup>16,17</sup> 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.<sup>18,19</sup> 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.,

## References

- Fisher GH, Rosenberg FJ, Straus SE, et al. Dominant interfering Fas gene mutations impair apoptosis in a human autoimmune lymphoproliferative syndrome. *Cell*. 1995;81(6):935-946.
- Teachey DT, Seif AE, Grupp SA. Advances in the management and understanding of autoimmune lymphoproliferative syndrome (ALPS). *Br J Haematol*. 2010;148(2):205-216.
- Oliveira JB, Bidere N, Niemela JE, et al. NRAS mutation causes a human autoimmune lymphoproliferative syndrome. *Proc Natl Acad Sci U S A*. 2007;104(21):8953-8958.
- Oliveira J, Bleesing J, Dianzani U, et al. Revised diagnostic criteria and classification for the autoimmune lymphoproliferative syndrome (ALPS): report from the 2009 NIH International Workshop. *Blood*. 2010;116(14):e35-e40.
- Miyauchi J, Asada M, Sasaki M, Tsunematsu Y, Kojima S, Mizutani S. Mutations of the N-ras gene in juvenile chronic myelogenous leukemia. *Blood*. 1994;83(8):2248-2254.
- Emanuel PD. Juvenile myelomonocytic leukemia and chronic myelomonocytic leukemia. *Leukemia*. 2008;22(7):1335-1342.
- Aoki Y, Niihori T, Narumi Y, Kure S, Matsubara Y. The RAS/MAPK syndromes: novel roles of the RAS pathway in human genetic disorders. *Hum Mutat*. 2008;29(8):992-1006.
- Kitahara M, Koike K, Kurokawa Y, et al. Lupus nephritis in juvenile myelomonocytic leukemia. *Clin Nephrol*. 1999;51(5):314-318.
- Oliver JW, Farnsworth B, Tonk VS. Juvenile myelomonocytic leukemia in a child with Crohn disease. *Cancer Genet Cytogenet*. 2006;167(1):70-73.
- Saif MW, Hopkins JL, Gore SD. Autoimmune phenomena in patients with myelodysplastic syndromes and chronic myelomonocytic leukemia. *Leuk Lymphoma*. 2002;43(11):2083-2092.
- Flotho C, Kratz CP, Bergstrasser E, et al. Genotype-phenotype correlation in cases of juvenile myelomonocytic leukemia with clonal RAS mutations. *Blood*. 2008;111(2):966-967.
- Matsuda K, Shimada A, Yoshida N, et al. Spontaneous improvement of hematologic abnormalities in patients having juvenile myelomonocytic leukemia with specific RAS mutations. *Blood*. 2007;109(12):5477-5480.
- Flotho C, Valcamonica S, Mach-Pascual S, et al. RAS mutations and clonality analysis in children with juvenile myelomonocytic leukemia (JMML). *Leukemia*. 1999;13(1):32-37.
- Matsuda K, Sakashita K, Taira C, et al. Quantitative assessment of PTPN11 or RAS mutations at the neonatal period and during the clinical course in patients with juvenile myelomonocytic leukemia. *Br J Haematol*. 2010;148(4):593-599.
- Imamura M, Imai C, Takachi T, Nemoto T, Tanaka A, Uchiyama M. Juvenile myelomonocytic leukemia with less aggressive clinical course and KRAS mutation. *Pediatr Blood Cancer*. 2008;51(4):569.
- Zhang J, Wang J, Liu Y, et al. Oncogenic Kras-induced leukemogenesis: hematopoietic stem cells as the initial target and lineage-specific progenitors as the potential targets for final leukemic transformation. *Blood*. 2009;113(6):1304-1314.
- Sabnis AJ, Cheung LS, Dail M, et al. Oncogenic Kras initiates leukemia in hematopoietic stem cells. *PLoS Biol*. 2009;7(3):0537-0548.
- Teachey DT, Manno CS, Axsom KM, et al. Unmasking Evans syndrome: T-cell phenotype and apoptotic response reveal autoimmune lymphoproliferative syndrome (ALPS). *Blood*. 2005;105(6):2443-2448.
- Seif AE, Manno CS, Sheen C, Grupp SA, Teachey DT. Identifying autoimmune lymphoproliferative syndrome in children with Evans syndrome: a multi-institutional study. *Blood*. 2010;115(11):2142-2145.

## Optimization of Therapy for Severe Aplastic Anemia Based on Clinical, Biologic, and Treatment Response Parameters: Conclusions of an International Working Group on Severe Aplastic Anemia Convened by the Blood and Marrow Transplant Clinical Trials Network, March 2010

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Although recent advances in therapy offer the promise for improving survival in patients with severe aplastic anemia (SAA), the small size of the patient population, lack of a mechanism in North America for longitudinal follow-up of patients, and inadequate cooperation among hematologists, scientists, and transplant physicians remain obstacles to conducting large studies that would advance the field. To address this issue, the Blood and Marrow Transplant Clinical Trials Network (BMT CTN) convened a group of international experts in March 2010 to define the most important questions in the basic science, immunosuppressive therapy (IST), and bone marrow transplantation (BMT) of SAA and propose initiatives to facilitate clinical and biologic research. Key conclusions of the working group were: (1) new patients should obtain accurate, expert diagnosis and early identification of biologic risk; (2) a population-based SAA outcomes registry should be established in North America to collect data on patients longitudinally from diagnosis through and after treatment; (3) a repository of biologic samples linked to the clinical data in the outcomes registry should be developed; (4) innovative approaches to unrelated donor BMT that decrease graft-versus-host disease are needed; and (5) alternative donor transplantation approaches for patients lacking HLA-matched unrelated donors must be improved. A partnership of BMT, IST, and basic science researchers will develop initiatives and partner with advocacy and funding organizations to address these challenges. Collaboration with similar study groups in Europe and Asia will be pursued.

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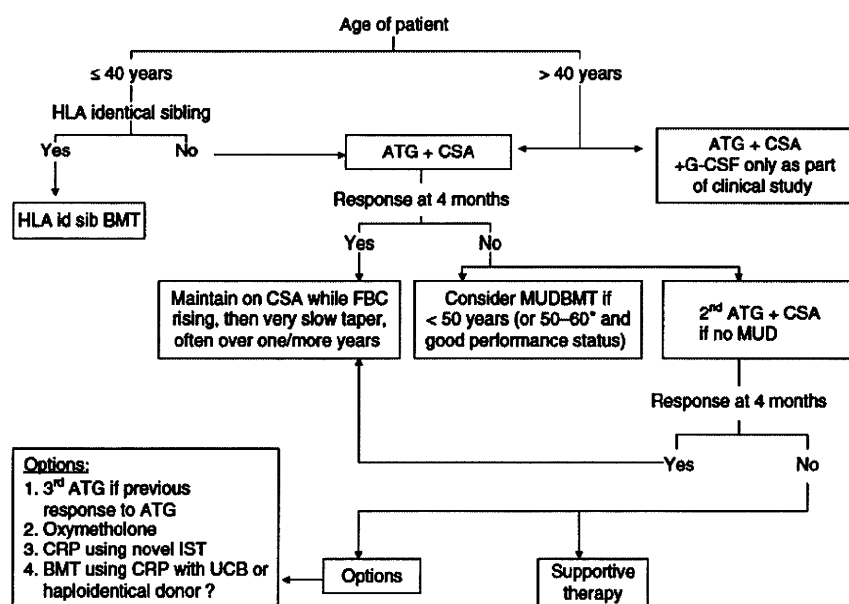
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## INTRODUCTION

Aplastic anemia is a marrow failure syndrome with an incidence of 2 per million in Western countries and 4-6 per million in Asia [1,2]. In the vast majority of patients, the disease results from T cell-mediated autoimmune destruction of marrow elements leading to life-threatening cytopenias. The preferred therapy for younger patients with severe aplastic anemia (SAA) is HLA-matched sibling allogeneic bone marrow transplantation (BMT), which results in long-term survival in 85% to 90% of recipients [3-5]. Only 20% to 30% of patients will have HLA-matched siblings, and some will not receive an upfront BMT approach because of patient choice, physician preference, or BMT access issues. Hence, most patients with SAA receive initial treatment with immunosuppressive therapy (IST), most commonly with a combination of antithymocyte globulin (ATG) and cyclosporine (CsA). Although 60% to 75% of patients respond with a decrease in or elimination of transfusion requirements, 10% to 35% of patients will relapse (require transfusions again), and the majority of patients will require long-term (5-year) therapy with CsA [6,7]. Others are at risk of clonal evolution to hemolytic paroxysmal nocturnal hemoglobinuria (PNH), myelodysplasia (MDS), or acute myeloid leukemia (AML) [8,9]. Well-matched unrelated donor (URD) BMT can be successful in patients failing immunosuppression, but because transplant-related mortality (TRM) and graft-versus-host disease (GVHD) are higher than with HLA-matched sibling BMT, there has been limited enthusiasm for this approach in the past.

With recent improvements in survival after URD BMT [10-12] SAA experts from the United Kingdom published guidelines recommending matched URD BMT if patients fail to respond to IST after 4-6 months (Figure 1) [13]. This approach is being adopted more widely in Europe and Japan, supported by a prospective study in Japanese pediatric patients [14]. Prospective validation of these guidelines in older adults is needed, and several key questions about URD transplantation in SAA remain: (1) It is not clear when to offer URD transplantation to patients who relapse after an initial response to IST, as most patients will respond to further treatment with IST. (2) Half of SAA patients will not have a well-matched URD, and the role of alternative donor procedures such as unrelated cord blood (UCB) or haploidentical related donor transplantation is unclear. (3) Finally, there are inadequate data regarding long-term quality of life after URD BMT for SAA. If survival is improved after transplant, does it come at a high cost?

Answering these questions is challenging, as it requires comprehensive tracking of patients from diagnosis through all therapies. Long-term follow up of SAA patients is especially important because many adverse events (MDS/AML) can occur 1-2 decades after diagnosis. In the United States, except in a few centers, long-term outcomes of patients with this rare disorder are not followed. The Center for Blood and Marrow Transplant Research (CIBMTR) collects long-term outcome data on the minority of patients who undergo BMT, but data collected regarding therapies prior to transplantation is often inadequate to address many issues. More importantly, there is no effective



**Figure 1.** Treatment of acquired severe aplastic anemia according to United Kingdom Guidelines [13]. ATG, antithymocyte globulin; CSA, cyclosporine; FBC, full blood count (or CBC); MUD, matched unrelated donor; CRP, clinical research protocol; IST, immunosuppressive therapy; UCB, umbilical cord blood.



mechanism to compare outcomes of BMT recipients with comparable patients receiving IST approaches.

In view of these challenges, an ad hoc SAA Committee was formed by the Steering Committee of the Blood and Marrow Transplant Clinical Trials Network (BMT CTN), a program sponsored by the National Heart, Lung, and Blood Institute and the National Cancer Institute, to consider potential research strategies in this disease. The Committee convened a working group of international experts in March 2010 in Bethesda, Maryland, in conjunction with an educational and scientific meeting sponsored by the Aplastic Anemia and Myelodysplastic Syndrome International Foundation. The purpose of the working group was to: (1) define the most pressing questions in the basic science and therapy of SAA that could be addressed through clinical trials; (2) establish an approach to identify biologic and clinical parameters of SAA that define risk, both with IST and BMT; and (3) initiate a process that will result in the identification of rational intervention points, where URD and alternative donor BMT approaches can be compared with IST.

The conclusions of this working group are summarized below.

### New Insights into SAA Biology, Key Issues for Study

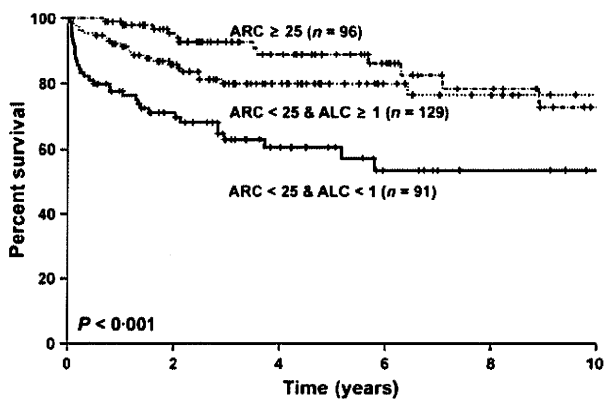
Idiopathic SAA patients have immune-mediated oligoclonal expansion of cytotoxic T cells targeting hematopoietic stem and progenitor cells. These T cells have a Th1 profile and secrete interferon- $\gamma$  [15]; potentially relevant polymorphisms in genes associated with an increased immune response have been identified [16]. Regulatory T cells (T-regs) are decreased in almost all patients with SAA [17], and infusion of T-regs abrogates lymphocyte-induced marrow dysplasia in mouse models [18].

A notable observation in a portion of patients with SAA is the presence of shortened telomeres [19,20]. Mutations of the telomerase enzyme complex (*TERT*, *TERC*, *DKC1*, *NOP10*, or *NHP2*) or in the shelterin telomere protection complex (*TINF2*) form the basis for the inherited marrow failure disorder dyskeratosis congenita. Just under 10% of SAA patients will have a mutation in either *TERT* or *TERC*. A smaller percentage of patients with SAA and no other clinical phenotype will have a mutation in *TINF2*. Genetic variants in *TERF1* may also contribute to risk of SAA, although to a lesser extent [21]. All of these genes are thought to contribute to telomere erosion, increasing risk of marrow failure and malignant transformation. Although telomere length does not predict response to immunosuppression in SAA patients (as opposed to dyskeratosis congenita patients who do not respond to IST), retrospective studies show that SAA patients with shorter telomeres at diagnosis are at higher risk of

relapse after IST and are also more likely to undergo clonal evolution to MDS or AML [22].

The impact of telomere dysfunction on BMT outcomes in SAA is not known. Patients with dyskeratosis congenita have a high incidence of organ toxicity, most notably hepatic and pulmonary, after BMT [23-25]. In telomerase knockout mice (*Terc*<sup>-/-</sup>), short and dysfunctional telomeres preclude appropriate engraftment of donor wild-type hematopoietic stem cells, possibly because of poor stromal function [26]. A large study correlating telomere length with engraftment, toxicity, and survival in patients who received unrelated donor BMT for SAA over the past decade is currently underway through the National Marrow Donor Program (NMDP) and CIBMTR. Although this study may define putative risks associated with shorter telomeres during URD BMT, prospective studies will be needed to test the applicability of these associations with modern BMT therapy.

Until recently, laboratory-based predictive biomarkers for IST response in SAA were lacking. Scheinberg [27] and the NIH group correlated absolute reticulocyte count (ARC) and absolute lymphocyte count (ALC) at initial diagnosis with response, identifying groups at low and higher risk of failure and early mortality (Figure 2). Further investigation showed that ARC combined with telomere length had better predictive power than either biomarker alone. Patients with both high ARC and longer telomeres appear to have excellent outcomes, whereas those with low ARC and shorter telomeres do poorly; patients with only 1 of the 2 adverse factors had intermediate outcomes [28]. Important follow-up questions to address include: (1) does the prognostic ability of these assays hold up in a prospective multicenter cohort; and (2) can intervention with URD BMT improve survival of patients with low ARC and shorter telomeres compared to IST? Other important goals for future trials are discovery of additional biologic factors with



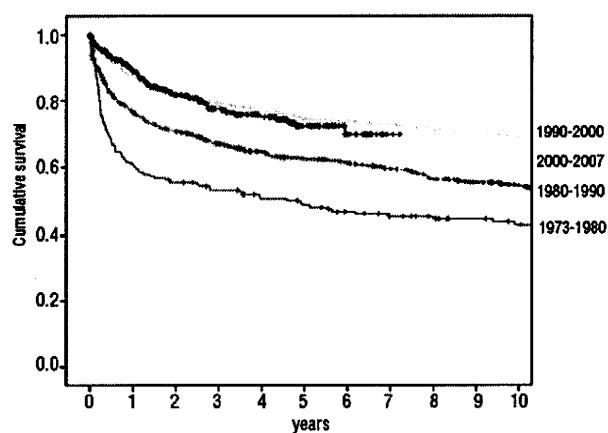
**Figure 2.** Probability of survival in patients treated with IST who had high versus low absolute reticulocyte counts (ARC) and high versus low absolute lymphocyte counts (ALC). Patients undergoing BMT were censored at the time of transplant [27].

prognostic value (cytokine polymorphism profiles, single nucleotide polymorphism [SNP-A] genotypes, etc.), or identification of genetic aberrations that contribute to the pathophysiology of SAA.

### Advances in Immunosuppression and Supportive Care: Next Steps

Initial therapy of SAA with horse ATG and CsA, standard for more than 2 decades, results in response rates of 50% at 3 months and 60% to 75% at 6 months [29-31]. A second course of rabbit ATG given after a minimum of 3 months may lead to response in about a third of patients who do not respond to the first course [32]. Among patients who respond initially but later relapse, most will have some response to subsequent courses of immunosuppressive therapy. Slowing the rate of taper of CsA appears to decrease the likelihood or delay the onset of relapse [33].

Over the past decade, researchers sought to increase initial response rates by increasing the intensity of IST through the addition of mycophenolate mofetil (MMF), sirolimus, or other agents to ATG/CsA [34,35]. These efforts were not successful, suggesting that even intense IST is insufficient to abrogate the autoimmune aggression in some patients, or that some of patients have more severe destruction of hematopoietic progenitors resulting in worse marrow reserve and insufficient stem cells to support renewed blood cell production after abrogating the autoimmune response. The possibility that we have reached a ceiling in the percentage of patients with the capacity to respond to immunosuppression was raised. Consistent with this idea, the EBMT group reported that although significant improvements in survival after IST occurred over each decade between the 1970s and the 1990s, unfortunately, survival of patients treated between 2000 and 2007 has remained unchanged compared to those treated between 1990 and 2000 (Figure 3) [36].



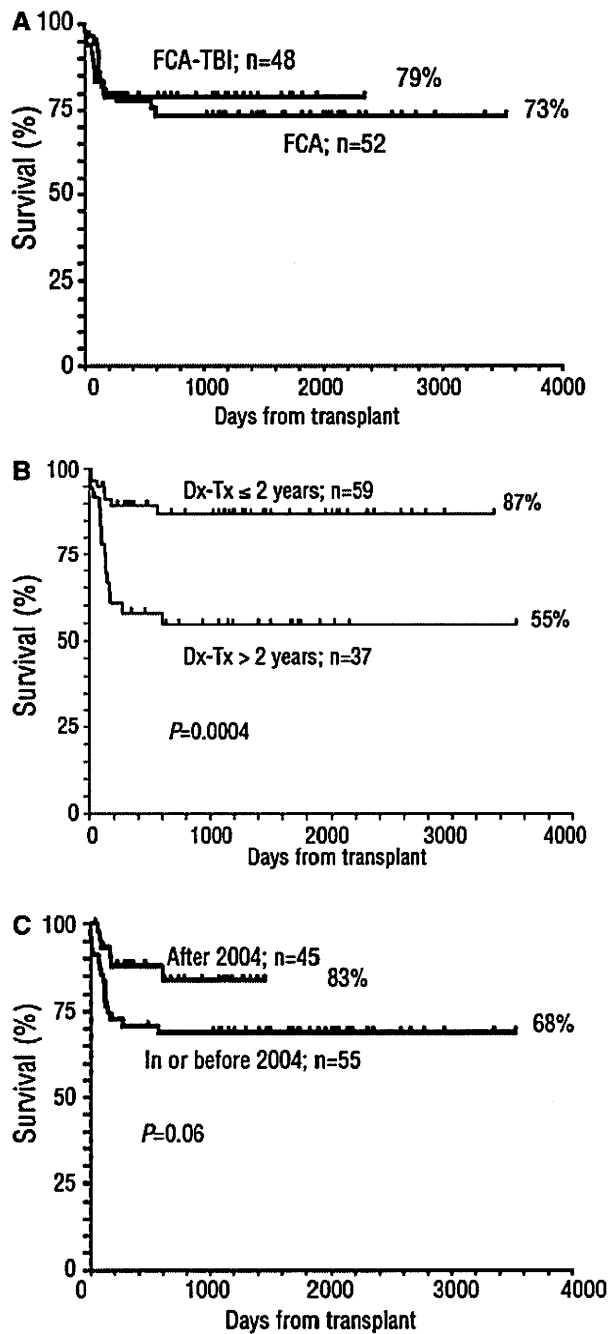
**Figure 3.** Survival among patients with severe aplastic anemia treated with ATG-based immunosuppression reported to the EBMT database (n = 2400) [36].

In the context of this lack of improvement in response to IST, what agents or approaches might improve survival or patient quality of life in the future? A randomized study of 120 patients in the United States comparing horse ATG with rabbit ATG (NCT00260689) has completed accrual and will soon offer insights into the quality and length of response with these 2 agents. A few pilot studies show responses to alemtuzumab, although this highly immunosuppressive agent requires attentive supportive care measures to avoid life-threatening infectious complications [37]. Other new immunosuppressive agents will be tested in patients with relapse of SAA to establish efficacy and toxicity. Finally, investigators at Johns Hopkins University using high-dose cyclophosphamide (Cy) without stem cell rescue have demonstrated a high response rate with relatively low toxicity in newly diagnosed patients [38]. The use of high-dose Cy by other groups was associated with high rates of early and late toxicities, leading to closure of randomized trials examining this approach [39]. There is recent renewed interest in this agent as investigators in China have shown high rates of response with manageable toxicity using lower doses of Cy than used by the Hopkins group [40].

### Improvements in BMT Outcomes: A Case for Earlier Intervention?

Survival after HLA-matched sibling BMT in patients with SAA less than 30 years old has exceeded 80% for the past 20 years, making this the preferred approach for these patients. In the last decade, survival of older BMT recipients improved significantly. Several factors likely contributed to this improvement. An EBMT analysis of HLA-matched sibling BMT outcomes in patients older than 30 years showed a statistically significant improvement in survival when a fludarabine (Flu)/Cy/ATG preparative regimen was used, compared with traditional Cy/ATG approaches. Five-year survival in the Flu/Cy/ATG cohort was 77%, compared to 60% in the Cy/ATG group, and patients between the ages of 30 and 40 years had a survival probability exceeding 80% [41].

Survival after URD BMT also improved dramatically in recent years (from 30%-40% in the 1990s [42] to 70%-80% currently [11]). EBMT data using Flu/Cy/ATG  $\pm$  low-dose total-body irradiation (TBI) showed that improvement was especially notable after 2004, and that patients have the best chance of survival after BMT when they undergo the procedure within 2 years of diagnosis (Figure 4). Unpublished data from the CIBMTR using similar approaches show that 2-year survival rates after 8/8 HLA-matched (using high-resolution typing) URD BMT for SAA exceeds 80% (personal communication, M. Eapen, CIBMTR). There are many possible reasons



**Figure 4.** Outcomes of URD BMT for SAA using fludarabine/cyclophosphamide/ATG ± low dose TBI reported to the EBMT. (A) Survival after Flu/Cy/ATG with TBI (median age 27 [7-53 years] versus Flu/Cy/ATG (median age 13 [3-51 years]). (B) Survival of patients transplanted ≤2 years from diagnosis versus those receiving transplantation later in their disease course. (C) Survival of patients transplanted in the most recent era (after 2004 versus those transplanted earlier) [11].

for these improvements: the advent of molecular HLA typing resulting in better HLA matching, modern supportive care, and optimization of reduced-intensity conditioning (RIC) approaches [12]. Patient selection is also a factor. In early studies, BMT was only offered to high-risk patients who had failed multiple rounds of

IST. There is now a tendency to offer BMT earlier in the course of therapy [11,12]. Patients undergoing transplantation earlier in their disease course are more likely to begin the procedure with a history of fewer infections and with a lower likelihood of iron overload, renal dysfunction from long-term CsA, transfusion-induced alloimmunization (which can increase risk of rejection), or platelet refractoriness.

The major focus of recent clinical trials in URD BMT for SAA has been optimizing preparative regimens to allow sustained engraftment while minimizing regimen-related toxicity. A study published by Deeg et al. [12] evaluated de-escalation of TBI doses and demonstrated better survival in patients receiving Cy (200 mg/kg)/ATG plus 200 cGy TBI compared to higher TBI doses. Five-year survival probabilities after HLA-matched URD BMT using the regimen containing 200 cGy of TBI were 78% for patients 20 years of age or younger and 50% for older patients. A second optimization trial is currently underway under the auspices of the BMT CTN. This trial (BMT CTN 0301; NCT00326417) is designed to determine the optimal dose of cyclophosphamide (0, 50, 100, 150 mg/kg) when given in combination with Flu, ATG, and a single dose of TBI (200 cGy). The 0- and 150-mg levels have been closed because of rejection and toxicity, respectively. The trial continues to accrue patients at the 50- and 100-mg dose levels. Thus, this type of conditioning regimen should be considered investigational, and caution should be exercised when selecting the cyclophosphamide dose in this setting.

There is some concern about TBI-based regimens increasing the risk of malignancies after URD BMT for SAA. Studies demonstrating an increased risk of second malignancies after related donor BMT with TBI-based regimens were published in the early 1990s. Those studies involved patients treated in the 1970-1980s using TBI doses >900 cGy or total abdominal irradiation (TAI) doses of 5-600 cGy [43,44]. Whether current approaches using a single dose of 200 cGy of TBI increase the risk of posttransplant malignancies is unknown. Long-term follow-up of these patients is very important.

The dramatic improvement in survival rates after URD BMT that has occurred over the past decade has raised an important question. In the context of current therapy, when should patients with SAA be offered URD transplantation? Although many groups now support offering this approach after failure of first IST, when should it be offered after subsequent failures? Can biologic risk factors for failure of IST help determine the timing of URD BMT? What level of HLA typing and matching is required? When can alternative graft sources (cord blood, haploidentical donors) be used? What is the quality of life of survivors after URD or alternative donor transplantation? Some insight into URD transplantation of younger

patients failing to respond to their initial round of IST was provided by Kosaka et al. [14]. In this study, 201 pediatric patients with SAA lacking HLA-matched sibling donors were treated initially with IST. Of 60 patients who failed to respond at 6 months, 31 underwent URD BMT if they had matched URDs (25 patients), single antigen-mismatched related donors (4 patients), or single antigen-mismatched cord blood units (2 patients). Patients who did not have donors received subsequent rounds of IST. Although overall survival (OS) at 5 years was not different between the transplantation and IST groups, failure-free survival was dramatically better in the BMT group at 84% versus 9% ( $P = .001$ ), and the majority of patients treated with subsequent courses of IST had continuing marrow failure.

An additional issue is the availability of suitable HLA-matched donors. Only about 70% of Caucasian patients will find a fully matched and available URD; patients from ethnic groups such as Hispanic, Black, or Asian-Pacific islander will find a fully matched and available donor less than half of the time [45]. Cord blood transplantation, which allows greater degrees of donor-recipient HLA-match, might be considered for patients without a suitable adult donor, but published experience from Japan and unpublished CIBMTR and European Group for Blood and Marrow Transplantation (EBMT) data show high rates of rejection and survival rates of less than 50% after cord blood transplantation for SAA [46]. Some small studies using combinations of Flu/Cy/ATG/TBI for conditioning show more promising survival rates after cord blood transplantation [47], but larger validation studies are required. Some groups have explored the feasibility of haploidentical transplants, but reports to date are anecdotal [48]. Novel approaches that improve survival with the use of cord blood or haploidentical donors are needed to allow all patients who do not have good options with IST to undergo transplantation.

### Should Age Determine the Choice of Immunosuppression versus BMT?

Age is a significant factor in both IST and BMT outcomes, with higher chances of failure and mortality, especially in patients >40 years of age. An analysis published in 1999 showed that the response rates to ATG/CsA IST among patients aged >60, 50-59, and <50 years were 37%, 49%, and 57%, respectively; corresponding 5-year survival rates were 50%, 57%, and 72% [49]. In the decade since this analysis was published, response rates to IST have not changed substantially, but there has been a steady improvement in supportive care leading to increased survival after both IST and BMT. Rates of GVHD are higher in older patients, however, and remain a major barrier

to successful outcomes. Whether URD BMT can offer a survival advantage over IST in older patients is unclear; however, older patients failing IST may benefit from BMT approaches aimed at reducing GVHD while maintaining donor engraftment.

### Longitudinal and Long-Term Outcomes: Vital Questions and Proposed Approaches

There are several barriers to advancing care of patients with SAA. First, the disease is rare. Only about 600 new cases/year are expected in the United States. This makes all types of studies difficult because any given center will only have a handful of patients. Second, the natural history of the disease plays out over more than a decade, with some patients failing multiple courses of therapy, but still surviving for 5 to 10 years, and others developing late-onset secondary MDS/AML. Third, referral to centers with specific expertise in SAA is sporadic and varies by patient location. Referral is important to have the latest testing performed to rule out inherited bone marrow failure syndromes, hypoplastic MDS, and other conditions that mimic SAA, and to enroll patients in studies. Fourth, timing and indications for referral for transplant vary considerably among centers, with many patients delayed excessively and referred to BMT only after they have developed active infections, significant transfusion burden, alloimmunization, and/or platelet refractoriness. Finally, because patients with SAA may be seen by local physicians or hematologists, referred to a regional academic center, and then referred a second time for BMT evaluation, it is difficult to follow patients from diagnosis through all of their therapeutic courses with an observation period sufficient to understand their long-term outcomes. In the United States, particularly, a mechanism to track patients through multiple care providers is lacking.

The SAA working group agreed that advances in biology and BMT survival warrant a series of initiatives to better understand the appropriate roles and timing of IST and BMT in treating SAA. One important effort that would greatly assist in moving the field forward would be to create a mechanism for identifying a high percentage of SAA patients at diagnosis, obtaining blood specimens to evaluate prognostic biomarkers, and following these patients through their treatment courses. Biologic samples for later studies could be obtained, with appropriate consent, and an SAA repository established. Patients could be offered participation in studies of related donor BMT, IST, URD BMT, and alternative donor BMT as they became eligible for such studies. BMT timing would be determined by patient age, the availability of well-matched related or unrelated donors, response to IST, and risk as determined by biologic markers. All patients would be followed long term, and quality-of-life studies

could carefully compare outcomes of surviving patients receiving URD BMT versus IST.

The working group felt that the most appropriate way to improve care and enroll patients into a large registry study would be to designate regional centers of excellence, where comprehensive evaluations, including key biologic assessments (telomere studies, etc.), could be performed at diagnosis and other key time points. Vital to this process is early assurance that the diagnosis of SAA is correct. A subcommittee headed by Dr. Richard Harris was appointed to assemble comprehensive diagnostic guidelines including tests to rule out inherited BM failure syndromes and other non-SAA diseases. Because the therapy of SAA patients sometimes involves transplantation, and the CIBMTR currently has a large database of information on patients receiving BMT, the CIBMTR is a possible choice to manage an SAA registry or longitudinal observational study. Trials of biology, IST, and BMT could be facilitated by a population-based SAA outcomes registry by identifying potential study subjects; patients would benefit by having wider access to studies of relevance to them. Patients in the registry could also be targeted for distribution of educational and support materials. The working group plans to seek funding from patient advocacy groups, private corporations, and governmental funding sources to establish a population-based outcomes registry and accompanying biologic sample repository to facilitate studies to address many of the issues discussed in this document.

The important questions in the therapy of acquired SAA can be addressed most effectively with collaboration among transplantation physicians, hematologists interested in IST, and scientists studying the biology of marrow failure. The relevant issues are interdependent. For instance, can telomere length and telomerase complex mutations help predict whether patients will fail IST and should, therefore, seek early transplantation therapy? Can selected biological factors (cytokine profiles, etc.) better define clinical risk groups? If we can define patients at high risk of IST failure, do the same or different factors predict outcomes after BMT? If a patient either fails to respond to IST or relapses afterward, can new agents induce or prolong responses (alemtuzumab, etc.)? Can cyclophosphamide, given at a modified dose, improve the durability of initial responses without excessive early morbidity? Finally, we need to know whether novel strategies for URD BMT that decrease GVHD and maintain engraftment can be developed and performed safely with reasonable survival in older patients. Can alternative donor grafts be used successfully so that more patients are able to go to transplantation if immunosuppression is unsuccessful?

These questions can be directly addressed through the proposed SAA registry/biology study, because at

given time points (first or subsequent failure of IST, etc.), patients who have appropriate donors and go to BMT can be compared with similar patients who undergo IST. Questions regarding access to BMT (inability to get BMT because of insurance, etc.) can be addressed by the registry study as well. Transplantation studies could be performed through the BMT CTN in cooperation with international study groups as needed to allow for sufficient accrual or to rapidly test highly promising ideas.

## CONCLUSIONS

Treatment for SAA has advanced in the past decade, most notably with (1) the development of biological measures that may allow clinical risk classification, and (2) improvement in survival after HLA-matched URD BMT. Creation of an SAA outcomes registry and specimen repository would allow investigators to follow patients from diagnosis through all of their therapies, would facilitate better studies comparing specific therapies, and thus may help optimize timing of URD BMT for patients failing IST. Cooperation among hematologists, transplant physicians, and basic scientists in the study and treatment of SAA patients should speed advances in clinical care and improve outcomes.

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## REFERENCES

1. Issaragrisil S, Kaufman DW, Anderson T, et al. The epidemiology of aplastic anemia in Thailand. *Blood*. 2006;107:1299-1307.
2. Young NS, Calado RT, Scheinberg P. Current concepts in the pathophysiology and treatment of aplastic anemia. *Blood*. 2006;108:2509-2519.
3. Marsh JC. Management of acquired aplastic anaemia. *Blood Rev*. 2005;19:143-151.
4. Schrezenmeier H, Passweg JR, Marsh JC, et al. Worse outcome and more chronic GVHD with peripheral blood progenitor cells than bone marrow in HLA-matched sibling donor transplants for young patients with severe acquired aplastic anemia. *Blood*. 2007;110:1397-1400.
5. Kahl C, Leisenring W, Deeg HJ, et al. Cyclophosphamide and antithymocyte globulin as a conditioning regimen for allogeneic marrow transplantation in patients with aplastic anaemia: a long-term follow-up. *Br J Haematol*. 2005;130:747-751.
6. Schrezenmeier H, Marin P, Raghavachar A, et al. Relapse of aplastic anaemia after immunosuppressive treatment: a report from the European Bone Marrow Transplantation Group SAA Working Party. *Br J Haematol*. 1993;85:371-377.

7. Bacigalupo A, Bruno B, Saracco P, et al. Antilymphocyte globulin, cyclosporine, prednisolone, and granulocyte colony-stimulating factor for severe aplastic anemia: an update of the GITMO/EBMT study on 100 patients. European Group for Blood and Marrow Transplantation (EBMT) Working Party on Severe Aplastic Anemia and the Gruppo Italiano Trapianti di Midollo Osseo (GITMO). *Blood*. 2000;95:1931-1934.
8. Socie G, Rosenfeld S, Frickhofen N, Gluckman E, Tichelli A. Late clonal diseases of treated aplastic anemia. *Semin Hematol*. 2000;37:91-101.
9. Frickhofen N, Heimpel H, Kaltwasser JP, Schrezenmeier H. Antithymocyte globulin with or without cyclosporin A: 11-year follow-up of a randomized trial comparing treatments of aplastic anemia. *Blood*. 2003;101:1236-1242.
10. Bacigalupo A, Locatelli F, Lanino E, Marsh J, Socie G, Passweg J. Fludarabine, cyclophosphamide with or without low dose TBI for alternative donor transplants in acquired aplastic anemia (SAA): a report from the EBMT-SAA Working Party. *Biol Blood Marrow Transplant*. 2009;15:5.
11. Bacigalupo A, Socie G, Lanino E, et al. Fludarabine, cyclophosphamide, antithymocyte globulin, with or without low dose total body irradiation, for alternative donor transplants, in acquired severe aplastic anemia: a retrospective study from the EBMT-SAA working party. *Haematologica*. 2010;95:976-982.
12. Deeg HJ, O'Donnell M, Tolar J, et al. Optimization of conditioning for marrow transplantation from unrelated donors for patients with aplastic anemia after failure of immunosuppressive therapy. *Blood*. 2006;108:1485-1491.
13. Marsh JC, Ball SE, Cavenagh J, et al. Guidelines for the diagnosis and management of aplastic anaemia. *Br J Haematol*. 2009;147:43-70.
14. Kosaka Y, Yagasaki H, Sano K, et al. Prospective multicenter trial comparing repeated immunosuppressive therapy with stem-cell transplantation from an alternative donor as second-line treatment for children with severe and very severe aplastic anemia. *Blood*. 2008;111:1054-1059.
15. Sloand E, Kim S, Maciejewski JP, Tisdale J, Follmann D, Young NS. Intracellular interferon-gamma in circulating and marrow T cells detected by flow cytometry and the response to immunosuppressive therapy in patients with aplastic anemia. *Blood*. 2002;100:1185-1191.
16. Gidvani V, Ramkissoon S, Sloand EM, Young NS. Cytokine gene polymorphisms in acquired bone marrow failure. *Am J Hematol*. 2007;82:721-724.
17. Solomou EE, Rezvani K, Mielke S, et al. Deficient CD4+ CD25+ FOXP3+ T regulatory cells in acquired aplastic anemia. *Blood*. 2007;110:1603-1606.
18. Chen J, Ellison FM, Eckhaus MA, et al. Minor antigen h60-mediated aplastic anemia is ameliorated by immunosuppression and the infusion of regulatory T cells. *J Immunol*. 2007;178:4159-4168.
19. Ball SE, Gibson FM, Rizzo S, Tooze JA, Marsh JC, Gordon-Smith EC. Progressive telomere shortening in aplastic anemia. *Blood*. 1998;91:3582-3592.
20. Brummendorf TH, Maciejewski JP, Mak J, Young NS, Lansdorp PM. Telomere length in leukocyte subpopulations of patients with aplastic anemia. *Blood*. 2001;97:895-900.
21. Calado RT, Young NS. Telomere maintenance and human bone marrow failure. *Blood*. 2008;111:4446-4455.
22. Calado RT. Telomeres and marrow failure. *Hematology Am Soc Hematol Educ Program*. 2009;338-343.
23. de la Fuente J, Dokal I. Dyskeratosis congenita: advances in the understanding of the telomerase defect and the role of stem cell transplantation. *Pediatr Transplant*. 2007;11:584-594.
24. Dietz AC, Orchard PJ, Baker KS, et al. Disease-specific hematopoietic cell transplantation: nonmyeloablative conditioning regimen for dyskeratosis congenita. *Bone Marrow Transplant*. 2010 Apr 12. [E-pub ahead of print].
25. Nobili B, Rossi G, De Stefano P, et al. Successful umbilical cord blood transplantation in a child with dyskeratosis congenita after a fludarabine-based reduced-intensity conditioning regimen. *Br J Haematol*. 2002;119:573-574.
26. Ju Z, Jiang H, Jaworski M, et al. Telomere dysfunction induces environmental alterations limiting hematopoietic stem cell function and engraftment. *Nat Med*. 2007;13:742-747.
27. Scheinberg P, Wu CO, Nunez O, Young NS. Predicting response to immunosuppressive therapy and survival in severe aplastic anaemia. *Br J Haematol*. 2009;144:206-216.
28. Scheinberg P, Cooper J, Sloand E, Wu C, Calado R, Young N. Association of telomere length of peripheral blood leukocytes with hematopoietic relapse, malignant transformation, and survival in severe aplastic anemia. *JAMA*. 304:1358-1364.
29. Rosenfeld SJ, Kimball J, Vining D, Young NS. Intensive immunosuppression with antithymocyte globulin and cyclosporine as treatment for severe acquired aplastic anemia. *Blood*. 1995;85:3058-3065.
30. Frickhofen N, Kaltwasser JP, Schrezenmeier H, et al. Treatment of aplastic anemia with antilymphocyte globulin and methylprednisolone with or without cyclosporine. The German Aplastic Anemia Study Group. *N Engl J Med*. 1991;324:1297-1304.
31. Marsh J, Schrezenmeier H, Marin P, et al. Prospective randomized multicenter study comparing cyclosporin alone versus the combination of antithymocyte globulin and cyclosporin for treatment of patients with nonsevere aplastic anemia: a report from the European Blood and Marrow Transplant (EBMT) Severe Aplastic Anaemia Working Party. *Blood*. 1999;93:2191-2195.
32. Scheinberg P, Nunez O, Young NS. Retreatment with rabbit anti-thymocyte globulin and cyclosporin for patients with relapsed or refractory severe aplastic anaemia. *Br J Haematol*. 2006;133:622-627.
33. Saracco P, Quarello P, Iori AP, et al. Cyclosporin A response and dependence in children with acquired aplastic anaemia: a multicentre retrospective study with long-term observation follow-up. *Br J Haematol*. 2008;140:197-205.
34. Scheinberg P, Nunez O, Wu C, Young NS. Treatment of severe aplastic anaemia with combined immunosuppression: antithymocyte globulin, cyclosporin and mycophenolate mofetil. *Br J Haematol*. 2006;133:606-611.
35. Scheinberg P, Wu CO, Nunez O, Boss C, Sloand EM, Young NS. Treatment of severe aplastic anemia with a combination of horse antithymocyte globulin and cyclosporine, with or without sirolimus: a prospective randomized study. *Haematologica*. 2009;94:348-354.
36. Passweg JR, Tichelli A. Immunosuppressive treatment for aplastic anemia: are we hitting the ceiling? *Haematologica*. 2009;94:310-312.
37. Risitano AM, Selleri C, Serio B, et al. Alemtuzumab is safe and effective as immunosuppressive treatment for aplastic anaemia and single-lineage marrow failure: a pilot study and a survey from the EBMT WPSAA. *Br J Haematol*. 2010;148:791-796.
38. Brodsky RA, Chen AR, Dorr D, et al. High-dose cyclophosphamide for severe aplastic anemia: long-term follow-up. *Blood*. 2010;115:2136-2141.
39. Tisdale JF, Maciejewski JP, Nunez O, Rosenfeld SJ, Young NS. Late complications following treatment for severe aplastic anemia (SAA) with high-dose cyclophosphamide (Cy): follow-up of a randomized trial. *Blood*. 2002;100:4668-4670.
40. Li Z, Yin S, Xie S, Ma L, Nie D, Xsu L. Treatment of severe aplastic anemia using high-dose cyclophosphamide alone in China. *Haematologica*. 2000;85:E06.
41. Maury S, Bacigalupo A, Anderlini P, et al. Improved outcome of patients older than 30 years receiving HLA-identical sibling hematopoietic stem cell transplantation for severe acquired aplastic anemia using fludarabine-based conditioning: a comparison with conventional conditioning regimen. *Haematologica*. 2009;94:1312-1315.
42. Passweg JR, Perez WS, Eapen M, et al. Bone marrow transplants from mismatched related and unrelated donors for severe aplastic anemia. *Bone Marrow Transplant*. 2006;37:641-649.

43. Deeg HJ, Socie G, Schoch G, et al. Malignancies after marrow transplantation for aplastic anemia and fanconi anemia: a joint Seattle and Paris analysis of results in 700 patients. *Blood*. 1996;87:386-392.
44. Socie G, Henry-Amar M, Bacigalupo A, et al. Malignant tumors occurring after treatment of aplastic anemia. European Bone Marrow Transplantation-Severe Aplastic Anaemia Working Party. *N Engl J Med*. 1993;329:1152-1157.
45. Kollman C, Abella E, Baitty RL, et al. Assessment of optimal size and composition of the U.S. National Registry of hematopoietic stem cell donors. *Transplantation*. 2004;78:89-95.
46. Yoshimi A, Kojima S, Taniguchi S, et al. Unrelated cord blood transplantation for severe aplastic anemia. *Biol Blood Marrow Transplant*. 2008;14:1057-1063.
47. Chan KW, McDonald L, Lim D, Grimley MS, Grayson G, Wall DA. Unrelated cord blood transplantation in children with idiopathic severe aplastic anemia. *Bone Marrow Transplant*. 2008;42:589-595.
48. Lacerda JF, Martins C, Carmo JA, et al. Haploidentical stem cell transplantation with purified CD34+ cells after a chemotherapy-alone conditioning regimen in heavily transfused severe aplastic anemia. *Biol Blood Marrow Transplant*. 2005;11:399-400.
49. Tichelli A, Socie G, Henry-Amar M, et al. Effectiveness of immunosuppressive therapy in older patients with aplastic anemia. European Group for Blood and Marrow Transplantation Severe Aplastic Anaemia Working Party. *Ann Intern Med*. 1999;130:193-201.

## Recent progress in dyskeratosis congenita

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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 multi-system 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 led 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

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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*)

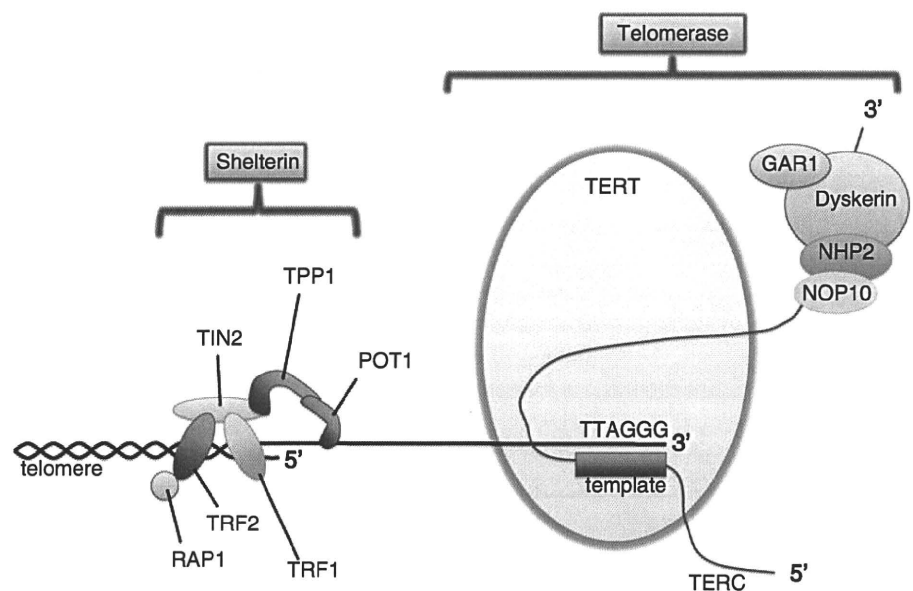
[22, 23], *NOP10* [24], and *NHP2* [25] in patients with rare autosomal recessive DC. Mutations of *TERT* were also reported in the autosomal dominant family [8]. More recently, heterozygous mutations of *TINF2* encoding TIN2, main component of shelterin which protects telomeres, have been identified 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).

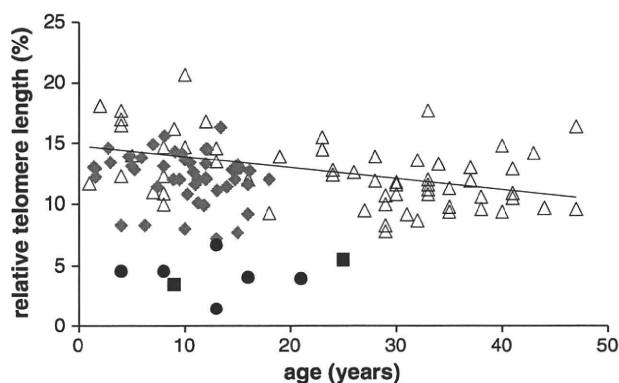
Among three methods of measuring telomere length, including southern blot, real-time polymerase chain reaction, and flow cytometry and fluorescence in situ

**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]	<i>TERC</i>	2/17 (12%)
Vulliamy et al. [8]	<i>TERT</i>	2/80 (2.5%)
Yamaguchi et al. [9]	<i>TERC</i>	2/150 (1.3%)
Yamaguchi et al. [7]	<i>TERT</i>	7/200 (3.5%)
Savage et al. [50]	<i>TERF1</i>	1/47 (2.1%)
	<i>TERF2</i>	1/47 (2.1%)
Liang et al. [6]	<i>TERT</i>	2/96 (2.1%)
Walne et al. [51]	<i>TINF2</i>	2/111 (1.8%)
Du et al. [3]	<i>TERT</i>	4/199 (2.0%)
Du et al. [4]	<i>TINF2</i>	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

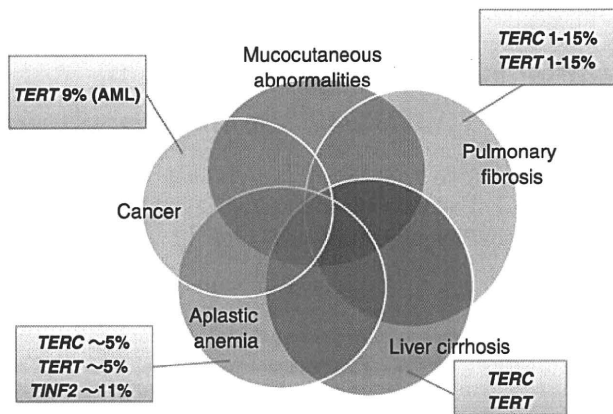
marrow failure syndromes. Although short telomeres are also found in patients with other congenital bone marrow failure syndromes, such as Shwachman–Diamond syndrome and Fanconi anemia, telomere length in patients with DC is the shortest compared with other bone marrow failure syndromes. In fact, telomere 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.

## References

- Greider CW, Blackburn EH. Identification of a specific telomere terminal transferase activity in *Tetrahymena* extracts. *Cell*. 1985;43:405–13.
- Szostak JW, Blackburn EH. Cloning yeast telomeres on linear plasmid vectors. *Cell*. 1982;29:245–55.
- Du HY, Pumbo E, Ivanovich J, An P, Maziarz RT, Reiss UM, et al. *TERC* and *TERT* gene mutations in patients with bone marrow failure and the significance of telomere length measurements. *Blood*. 2009;113:309–16.
- Du HY, Mason PJ, Bessler M, Wilson DB. *TINF2* mutations in children with severe aplastic anemia. *Pediatr Blood Cancer*. 2009;52:687.
- Walne AJ, Dokal I. Dyskeratosis congenita: a historical perspective. *Mech Ageing Dev*. 2008;129:48–59.
- Liang J, Yagasaki H, Kamachi Y, Hama A, Matsumoto K, Kato K, et al. Mutations in telomerase catalytic protein in Japanese children with aplastic anemia. *Haematologica*. 2006;91:656–8.
- Yamaguchi H, Calado RT, Ly H, Kajigaya S, Baerlocher GM, Chanock SJ, et al. Mutations in *TERT*, the gene for telomerase reverse transcriptase, in aplastic anemia. *N Engl J Med*. 2005;352:1413–24.
- Vulliamy TJ, Walne A, Baskaradas A, Mason PJ, Marrone A, Dokal I. Mutations in the reverse transcriptase component of telomerase (*TERT*) in patients with bone marrow failure. *Blood Cells Mol Dis*. 2005;34:257–63.
- Yamaguchi H, Baerlocher GM, Lansdorp PM, Chanock SJ, Nunez O, Sloand E, et al. Mutations of the human telomerase RNA gene (*TERC*) in aplastic anemia and myelodysplastic syndrome. *Blood*. 2003;102:916–8.
- Vulliamy T, Marrone A, Dokal I, Mason PJ. Association between aplastic anaemia and mutations in telomerase RNA. *Lancet*. 2002;359:2168–70.
- Tsakiri KD, Cronkhite JT, Kuan PJ, Xing C, Raghu G, Weissler JC, et al. Adult-onset pulmonary fibrosis caused by mutations in telomerase. *Proc Natl Acad Sci USA*. 2007;104:7552–7.
- Armanios MY, Chen JJ, Cogan JD, Alder JK, Ingersoll RG, Markin C, et al. Telomerase mutations in families with idiopathic pulmonary fibrosis. *N Engl J Med*. 2007;356:1317–26.
- Calado RT, Regal JA, DE Kleiner, Schrupp DS, Peterson NR, Pons V, et al. A spectrum of severe familial liver disorders associate with telomerase mutations. *PLoS ONE*. 2009;4:e7926.
- Walne AJ, Marrone A, Dokal I. Dyskeratosis congenita: a disorder of defective telomere maintenance? *Int J Hematol*. 2005;82:184–9.
- Kirwan M, Dokal I. Dyskeratosis congenita, stem cells and telomeres. *Biochim Biophys Acta*. 2009;1792:371–9.
- Vulliamy TJ, Marrone A, Knight SW, Walne A, Mason PJ, Dokal I. Mutations in dyskeratosis congenita: their impact on telomere length and the diversity of clinical presentation. *Blood*. 2006;107:2680–5.
- Dokal I. Dyskeratosis congenita in all its forms. *Br J Haematol*. 2000;110:768–79.
- Walne AJ, Dokal I. Advances in the understanding of dyskeratosis congenita. *Br J Haematol*. 2009;145:164–72.
- Heiss NS, Knight SW, Vulliamy TJ, Klauck SM, Wiemann S, Mason PJ, et al. X-linked dyskeratosis congenita is caused by mutations in a highly conserved gene with putative nucleolar functions. *Nat Genet*. 1998;19:32–8.
- Mitchell JR, Wood E, Collins K. A telomerase component is defective in the human disease dyskeratosis congenita. *Nature*. 1999;402:551–5.
- Vulliamy T, Marrone A, Goldman F, Dearlove A, Bessler M, Mason PJ, et al. The RNA component of telomerase is mutated in autosomal dominant dyskeratosis congenita. *Nature*. 2001;413:432–5.
- Armanios M, Chen JL, Chang YP, Brodsky RA, Hawkins A, Griffin CA, et al. Haploinsufficiency of telomerase reverse transcriptase leads to anticipation in autosomal dominant dyskeratosis congenita. *Proc Natl Acad Sci USA*. 2005;102:15960–4.
- Marrone A, Walne A, Tamary H, Masunari Y, Kirwan M, Beswick R, et al. Telomerase reverse-transcriptase homozygous mutations in autosomal recessive dyskeratosis congenita and Hoyeraal–Hreidarsson syndrome. *Blood*. 2007;110:4198–205.
- Walne AJ, Vulliamy T, Marrone A, Beswick R, Kirwan M, Masunari Y, et al. Genetic heterogeneity in autosomal recessive dyskeratosis congenita with one subtype due to mutations in the telomerase-associated protein NOP10. *Hum Mol Genet*. 2007;16:1619–29.
- Vulliamy T, Beswick R, Kirwan M, Marrone A, Digweed M, Walne A, et al. Mutations in the telomerase component NHP2 cause the premature ageing syndrome dyskeratosis congenita. *Proc Natl Acad Sci USA*. 2008;105:8073–8.

26. Savage SA, Giri N, Baerlocher GM, Orr N, Lansdorp PM, Alter BP. TINF2, a component of the shelterin telomere protection complex, is mutated in dyskeratosis congenita. *Am J Hum Genet.* 2008;82:501–9.
27. Ball SE, Gibson FM, Rizzo S, Tooze JA, Marsh JC, Gordon-Smith EC. Progressive telomere shortening in aplastic anemia. *Blood.* 1998;91:3582–92.
28. Lee JJ, Kook H, Chung IJ, Na JA, Park MR, Hwang TJ, et al. Telomere length changes in patients with aplastic anaemia. *Br J Haematol.* 2001;112:1025–30.
29. Baerlocher GM, Vulto I, de Jong G, Lansdorp PM. Flow cytometry and FISH to measure the average length of telomeres (flow FISH). *Nat Protoc.* 2006;1:2365–76.
30. Canela A, Klatt P, Blasco MA. Telomere length analysis. *Methods Mol Biol.* 2007;371:45–72.
31. Alter BP, Baerlocher GM, Savage SA, Chanock SJ, Weksler BB, Willner JP, et al. Very short telomere length by flow fluorescence in situ hybridization identifies patients with dyskeratosis congenita. *Blood.* 2007;110:1439–47.
32. Lawson WE, Loyd JE. The genetic approach in pulmonary fibrosis: can it provide clues to this complex disease? *Proc Am Thorac Soc.* 2006;3:345–9.
33. Rocha V, Devergie A, Socie G, Ribaud P, Esperou H, Parquet N, et al. Unusual complications after bone marrow transplantation for dyskeratosis congenita. *Br J Haematol.* 1998;103:243–8.
34. Hackett JA, Greider CW. Balancing instability: dual roles for telomerase and telomere dysfunction in tumorigenesis. *Oncogene.* 2002;21:619–26.
35. Artandi SE, Chang S, Lee SL, Alson S, Gottlieb GJ, Chin L, et al. Telomere dysfunction promotes non-reciprocal translocations and epithelial cancers in mice. *Nature.* 2000;406:641–5.
36. Alter BP, Giri N, Savage SA, Rosenberg PS. Cancer in dyskeratosis congenita. *Blood.* 2009;113:6549–57.
37. Calado RT, Cooper JN, Scheinberg P, Wu C, Zago MA, Padilla-Nash H, et al. Telomere shortening promotes chromosomal instability and predicts malignant clonal evolution in aplastic anemia. *ASH Ann Meet Abstr.* 2009;114:3208.
38. Calado RT, Regal JA, Hills M, Yewdell WT, Dalmazzo LF, Zago MA, et al. Constitutional hypomorphic telomerase mutations in patients with acute myeloid leukemia. *Proc Natl Acad Sci USA.* 2009;106:1187–92.
39. Liu X, Bao G, Huo T, Wang Z, He X, Dong G. Constitutive telomere length and gastric cancer risk: case-control analysis in Chinese Han population. *Cancer Sci.* 2009;100:1300–5.
40. Jang JS, Choi YY, Lee WK, Choi JE, Cha SI, Kim YJ, et al. Telomere length and the risk of lung cancer. *Cancer Sci.* 2008;99:1385–9.
41. Rafnar T, Sulem P, Stacey SN, Geller F, Gudmundsson J, Sigurdsson A, et al. Sequence variants at the TERT-CLPTMIL locus associate with many cancer types. *Nat Genet.* 2009;41:221–7.
42. McKay JD, Hung RJ, Gaborieau V, Boffetta P, Chabrier A, Byrnes G, et al. Lung cancer susceptibility locus at 5p15.33. *Nat Genet.* 2008;40:1404–6.
43. Calado RT, Yewdell WT, Wilkerson KL, Regal JA, Kajigaya S, Stratakis CA, et al. Sex hormones, acting on the TERT gene, increase telomerase activity in human primary hematopoietic cells. *Blood.* 2009;114:2236–43.
44. Alter BP, Gardner FH, Hall RE. Treatment of dyskeratosis congenita with granulocyte colony-stimulating factor and erythropoietin. *Br J Haematol.* 1997;97:309–11.
45. Giri N, Pitel PA, Green D, Alter BP. Splenic peliosis and rupture in patients with dyskeratosis congenita on androgens and granulocyte colony-stimulating factor. *Br J Haematol.* 2007;138:815–7.
46. de la Fuente J, Dokal I. Dyskeratosis congenita: advances in the understanding of the telomerase defect and the role of stem cell transplantation. *Pediatr Transplant.* 2007;11:584–94.
47. Dietz AC, Orchard PJ, Baker KS, Giller RH, Savage SA, Alter BP et al. Disease-specific hematopoietic cell transplantation: nonmyeloablative conditioning regimen for dyskeratosis congenita. *Bone Marrow Transplant.* 2010.
48. Kirwan M, Beswick R, Vulliamy T, Nathwani AC, Walne AJ, Casimir C, et al. Exogenous TERC alone can enhance proliferative potential, telomerase activity and telomere length in lymphocytes from dyskeratosis congenita patients. *Br J Haematol.* 2009;144:771–81.
49. Agarwal S, Loh YH, McLoughlin EM, Huang J, Park IH, Miller JD, et al. Telomere elongation in induced pluripotent stem cells from dyskeratosis congenita patients. *Nature.* 2010;464:292–6.
50. Savage SA, Calado RT, Xin ZT, Ly H, Young NS, Chanock SJ. Genetic variation in telomeric repeat binding factors 1 and 2 in aplastic anemia. *Exp Hematol.* 2006;34:664–71.
51. Walne AJ, Vulliamy T, Beswick R, Kirwan M, Dokal I. TINF2 mutations result in very short telomeres: analysis of a large cohort of patients with dyskeratosis congenita and related bone marrow failure syndromes. *Blood.* 2008;112:3594–600.



## 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 analy-**

**ses 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.<sup>1-4</sup>

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.<sup>5-7</sup> 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*,<sup>8-14</sup> which is essential for normal development of erythroid and megakaryocytic cells.<sup>15-18</sup> 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.<sup>19,20</sup> Mice harboring a heterozygous *GATA1* knockdown allele frequently develop erythroblastic leukemia.<sup>21</sup> 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.<sup>22</sup> 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.<sup>13</sup> 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.<sup>23</sup>

### 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.<sup>13</sup> 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  $\chi^2$  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.<sup>5-7</sup> 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 *GATA1*s 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 *GATA1*s 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 *GATA1*s 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, *GATA1*s 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 *GATA1*s 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, ×10 <sup>9</sup> /L	Outcome	GATA1 mutation*	Consequence of mutation	Mutation type
113 <sup>24</sup>	F	63.9	CR	207 C>G	Tyr69stop	PTC 1-3'
2 <sup>13</sup>	F	89.0	Early death	199 G>T	Glu67stop	PTC 1-3'
3 <sup>13</sup>	F	NA	NA	174 ins 19 bp CAGCCACCCTGCAGCTGC	Frame shift at codon58, stop at codon 73	PTC 1-3'
4 <sup>13</sup>	F	128.8	CR	IVS1 to IVS2 del 1415 bp	Splice mutant	Splicing error
5 <sup>13</sup>	F	NA	NA	49 C>T	Gln17stop	PTC 1-5'
6 <sup>13</sup>	F	248.6	NA	Loss of 2nd exon	Splice mutant	Splicing error
7 <sup>13</sup>	F	31.2	CR	Loss of 2nd exon	Splice mutant	Splicing error
8 <sup>13</sup>	M	199.6	CR	-11 to +33 del 44 bp	No translation from Met1	Loss of 1st Met
9 <sup>13</sup>	M	44.9	Early death	45 ins C	Frame shift at codon15, stop at codon 39	PTC 1-5'
10 <sup>13</sup>	M	50.9	CR	37 G>T	Glu13stop	PTC 1-5'
11 <sup>13</sup>	F	103.0	Early death	90-91 del AG	Frame shift at codon 30, stop at codon 38	PTC 1-5'
12 <sup>13</sup>	F	14.6	Evolved to ML-DS	116 del A	Frame shift at codon 39, stop at codon 136	PTC 2
13 <sup>13</sup>	M	423.0	CR	185 ins 22 bp GCTGCAGCTGCGGCACTGGCCT	Frame shift at codon 62, stop at codon 74	PTC 1-3'
14 <sup>13</sup>	M	201.2	CR	189 C>A	Tyr63stop	PTC 1-3'
15 <sup>13</sup>	M	NA	NA	1 A>G	No translation from Met1	Loss of 1st Met
16 <sup>13</sup>	F	28.3	CR	189 C>A	Tyr63stop	PTC 1-3'
17 <sup>13</sup>	M	203.0	Evolved to ML-DS	38-39 del AG	Frame shift at codon 13, stop at codon 38	PTC 1-5'
18 <sup>13</sup>	M	31.3	CR	189 C>A	Tyr63stop	PTC 1-3'
19 <sup>13</sup>	M	NA	NA	90-91 del AG	Frame shift at codon 30, stop at codon 38	PTC 1-5'
20 <sup>13</sup>	F	114.0	Early death	187 ins T	Frame shift at codon 63, stop at codon 67	PTC 1-3'
21 <sup>25</sup>	F	26.0	Evolved to ML-DS	194 ins 20 bp GGCCTGGCCTACTACAGGG	Frame shift at codon 65, stop at codon 143	PTC 2
22 <sup>25</sup>	F	25.0	Evolved to ML-DS	194 ins 20 bp GGCCTGGCCTACTACAGGG	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 <sup>24</sup>	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 <sup>24</sup>	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 <sup>24</sup>	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 <sup>24</sup>	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 <sup>24</sup>	F	220.0	Early death	90-91 del AG	Frame shift at codon 30, stop at codon 38	PTC 1-5'
35 <sup>24</sup>	M	166.0	Early death	IVS2 5' boundary GT>CT	Splice mutant	Splicing error
36 <sup>24</sup>	M	57.6	Early death	193-199 GACGCTG>TAGTAGT	Asp65stop	PTC 1-3'
37 <sup>24</sup>	M	247.6	Early death	Exon2 to IVS2 del 218 bp	Splice mutant	Splicing error
38 <sup>24</sup>	M	93.3	Early death	IVS1 3' boundary AG>AA	Splice mutant	Splicing error
39 <sup>24</sup>	M	290.8	Early death	186 ins 12 bp GGCCTGGCCTA	Tyr62stop	PTC 1-3'
40	F	7.8	CR	2 T>C	No translation from Met1	Loss of 1st Met
41 <sup>24</sup>	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 AGCAGCTTCTCCTACTGCC	Frame shift at codon 50, stop at codon 143	PTC 2
45 <sup>24</sup>	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.