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

Detailed methods for experiments are described in Supplemental data 2

Results and Discussion

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

NRAS mutated Type IV ALPS was previously characterized by apoptosis

resistance of T-cells in IL-2 depeletion[3]. Then, activated T cells were subjected to an apoptosis assay by FAS stimulation or IL-2 depletion. Remarkable resistance to IL-2 depletion but not to FAS-dependent apoptosis (Fig. 1d and 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 (Fig. 1d and e). Western blotting analysis of activated T cells or Epstein-Barr virus-transformed B cells showed reduced expression of Bim (Fig. 1f).

In case 2, autoimmune phenotype and hepatosplenomegaly were remarkable as shown in Supplementary data 1. The patient was initially diagnosed as Evans Syndrome based on presence of hemolytic anemia and autoimmune thrombocytopenia. DNT cells were 1.1% of TCRαβ cells in the peripheral blood, which did not meet with the criteria of ALPS. Although spontaneous colony formation was shown in PB- and BM-MNC, and GM-CSF hypersensitivity was demonstrated in BM-MNC derived CD34 positive cell (Supplemental data 1 Table2), she showed no massive monocytosis or increased HbF. 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 (Fig. 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 PCR revealed mutated allele was almost equally present in granulocytes and T cells, but barely in B cells (Fig. 1c). Activated T cells showed resistance to IL-2 depletion but not to FAS-dependent apoptosis (Fig. 1d and 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 defied diagnostic

criteria of ALPS[2] or JMML[6]. It is interesting that Case 2 presented GM-CSF hypersensitivity, which is one of the hallmarks of JMML. Given the strict clinical and laboratory criteria of JMML and ALPS, our two cases should be defined as a new disease entity, like RAS associated ALPS like disease (RALD). Recently defined NRAS mutated ALPS type IV may also be included in a similar disease entity.

There are several cases of JMML reported simultaneously having clinical and laboratory findings compatible with autoimmune disease[8,9]. Autoimmune syndromes are occasionally seen in patients with myelodysplastic syndromes, including chronic myelomonocytic leukemia[10]. These previous findings may suggest a close relationship of autoimmune disease and JMML. Since KRAS G13D has been identified in JMML[11-13], it is tempting to speculate that KRAS G13D mutation is involved in JMML as well as RAS associated ALPS like disease (RALD). It should be noted in JMML, erythroid cells reportedly carry mutant RAS, while B and T cell involvement was variable[13]. In both of our cases, myeloid cells and T cells carried mutant RAS, while B cells were affected variably. These findings would support a hypothesis that the clinical and hematological features are related to the differentiation stages of hematopoietic stem cells where RAS mutation is acquired. JMML-like myelo-monocytic 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 two cases presented, their hematological 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, while 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 points of view with regard to the clinicopathological features of these disorders. First, transformation in a fetal HSC might be obligatory for the development of JMML[14] and that in HSC later in life may not have the same consequences. Second, certain KRAS mutations may be more potent than the others. Codon 13 mutations are generally less deleterious biochemically than codon 12 substitutions, and patients with JMML with codon 13 mutations have been reported to show spontaneous hematologic improvement[12,15]. Thus further studies are needed to reveal in-depth clinicopathological characteristics in this type of lympho-myelo proliferative disorders.

KRAS mutation may initiate the oncogenic pathway as one of the first genetic hits, but is insufficient to cause frank malignancy by itself[16,17]. Considering recent findings that additional mutations of the genes involved in DNA repair, cell cycle arrest, and apoptosis are required for full malignant transformation, one can argue that RAS associated ALPS like disease (RALD) patients will also develop malignancies during the course of the diseases. Occasional association of myeloid blast crisis in JMML and that of lymphoid malignancies in ALPS will support this notion. Thus the two 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, meet the criteria for ALPS diagnosis[18,19]. In this study, FAS-mediated apoptosis analysis was utilized for the screening. Considering the cases we presented, it will be intriguing to re-evaluate Evans syndrome by IL-2 depletion-dependent apoptosis assay focusing on the overlapping autoimmunity with RAS associated ALPS like disease (RALD).

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Author ship

MT and SM designed entire experiments and wrote this manuscript. KS, NM, and MT treat those patients, and designed clinical laboratory test. JP performed experiments described in Fig.1b-f. KM, HM, and SD performed colony and mutational analysis. MN, TM, KK, SK, YK and AT supervised clinical and immunological experiments, or coordinated clinical information.

Conflict of interest disclosure

The authors declare no conflict of interest.

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Figure Legends

Figure 1

a. Flow cytometric analysis of DNT cells. CD8 and CD4 double staining was performed in TCRαβ-expressing cells.

b. Electropherogram showing KRAS G13D mutation in BM-MNC in case 1 (left panel) and case 2 (right panel).

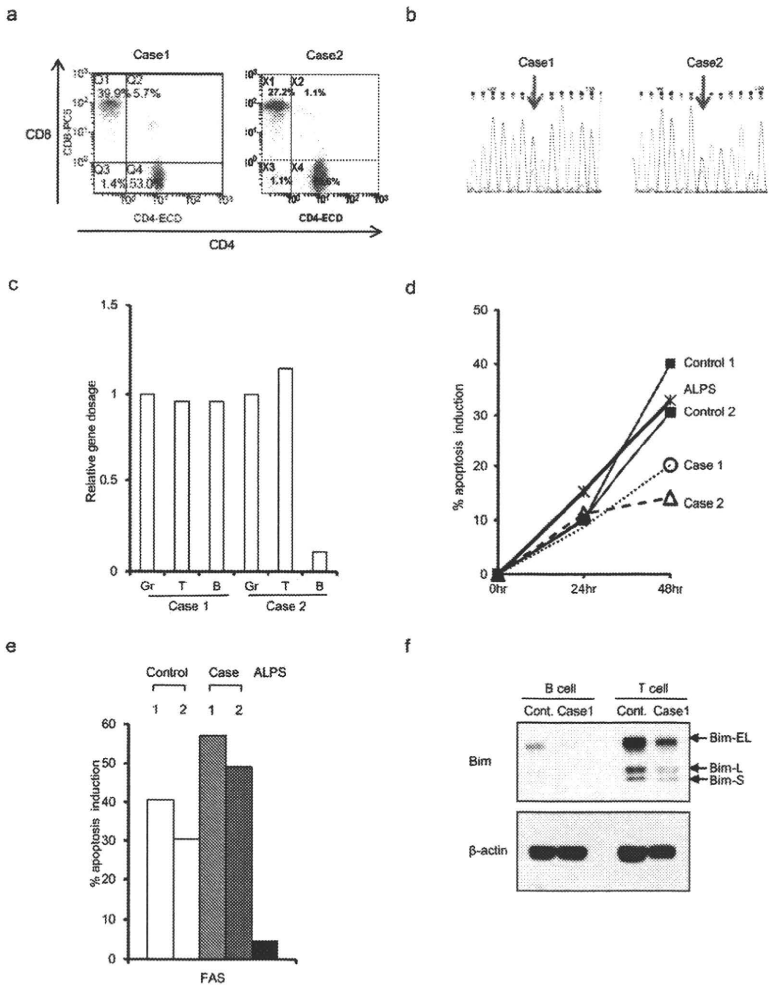
c. Gene dosage of mutated allele in granulocyte (Gr), T cell (T) and B cell (B). Relative gene dosage was estimated by a mutant allele specific PCR method in case 1 and 2 using albumin gene as internal control.

d. Apoptosis assay using activated T cells. Apoptosis percent was measured by flow cytometry with Annexin V staining 24 and 48 hr after IL-2 depletion

e. Apoptosis percent was measured 24hr after addition of anti-FAS CH11 antibody (final 100ng/ml)

f. Western blotting analysis of Bim expression.

Figure 1


a
Case1
Case2
CD8
CD8-PC5
Q1
Q2
39.9%
5.7%
Q3
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Q4
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X1
X2
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X3
1.1%
X4
CD4-ECD
CD4-ECD
CD4
b
Case1
Case2
c
Relative gene dosage
1.5
1
0.5
0
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Gr
T
B
Case 1
Case 2
d
% apoptosis induction
50
40
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20
10
0
0hr
24hr
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Case1
Bim
Bim-EL
Bim-L
Bim-S
β-actin

REPORT

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

Michael A. Pulsipher,[1] Neal S. Young,[2] Jakub Tolar,[3] Antonio M. Risitano,[4] H. Joachim Deeg,[5] Paolo Anderlini,[6] Rodrigo Calado,[2] Seiji Kojima,[7] Mary Eapen,[8] Richard Harris,[9] Phillip Scheinberg,[2] Sharon Savage,[10] Jaroslaw P. Maciejewski,[11] Ramon V. Tiu,[11] Nancy DiFronzo,[12] Mary M. Horowitz,[8] Joseph H. Antin[13]

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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KEY WORDS: Severe aplastic anemia, Blood and marrow transplantation, Immunosuppressive therapy, Telomeres

From the [1]Primary Children's Medical Center, University of Utah School of Medicine, Salt Lake City, Utah; [2]Hematology Branch, National Heart, Lung, and Blood Institute, Bethesda, Maryland; [3]University of Minnesota, Minneapolis, Minnesota; [4]Federico II University of Naples, Naples, Italy; [5]Fred Hutchinson Cancer Research Center, Seattle, Washington; [6]University of Texas, M.D. Anderson Cancer Center, Houston, Texas; [7]Children's Medical Center, Nagoya, Japan; [8]Medical College of Wisconsin, Milwaukee, Wisconsin; [9]Cincinnati Children's Hospital, Cincinnati, Ohio; [10]Division of Cancer Epidemiology and Genetics, National Cancer Institute, Bethesda, Maryland; [11]Cleveland Clinic Taussig Cancer Institute, Cleveland, Ohio; [12]National Heart, Lung, and Blood Institute, Bethesda, Marylan; and [13]Dana-Farber Cancer Institute, Boston, Massachusetts.

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Correspondence and reprint requests: Michael A. Pulsipher, MD, Associate Professor of Pediatrics, University of Utah School of Medicine, Primary Children's Medical Center, 100 North Mario Capecchi Drive, Salt Lake City, UT 84113 (e-mail: michael.pulsipher@hsc.utah.edu).

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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

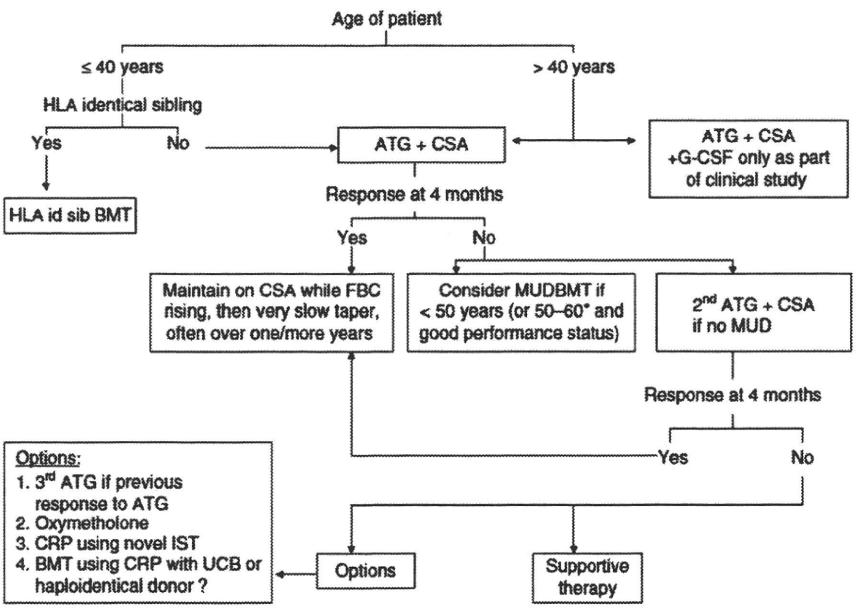

Figure 1. Treatment of acquired severe aplastic anemia according to United Kingdom Guidelines [13]. ATG, antithymocyte globulin; CSA, cyclosporine; FBC, full blood count (or CBC); MUD, matched unrelated donor; CRP, clinical research protocol; IST, immunosuppressive therapy; UCB, umbilical cord blood.

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-γ [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 ($Terc^{-/-}$), 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

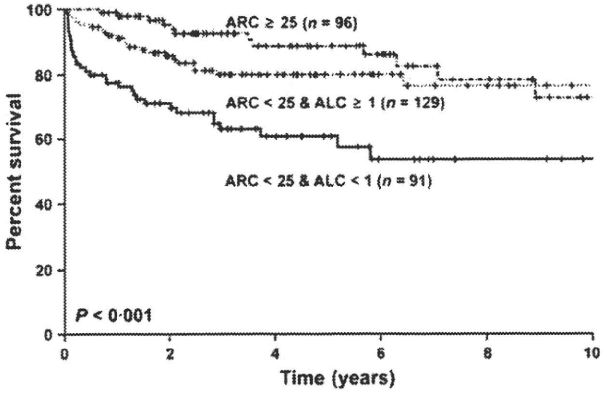

Figure 2. Probability of survival in patients treated with IST who had high versus low absolute reticulocyte counts (ARC) and high versus low absolute lymphocyte counts (ALC). Patients undergoing BMT were censored at the time of transplant [27].

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].

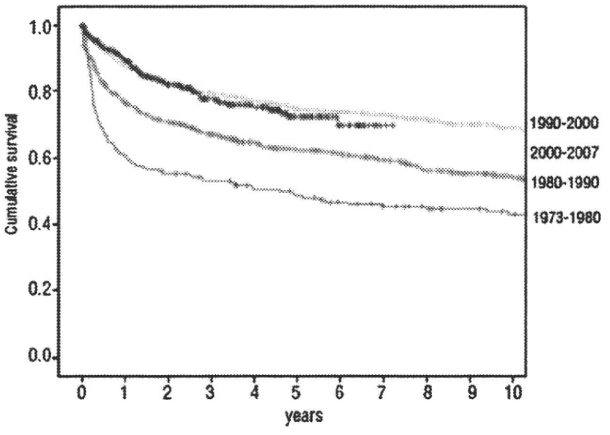

Figure 3. Survival among patients with severe aplastic anemia treated with ATG-based immunosuppression reported to the EBMT database (n = 2400) [36].

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 ± 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

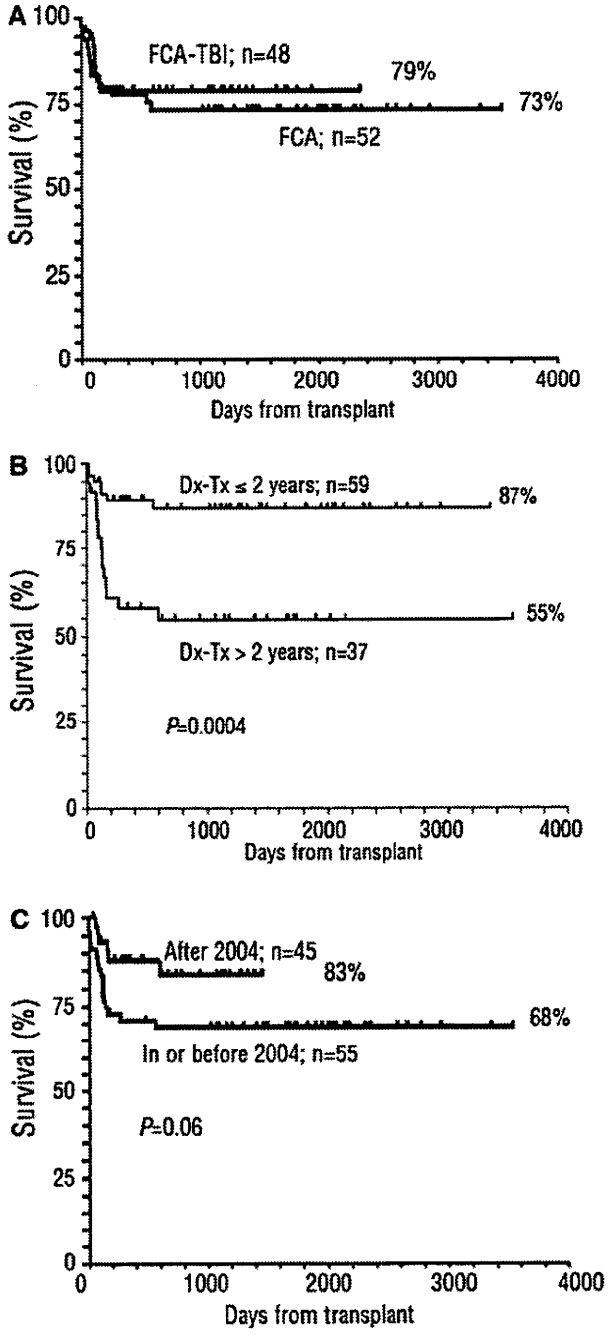

Figure 4. Outcomes of URD BMT for SAA using fludarabine/cyclophosphamide/ATG ± low dose TBI reported to the EBMT. (A) Survival after Flu/Cy/ATG with TBI (median age 27 (7-53 years) versus Flu/Cy/ATG (median age 13 [3-51 years]). (B) Survival of patients transplanted ≤2 years from diagnosis versus those 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 established 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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Recent progress in dyskeratosis congenita

Nobuhiro Nishio · Seiji Kojima

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

Keywords Dyskeratosis congenita · Telomere · Telomerase · Bone marrow failure

1 Introduction

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

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

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

2 Dyskeratosis congenita

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

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

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

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

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

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

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

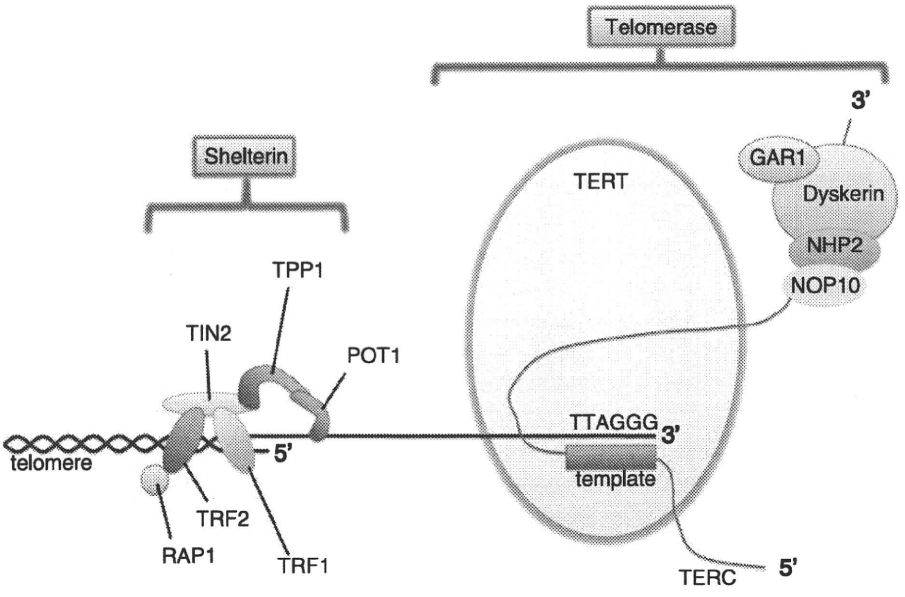

Fig. 1 Schema of telomerase and shelterin complex. Telomerase complex consists of the enzyme telomerase transcriptase (TERT), RNA component (TERC), and dyskerin protein complex (dyskerin, NOP10, NHP2, and GAR1). TERT adds new telomeres (TTAGGG repeats) onto the chromosome end by using the template provided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