Table 2. GATA1 expression vectors used in this study

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

– indicates not applicable.

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

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

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

To investigate the precise relationship between PTC type 1 mutations and GATA1s protein levels, we examined more type 1 mutations using the minigene constructs. Western blot analysis showed relatively higher expression of the proteins in samples expressing P1-5, P1-7, P1-8, P1-2, and P1-3 than the other constructs (Figure 2Bi). Each mutation in the mutant minigene construct is described in Table 2. Interestingly, all samples that expressed higher levels of GATA1s protein exhibited intense signals at lower molecular weights than the dominant GATA1 signal (Figure 2Biii). Because the size of the lower molecular weight band was identical to that observed in the splicing error mutant (Figure 2Biii), we speculated that the signal might be derived from a transcript lacking exon 2 (Δexon 2) by alternative splicing. To examine that possibility, we attempted Northern blot analysis using the *GATA1* exon 2 fragment as a probe, and as expected, only the longer transcript was detected (Figure 2Biv). To confirm the correlation between the amount of Δexon 2 transcript and GATA1s protein, we performed a quantitative assessment by densitometric analysis. The results showed a strong correlation between Δexon 2 transcript and GATA1s protein

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

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

Some clinical data were not available. We defined the number of patients for whom clinical data was available as (n).
LBW indicates low birth weight; AST, aspartate transaminase; ALT, alanine transaminase; and T-Bil, total bilirubin.

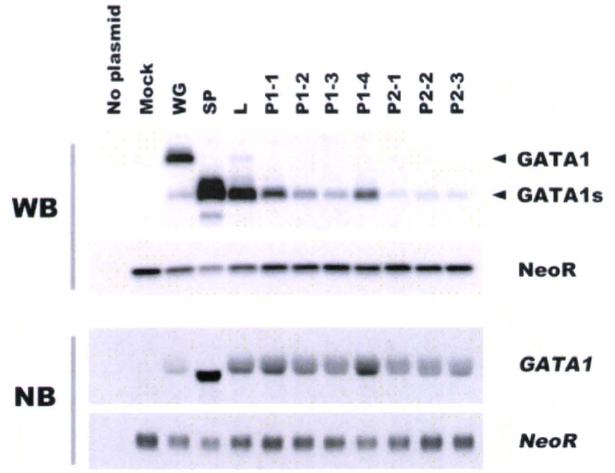

Figure 1. Effects of mutant transcripts of *GATA1* on the expression level of the truncated protein. The *GATA1* mutations observed in TAM patients are classified according to the types of transcripts. The translational efficiency of each transcript was assessed by Western blot analysis in BHK-21 cells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

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

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

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

Based on these results, *GATA1* mutations were classified into 2 groups: a high GATA1s expression group (GATA1s high group) including the loss of first methionine type, the splicing error type, and PTC type 1-3′, and a low GATA1s expression group (GATA1s low group) including PTC type 1-5′ and PTC type 2. We classified TAM patients into these 2 groups in accordance with the GATA1s expression levels estimated from the mutations and compared their clinical data. High counts of WBC and blast cells were significantly associated with the GATA1s high group ($P = .004$ and $P = .008$, respectively; Table 4). Although high WBC count was correlated with early death, there were no significant differences in the cumulative incidence of early death between the 2 groups (Figure 4). Importantly, TAM patients in the GATA1s low group had a significantly higher risk for the development of leukemia ($P < .001$; Figure 4). Of 11 TAM patients who progressed to ML-DS, 10 belonged to the GATA1s low group. Notably, 8 patients among them had PTC type 2 mutations (Tables 1, 5).

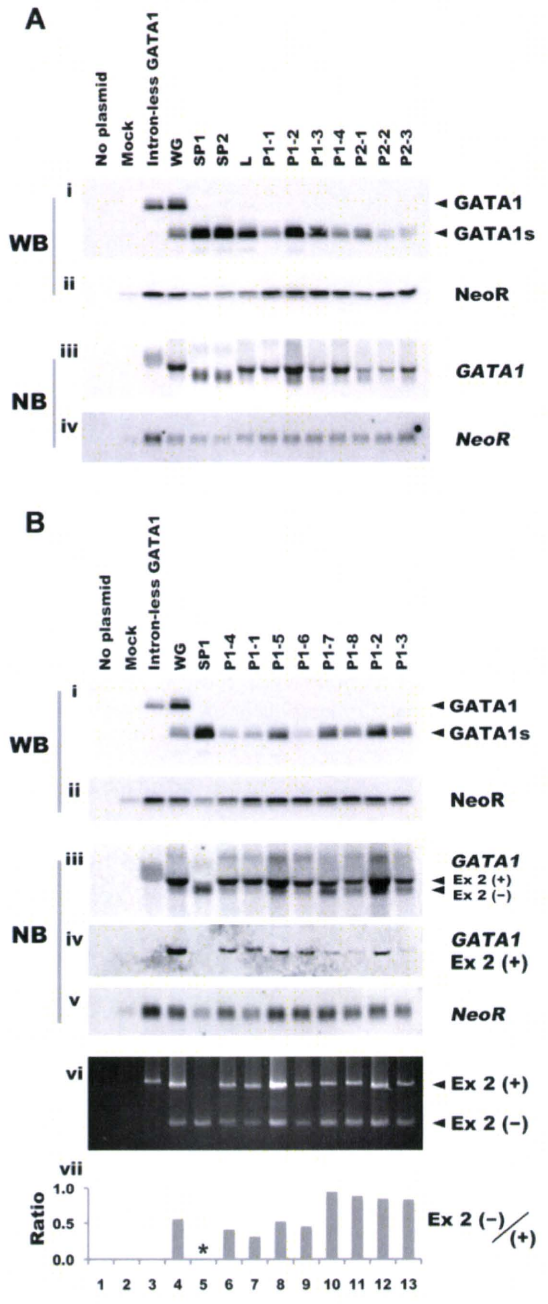

Figure 2. GATA1 mutations affect the expression level of the truncated protein. (A) The expression levels of GATA1s protein and mRNA were assessed in BHK-21 cells transfected with human *GATA1* minigene expression vectors carrying mutations observed in TAM patients. Western blot analysis was performed with anti-GATA1 (i) or anti-NeoR antibody (ii). Northern blot analysis was carried out with *GATA1* exon 3-6 fragment (iii) or *NeoR* cDNA (iv) as probe. (B) The expression levels of GATA1s protein and mRNA in BHK-21 cells transfected with human *GATA1* minigene expression vectors with PTC type 1 mutation. Levels were assessed by Western blot analysis with anti-GATA1 antibody (i), anti-NeoR antibody (ii). Northern blot analysis was performed with GATA1 exon 3-6 (iii), exon 2 (iv), or NeoR cDNA (v). To detect the transcripts derived from the human *GATA1* minigene expression construct, RT-PCR analysis was carried out using primers described in "RT-PCR" (vi). Ex 2(+) and Ex 2(−) indicate PCR products or transcripts with or without exon 2, respectively. Ratio of Ex 2(−)/(+) was calculated from the results of a densitometric analysis of the RT-PCR. The asterisk denotes unavailable data (vii).

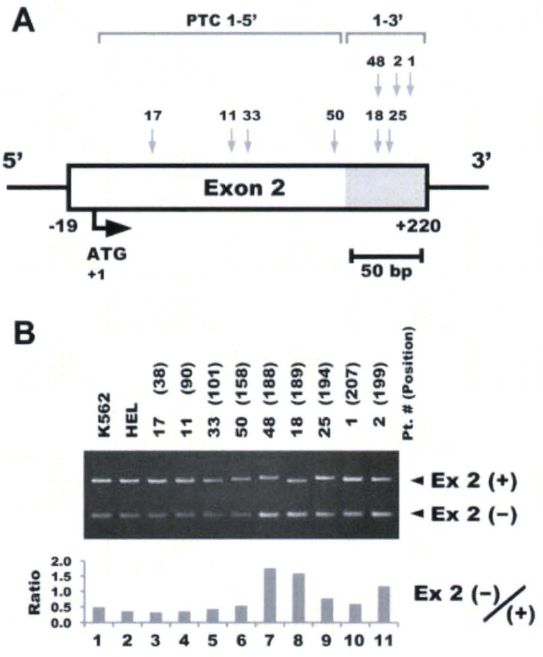

Figure 3. The location of the PTC type 1 mutation affects the efficiency of alternative splicing in TAM blast cells. (A) The location of the *GATA1* mutation in each TAM patient. Details of the mutation in each sample are described in Table 1. (B) RT-PCR analysis of *GATA1* in TAM blast cells harboring PTC type 1 mutations. RT-PCR was performed using primers recognizing both the long transcript including exon 2 and Δexon 2 (top). All of the patient samples consisted of mononuclear cells from peripheral blood. The numbers in parentheses indicate the number of nucleotides in mRNA from the translation initiation codon. Ex 2(+) and Ex 2(−) indicate PCR products with or without exon 2, respectively (middle). Ratio of Ex 2(−)/(+) was calculated from the results of a densitometric analysis of the RT-PCR (bottom). Note that the intense bands of the short form were observed in the samples from the patients who have *GATA1* mutations located on the 3′ side of exon 2 (lanes 7-11).

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

Table 4. Correlations between patient covariates and GATA1 expression levels

	GATA1s expression group		
	High (n = 40)	Low (n = 26)	*P*
Sex: male/female, n	19/21	13/13	.843*
Gestational age, wk	37.3 (30.0-40.0)	37.9 (32.6-40.6)	.487
Birth weight, kg	2.5 (1.6-3.3)	2.5 (1.4-3.5)	.698
WBC, $\times 10^9$/L	105.65 (7.8-423.0)	39.0 (9.0-220.0)	.004
Number of blasts, $\times 10^9$/L	72.1 (0.42-301.6)	13.4 (0.45-189.2)	.008
AST, IU/L	68.5 (23-501)	46.5 (16-4341)	.113
ALT, IU/L	41.0 (5-407)	12.5 (4-653)	.075
T-Bil mg/dL	6.7 (0.6-15.3)	4.65 (1.82-46.0)	.270
Effusions, n (%)	11 of 27 (40.7)	5 of 17 (29.4)	.447†
Bleeding diatheses, n (%)	8 of 29 (27.6)	5 of 16 (31.3)	.528†

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

*Pearson χ^2 test.

†Fisher exact test.

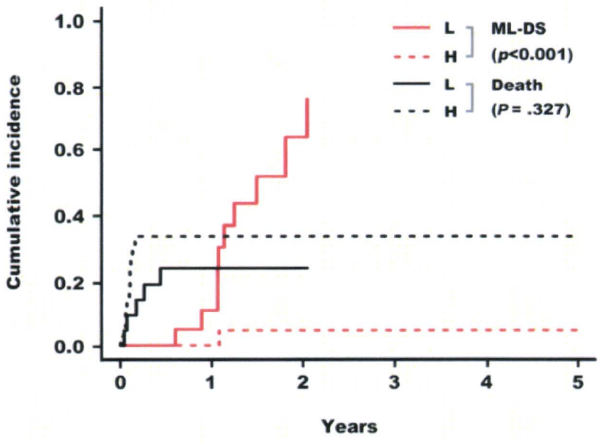

Figure 4. Cumulative incidence of early death and of ML-DS in children with TAM. Based on the estimated GATA1s expression levels, patients were classified in 2 groups: GATA1s high and low groups. TAM patients in the GATA1s low group had a significantly higher risk for the development of leukemia (P (gray)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

Discussion

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

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

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

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

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

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

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

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

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

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Authorship

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

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

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REPORT

ASBMT
American Society for Blood and Marrow Transplantation

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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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

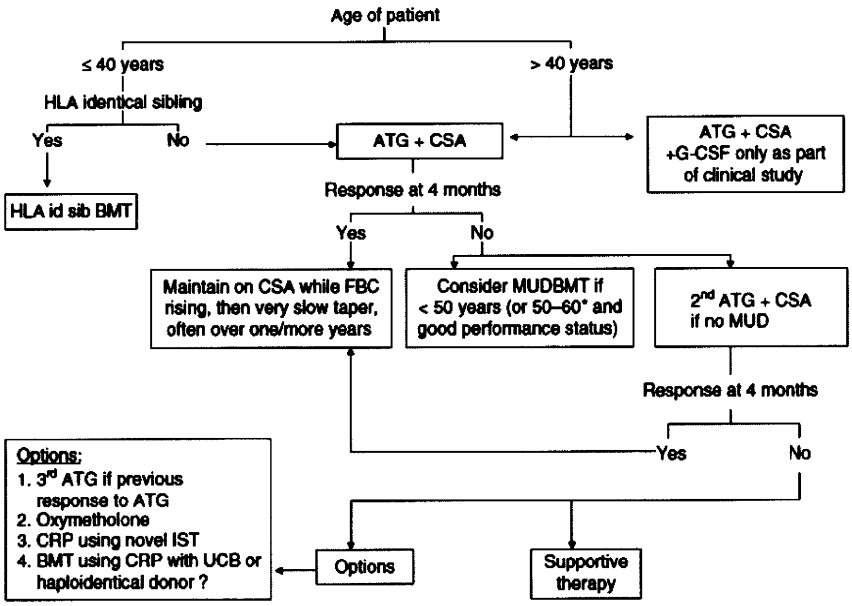

Figure 1. Treatment of acquired severe aplastic anemia according to United Kingdom Guidelines [13]. ATG, antithymocyte globulin; CSA, cyclosporine; FBC, full blood count (or CBC); MUD, matched unrelated donor; CRP, clinical research protocol; IST, immunosuppressive therapy; UCB, umbilical cord blood.

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

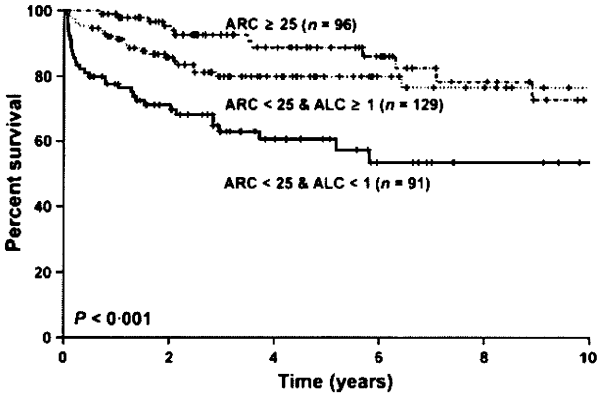

Figure 2. Probability of survival in patients treated with IST who had high versus low absolute reticulocyte counts (ARC) and high versus low absolute lymphocyte counts (ALC). Patients undergoing BMT were censored at the time of transplant [27].

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].

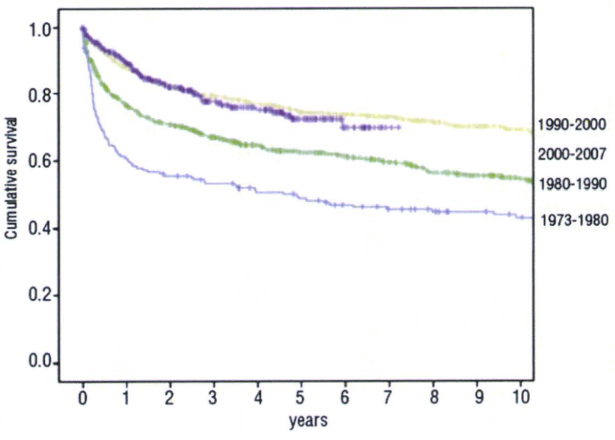

Figure 3. Survival among patients with severe aplastic anemia treated with ATG-based immunosuppression reported to the EBMT database (n = 2400) [36].

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

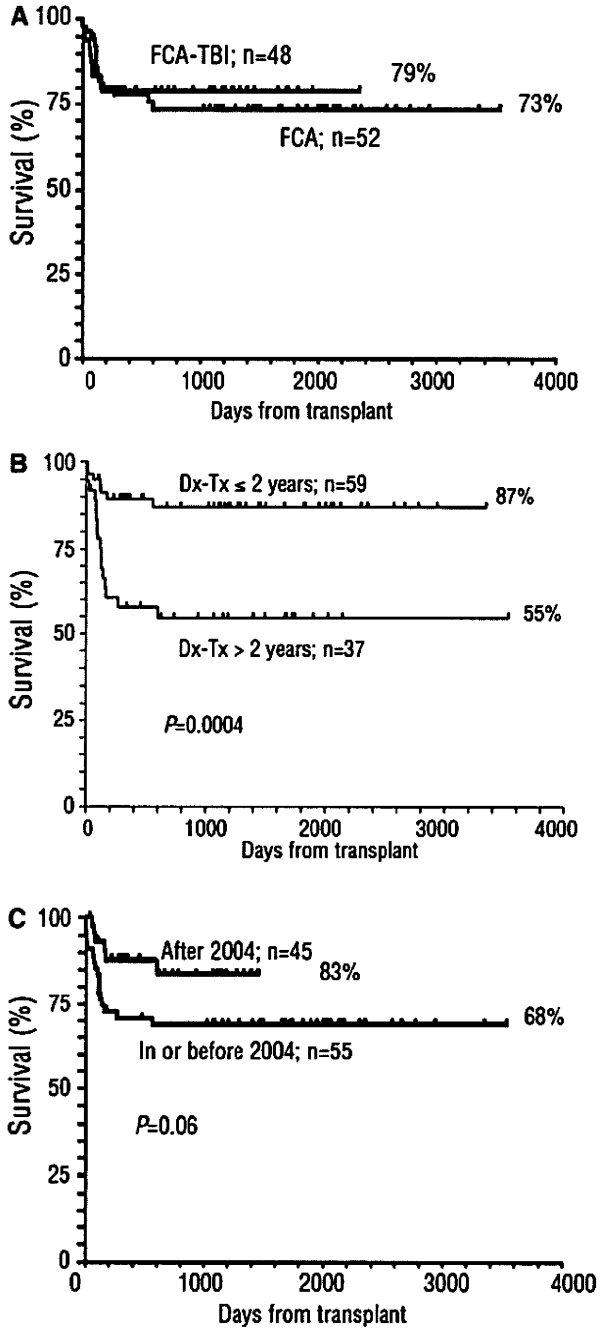

Figure 4. Outcomes of URD BMT for SAA using fludarabine/cyclophosphamide/ATG ± low dose TBI reported to the EBMT. (A) Survival after Flu/Cy/ATG with TBI (median age 27 (7-53 years) versus Flu/Cy/ATG (median age 13 [3-51 years]). (B) Survival of patients transplanted ≤2 years from diagnosis versus those 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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Masatoshi Takagi, Kunihiro Shinoda, Jinhua Piao, Noriko Mitsuiki, Mari Takagi, Kazuyuki Matsuda, Hideki Muramatsu, Sayoko Doisaki, Masayuki Nagasawa, Tomohiro Morio, Yoshihito Kasahara, Kenichi Koike, Seiji Kojima, Akira Takao and Shuki Mizutani

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Autoimmune Lymphoproliferative Syndrome Like Disease With Somatic *KRAS* Mutation

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

[1]Department of Pediatrics and Developmental Biology, Graduate School of Medicine, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo, 113-8519, Japan

[2]Department of Pediatrics, Gifu Municipal Hospital
7-1 Kashimacho, Gifu-shi, Gifu, 500-8513, Japan

[3]Department of Laboratory Medicine, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto-shi, Nagano, 390-8621, Japan

[4]Department of Pediatrics, Nagoya University, Graduate School of Medicine, 65 Tsurumaicho, Showa-ku, Nagoya-shi, Aichi, 466-8550, Japan

[5]Department of Laboratory Sciences, Kanazawa University School of Health Sciences, 5-11-80 Kodatsuno, Kanazawa, Ishikawa, 920-0942, Japan

[6]Department of Pediatrics, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto-shi, Nagano, 390-8621, Japan

[*]Correspondence

Masatoshi Takagi and Shuki Mizutani

Department of Pediatrics and Developmental Biology, Graduate School of Medicine, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo, 113-8519, Japan

Abstract

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

Introduction

ALPS is a disease characterized by dysfunction of the FAS-mediated apoptotic pathway[1,2], currently categorized as Type Ia, germline *TNFRSF6/FAS* mutation; Type Ib, germline *FAS ligand* mutation; Type Is, somatic *TNFRSF6/FAS* mutation; and Type II, germline *Caspase 10* mutation. Patients exhibit lymphadenopathy, hepatosplenomegaly, and autoimmune diseases such as immune cytopenia and hyper-γ-globulinemia. An additional subclassification has been proposed that includes Types III and IV, whereby Type III has been defined as that with no known mutation but with a defect in FAS-mediated apoptosis, and Type IV as one showing germline *NRAS* mutation[3]. Type IV is considered exceptional because the FAS-dependent apoptosis pathway is not involved in the pathogenesis, and this subclass is characterized by a resistance to IL-2 depletion-dependent apoptosis. Recent updated criteria and classification of ALPS suggested type IV ALPS as a RAS associated leukoproliferative disease [4].

JMML is a chronic leukemia in children. Patients show lymphadenopathy, hepatosplenomegaly, leukocytosis associated with monocytosis, anemia, thrombocytopenia, and occasional autoimmune phenotypes. About 80% of patients with JMML have been shown to have a genetic abnormality in their leukemia cells including mutations of *NF1*, *RAS* family[5], *CBL*, or *PTPN11*. The hallmarks of the laboratory findings of JMML include spontaneous colony formation in bone marrow (BM) or peripheral blood (PB) mononuclear cells (MNC) and hypersensitivity to granulocyte-macrophage colony-stimulating factor (GM-CSF) of CD34 positive BM-MNC[6].

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

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

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

Patients and Methods

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

Case 1

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

Case 2

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

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