the PU.1-induced upregulation of CSF1R. Notably, 5 d after exposure to 4-HT, we detected CSF1R^{high} and CSF1R^{low} cells in the population of PUER cells expressing MOZ-TIF2, but only CSF1R^{low} cells were in the control PUER cell population (**Fig. 4d**). We did not detect CSF1R expression before addition of 4-HT, even in PUER cells expressing MOZ-TIF2 (**Fig. 4d**), indicating that functional PU.1 is required for MOZ-TIF2–induced CSF1R expression. Chromatin immunoprecipitation (ChIP) analysis indicated that PU.1, MOZ-TIF2 and possibly endogenous MOZ were recruited to the *Csf1r* promoter in the bone marrow cells of mice with MOZ-TIF2–induced AML (**Supplementary Fig. 11a**). In PUER cells expressing MOZ-TIF2, recruitment of MOZ-TIF2 and MOZ to the *Csf1r* promoter was detected after 4-HT treatment, but not before the treatment (**Supplementary Fig. 11b**), suggesting that the recruitment of MOZ-TIF2 and MOZ is dependent upon functional PU.1.

To determine whether PU.1 is essential for the development of MOZ-TIF2–induced AML, we infected wild-type and *Sfpi1*$^{-/-}$ fetal liver cells of E12.5 littermates with retroviruses encoding MOZ-TIF2 or N-Myc and transplanted them into irradiated mice. Although mice transplanted with *Sfpi1*$^{+/+}$ cells expressing MOZ-TIF2 developed AML 8–14 weeks after transplantation, mice transplanted with *Sfpi1*$^{-/-}$ cells were healthy for at least 6 months (**Fig. 4e**). In contrast, all mice transplanted with either wild-type or *Sfpi1*$^{-/-}$ cells expressing N-Myc developed AML 6–10 weeks after transplantation (**Fig. 4f**). When both PU.1 and MOZ-TIF2 were introduced into PU.1-deficient fetal liver cells, the transplanted mice developed leukemia (**Fig. 4g**). However, introduction of either PU.1 or MOZ-TIF2 alone was not sufficient for AML induction. Thus, we conclude that PU.1 is required for the initiation of MOZ-TIF2–induced AML.

To determine whether PU.1 is also required for the maintenance of MOZ-TIF2–induced AML, we infected fetal liver cells of PU.1 conditional knockout mice (*Sfpi1*$^{flox/flox}$ and expressing estrogen receptor (ER)-Cre) with MOZ-TIF2 and transplanted them into irradiated recipient mice, which developed AML. We next transplanted bone marrow cells of these mice into irradiated secondary recipients and then treated half of the mice with tamoxifen to induce PU.1 deletion. All of the control mice died of AML within 6 weeks, but none of the tamoxifen-treated mice developed AML for at least for 6 months (**Fig. 4h**). These results indicate that PU.1 is also required for the maintenance of MOZ-TIF2–induced AML stem cells.

Taken together, our results indicate that MOZ and its leukemia-associated fusion proteins activate PU.1-mediated transcription of the monocyte-specific gene *Csf1r*. MOZ fusion proteins might constitutively stimulate high *Csf1r* expression to induce AML (**Fig. 4i**). In contrast, we previously found that MOZ fusion proteins inhibit AML1-mediated activation of granulocyte-specific *Mpo* gene transcription[18]. Because MOZ fusion proteins are associated with monocytic leukemia, commitment to the monocytic lineage may be determined by differential regulation of target genes by MOZ fusion proteins (that is, upregulation of monocyte-specific genes such as *Csf1r* and downregulation of granulocyte-specific genes such as that encoding myeloperoxidase). It is also likely that the normal MOZ protein modulates *Csf1r* expression to an appropriate level to regulate normal hematopoiesis (**Fig. 4i**), as *Csf1r* expression was impaired in *MOZ*$^{-/-}$ fetal liver cells (**Supplementary Fig. 12**).

Although AML induction was suppressed in mice transplanted with *Csf1r*$^{-/-}$ cells, half of these mice developed AML, albeit at a longer latency. Thus, MOZ-TIF2 can provoke either a rapid induction of AML in a CSF1R-dependent manner or a slower induction in a CSF1R-independent manner. There are several possibilities to explain this CSF1R independence. First, we observed increased HoxA9 expression in both CSF1R^{high} and CSF1R^{low} cells. HoxA9 overexpression is reportedly not sufficient to induce AML and additional mutations or oncogene activation is required for AML induction in this context[21,22]. Thus, MOZ-TIF2–transfected *Csf1r*$^{-/-}$ cells might require additional mutations to induce leukemia. Second, because we used a retrovirus vector to introduce MOZ-TIF2, it is possible that oncogene activation by retroviral integration might mediate AML pathogenesis.

In conclusion, our results indicate that PU.1-mediated upregulation of *Csf1r* is crucial for leukemia stem cell potential induced by MOZ-TIF2. Our findings add to previous work associating CSF1R with AML. CSF1R upregulation has been reported in human[23–25] and mouse[26] AML. CSF1R is also known as the oncoprotein c-Fms, and transplantation of bone marrow cells expressing the v-fms oncoprotein induces multilineage hematopoietic disorders[27]. A chromosomal translocation resulting in expression of a fusion protein in which RNA-binding motif protein-6 (RBM6) is fused to CSF1R has recently been reported to be associated with AML[28]. CSF1R may thus be crucial for not only leukemia induced by MOZ fusions but also a wider subset of AML.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

Y.A., I.K., T.K. and M.S. conducted experiments in AML mice. Y.A., H. Shima and I.K. performed western blotting, immunoprecipitation, GST pull down, ChIP and reporter assays. P.Z. and D.G.T. conducted experiments in PU.1-deficient mice. E.R.S. designed and performed experiments in CSF1R-deficient mice. K.T. and E.I. analyzed expression of CSF1R in human AML cells. H. Singh designed and performed experiments in PUER cells. H.O. prepared Ki20227. I.K. and Y.A. analyzed data and edited the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Human subjects, mice and cells. The study involving human samples was approved by the Ethics Committee of Hirosaki University Graduate School of Medicine, and all clinical samples were obtained with informed consent. C57BL/6 mice were purchased from CREA Japan. NGF-FKBP-Fas transgenic mice[10] (Jackson Laboratories), *Csf1r*-deficient mice[12] (provided by E.R.S.), PU.1-null (*Sfpi1*$^{-/-}$) and PU.1 conditionally deficient (*Sfpi1* floxed) mice[29] (provided by D.G.T.), CreERT2 knock-in mice (TaconicArtemis GmbH)[30] and MOZ-deficient mice[5] were backcrossed to C57BL/6 mice at least five times. Mouse experiments were performed in a specific pathogen–free environment at the Japan National Cancer Center animal facility according to institutional guidelines and with approval of the Japan National Cancer Center Animal Ethics Committee. PUER cells[20] were provided by H. Singh.

Generation of acute myeloid leukemia mouse models. MSCV-MOZ-TIF2-IRES-EGFP, MSCV-N-Myc-IRES-EGFP, MSCV-CSF1R-pgk-pac and MSCV-PU.1-pgk-pac constructs were generated by inserting cDNAs encoding MOZ-TIF2, N-Myc, CSF1R or PU.1 into the appropriate vector. The constructs were transfected into Plat-E cells[31] cells using the FuGENE 6 reagent (Roche Diagnostics) and supernatants containing retrovirus were collected 48 h after transfection. c-Kit$^+$ cells (1×10^5 cells) were selected from bone marrow or fetal liver cells using CD117-specific MicroBeads (Miltenyi Biotec); the cells were then incubated with retroviruses using RetroNectin (Takara Bio) for 24 h in StemPro-34 serum-free medium (Invitrogen) containing cytokines (20 ng ml^{-1} stem cell factor (PeproTech), 10 ng ml^{-1} interleukin-6 (PeproTech), 10 ng ml^{-1} interleukin-3 (a gift from Kirin Pharmaceuticals)). The infected cells were then transplanted together with bone marrow cells (2×10^5) into lethally irradiated (9 Gy) 6- to 8-week-old C57BL/6 mice by intravenous injection. Secondary transplants were performed by intravenous injection of bone marrow cells from primary AML mice into sublethally irradiated (6 Gy) C57BL/6 mice.

Administration of AP20187, imatinib or Ki20227. AP20187 (a gift from Ariad Pharmaceuticals; 10 mg per kg body weight) was administered daily by intravenous injection for 5 d, and then 1 mg per kg body weight AP20187 was administered every 3 d thereafter as described previously[10]. Mice were orally administered imatinib mesylate (Novartis Pharmaceuticals; 100 mg per kg body weight), Ki20227 (ref. 13) (a gift from Kirin Pharmaceuticals; 20 mg per kg body weight) or solvent twice daily from 7 d after transplantation.

Immunofluorescent staining, detection of side population cells, flow cytometric analysis and cell sorting. Bone marrow cells from mice with AML were preincubated with rat IgG and then incubated on ice with the following staining reagents: antibody to CD115 (AFS98) conjugated to phycoerythrin (PE) (eBioscience), antibody to Mac-1 (M1/70) conjugated to PE-Cy7 (eBioscience), antibody to Gr-1(RB6-8C5) conjugated to allophycocyanin (APC) (BD Pharmingen) and antibody to c-Kit (2B8) conjugated to APC (BD Pharmingen). For the detection of side population cells, bone marrow cells were stained with 5 μg ml^{-1} Hoechst 33342 in the presence or absence of 50 μM verapamil at 37 °C for 60 min. Flow cytometric analysis and cell sorting were performed using the JSAN cell sorter (Baybioscience) and the results were analyzed with FlowJo software (Tree Star).

Reporter analysis. *CSF1R*-luciferase constructs were generated by insertion of *CSF1R* promoter constructs, either wild type or lacking the PU.1-binding site[32], into pGL4.10 (luc2) (Promega). SaOS2 cells (a gift from T. Taya) were transfected with *CSF1R*-luciferase constructs and pGL4.75 (hRL-CMV) (Promega) together with various expression constructs (pLNCX-AML1 (ref. 18), pLNCX-PU.1 (ref. 33), pLNCX-MOZ[18], pLNCX-MOZ-TIF2 (ref. 18) and pLNCX-MOZ-CBP[18]) in 24-well plates, and luciferase activity was assayed 24 h after transfection using the microplate luminometer GLOMAX (Promega). The results shown for the reporter assays represent average values for relative luciferase activity generated from at least three independent experiments; relative values were obtained by normalizing to the luciferase activity of phRL-CMV, which served as an internal control.

Immunoprecipitation and immunoblotting. For Flag tag immunoprecipitation experiments, cells were lysed in a lysis buffer containing 250 mM NaCl, 20 mM sodium phosphate (pH 7.0), 30 mM sodium pyrophosphate, 10 mM NaF, 0.1% NP-40, 5 mM dithiothreitol, 1 mM phenylmethanesulfonylfluoride and Complete protease inhibitor (Roche). Cell lysates were incubated with Flag-specific antibody–conjugated agarose beads (Sigma) and rotated at 10 r.p.m. (TAITEC RT-50) at 4 °C overnight. The adsorbed beads were washed three times with lysis buffer. Precipitated proteins were eluted from the beads by Flag peptide and dissolved with the same volume of 2× SDS sample buffer. When immunoprecipitation was not performed, total protein lysates were prepared in 2× SDS sample buffer. Antibodies were detected by chemiluminescence with ECL plus Detection Reagents (Amersham Biosciences). The primary antibodies used in this study were Flag-specific antibody (M2) (Sigma), hemagglutinin-specific antibody (3F10) (Roche) and MOZ-specific antibody[18], which was generated by immunizing rabbit with peptides corresponding residue 441–460 of human MOZ.

GST pull-down assay. The HindIII-ClaI fragment corresponding to the N-terminal region (1–664) of MOZ was cloned into the pSP64polyA vector. [^{35}S]-MOZ (1–664) was produced by incubating pSP64polyA-MOZ with [^{35}S]-methionine using the TNT Coupled Rabbit Reticulocyte Lysate System (Promega). pGEX-6P-PU.1 and pGEX-6P-AML1 were generated by subcloning full-length human PU.1 and AML1 cDNAs into pGEX-6P (GE Healthcare). GST, GST-PU.1 and GST-AML1 were produced in *Escherichia coli* BL21 containing pGEX-6P, pGEX-6P-PU.1 and pGEX-6P-AML1, respectively. The [^{35}S]-MOZ (1-664) protein was incubated with GST-, GST-PU.1– or GST-AML1–conjugated glutathione-agarose at 4 °C for 60 min in lysis buffer, washed three times with lysis buffer, analyzed by SDS-PAGE and detected by autoradiography.

Statistical analyses. We performed unpaired two-tailed Student's *t* tests for comparisons and a log-rank test for survival data with JMP8 software (SAS Institute).

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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, Maryland; and [13]Dana-Farber Cancer Institute, Boston, Massachusetts.

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Correspondence and reprint requests: Michael 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

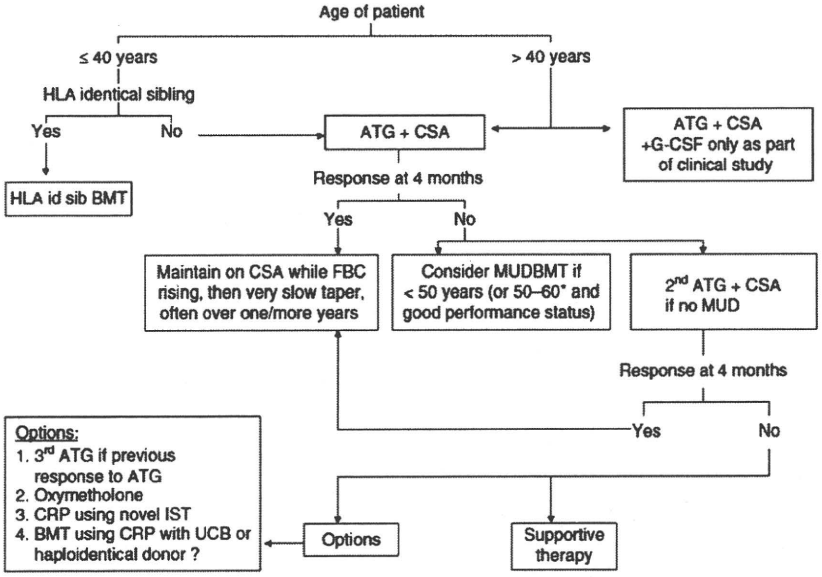

Figure 1. Treatment of acquired severe aplastic anemia according to United Kingdom Guidelines [13]. ATG, antithymocyte globulin; CSA, cyclosporine; FBC, full blood count (or CBC); MUD, matched unrelated donor; CRP, clinical research protocol; IST, immunosuppressive therapy; UCB, umbilical cord blood.

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

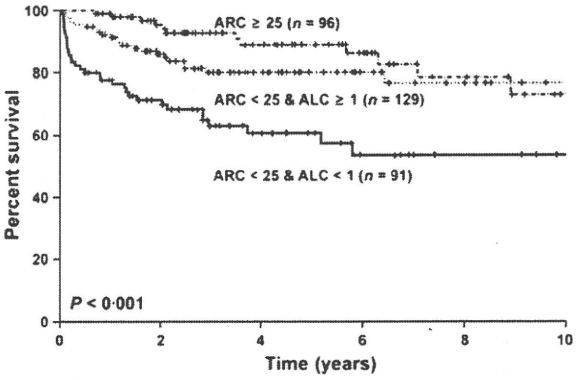

Figure 2. Probability of survival in patients treated with IST who had high versus low absolute reticulocyte counts (ARC) and high versus low absolute lymphocyte counts (ALC). Patients undergoing BMT were censored at the time of transplant [27].

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].

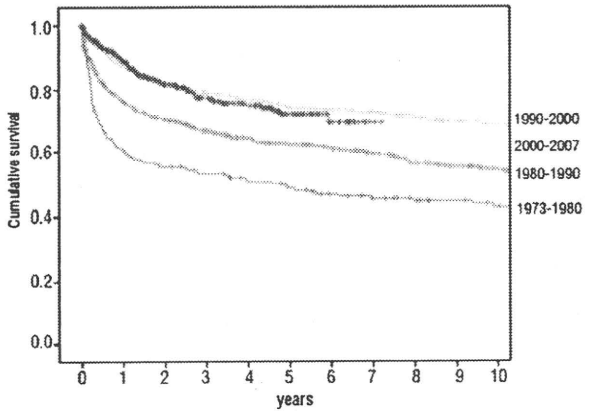

Figure 3. Survival among patients with severe aplastic anemia treated with ATG-based immunosuppression reported to the EBMT database (n = 2400) [36].

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

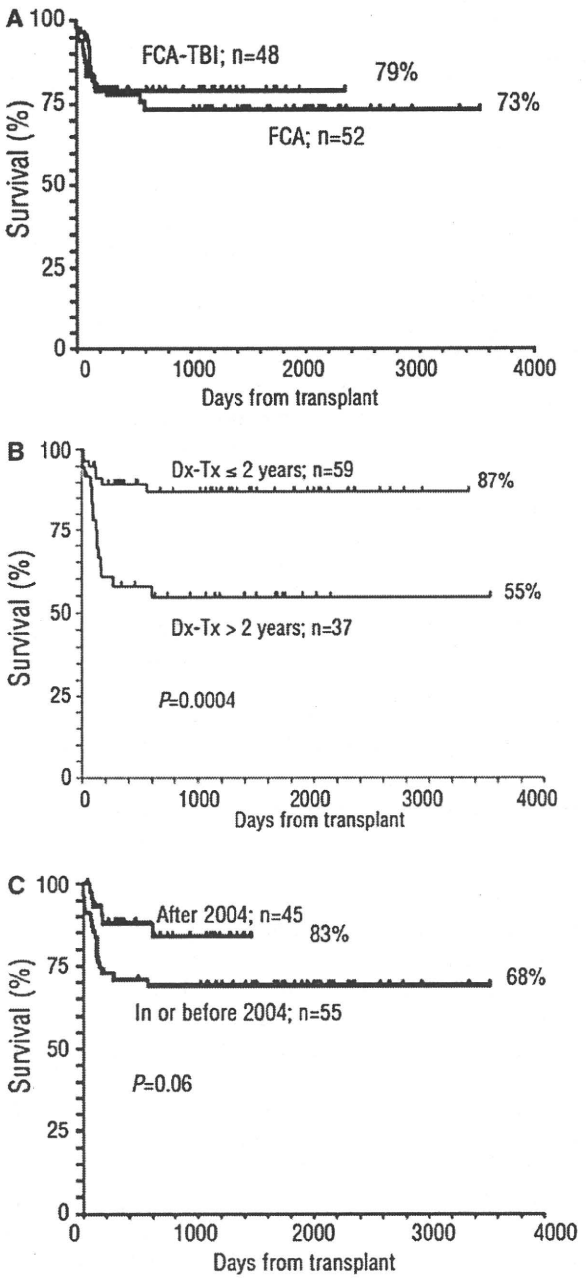

Figure 4. Outcomes of URD BMT for SAA using fludarabine/cyclophosphamide/ATG ± low dose TBI reported to the EBMT. (A) Survival after Flu/Cy/ATG with TBI (median age 27 (7-53 years) versus Flu/Cy/ATG (median age 13 [3-51 years]). (B) Survival of patients transplanted ≤2 years from diagnosis versus those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

Genetic variation of vascular endothelial growth factor pathway does not correlate with the severity of retinopathy of prematurity

T Kusuda[1], S Hikino[1], S Ohga[1], T Kinjo[1], M Ochiai[1], Y Takahata[1], S Tokunaga[2], K Ihara[1], Y Hata[3] and T Hara[1]

[1]Department of Pediatrics, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan; [2]Department of Medical Informatics, Kyushu University Hospital, Fukuoka, Japan and [3]Department of Ophthalmology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan

Objective: The aim of this study was to assess the genetic effects of the vascular endothelial growth factor (VEGF) pathway on retinopathy of prematurity (ROP).

Study Design: A prospective study from a tertiary center that enrolled 204 Japanese infants (<35 weeks of gestational age (GA)) having no anomalies. ROP developed in 127, but not in 77 infants. The relative severity was defined as non-severe, moderate and severe ROP for GA, based on the staging criteria. VEGF (g.−634G>C, g.+13553C>T) and VEGF-receptor (KDR g.+4422(AC)11 to 14, Flt-1 c.+6724(TG)13 to 23) gene polymorphisms and clinical variables were assessed by uni/multivariate analyses.

Result: The frequency of polymorphisms did not differ between ROP and non-ROP patients. The TT genotype of g.+13553 showed a higher odds ratio for non-severe ROP than CC genotype ($P=0.006$). Multivariate analyses indicated that low birth weight, blood transfusion and respiratory distress syndrome, but not polymorphisms, were the risk factors of advanced ROP (≥stage 3).

Conclusion: A genotype of the VEGF pathway weakly affects the severity of ROP compared with other clinical factors.
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Keywords: vascular endothelial growth factor; retinopathy of prematurity; neovascularization; preterm infants

Introduction

Retinopathy of prematurity (ROP) is one of the critical complications in preterm infants because of the most common cause of blindness in childhood. The formation of retinal and vitreous membranes leads to retinal detachments in severely affected infants. Abnormal and progressive neovascularization is the most important pathophysiological mechanism in this setting. Recent experimental studies indicated that growth factors including growth hormone, insulin-like growth factor-1 and vascular endothelial growth factor (VEGF) are involved in the pathogenesis of ROP.[1] VEGF, a specific mitogen for vascular endothelial cells, is essential for neovascularization in the tissue. VEGF gene polymorphisms have been reportedly associated with proliferative diabetic retinopathy[2] and age-related degeneration.[3] These retinal diseases might share the disease process of vasculopathy with ROP. Several lines of studies reported that the gene polymorphisms of VEGF and its associated molecules would be involved in development of ROP.[4,5] On the other hand, a lack of or negligible association has been indicated between the genetic background and the evolution of ROP.[6,7] To the best of our knowledge, the clinical impact of gene polymorphisms on the development of ROP has not yet been confirmed.

Besides the genetic predisposition, a variety of causative factors have been proposed for the development of ROP. Low birth weight, low gestational age and prolonged oxygen therapy have been consistently believed to raise the substantial risk of ROP.[8] Other putative factors have been reported in the association with ROP, including mechanical ventilation,[8] low Apgar score,[8] steroid therapy,[9] small-for-gestational age,[10] male gender,[11] blood transfusion[12] and multiple birth.[11] However, it remains unknown how and which factors contribute to the initiation and/or progression of ROP under the mutual interaction. Insulin-like growth factor-1 and VEGF cooperatively control the physiological development of ocular blood vessels of the fetus and the infants. To minimize the late effects on the preterm survivors, the prophylactic and therapeutic modality for ROP should be optimized according to the individual infants.

In this study, we analyzed VEGF and VEGF-receptor gene polymorphisms in preterm infants who developed or did not develop ROP. To establish the best practice for the control of ROP, the relationship between the gene polymorphisms and clinical factors for the evolution and severity of ROP was assessed in a Japanese population.

Correspondence: Professor S Ohga, Department of Pediatrics, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan.
E-mail: ohgas@pediatr.med.kyushu-u.ac.jp
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Methods

Patients

Of the 614 infants admitted to the tertiary center of a neonatal intensive care unit at Kyushu University Hospital between 2004 and 2007, 247 infants were born within less than 35 weeks of gestational age. Of the preterm infants, 204 were prospectively enrolled in this study. Exclusion criteria were chromosomal aberrations, major anomalies and inherited diseases. Informed consent, approved by Kyushu University Hospital Ethical Committee, was obtained from their guardians. Table 1 shows the clinical characteristics of patients studied. Patients who suffered from ROP had lower Apgar scores at 1 and 5 min, lower gestational ages and lower birth weights than those who did not. The frequencies of respiratory distress syndrome, oxygen dependency at 36 weeks of post-conceptual age, bronchopulmonary dysplasia and infections were higher in ROP infants than in non-ROP infants. As for the treatment, ROP patients frequently received more blood transfusions, dexamethasone therapy, management for patent ductus arteriosus and surgery for intestinal perforation, and also required longer oxygen therapy and intermittent mechanical ventilation than non-ROP patients. There were no differences in the sex, single or multiple birth and mode of delivery, small-for-gestational age and periventricular leukomalacia between ROP and non-ROP patients. All infants underwent ophthalmological examinations to assess neovascular proliferation and stage according to the international classification for ROP.[13]

Analyses of gene polymorphisms

Peripheral blood samples were collected at the time of routine examinations during hospitalization. Genomic DNA was conventionally extracted from peripheral blood mononuclear cells with the QIAamp DNA Blood Kit (QIAGEN, Tokyo, Japan). Restriction fragment length polymorphism analysis of VEGF g.−634 G>C and g.+13553 C>T single-nucleotide polymorphisms was carried out according to the established methods.[14] The KDR g.−4422(AC) 11 to 14 dinucleotide repeat polymorphism in all subjects was determined as described in our previous report.[15] The Flt-1 c.−6724(TG) 13 to 23 site was amplified by PCR, and the genotyping of gene microsatellite polymorphisms was determined by a fluorescence-based technique as described previously.[16]

Relative severity of ROP according to gestational age

ROP stages were most closely linked with gestational age. Median ROP stages in each gestational age were as follows: 3 in infants who were born at 22 to 25 weeks of gestational age, 2 in infants at 26 weeks of gestational age, 1 in infants at 27 to 29 weeks of gestational age and 0 in infants at 30 to 34 weeks of gestational age. The relative severity was defined according to the gestational age. Based on the equation score = ROP stage−the median stage of each gestational age group, $\geqslant 1$ was classified as 'severe', 0 as 'moderate' and $\leqslant -1$ as 'non-severe ROP'.

Table 1 Clinical characteristics of the patients

Number	*Non-ROP*	*ROP*	P-*value*
	77	*127*	
Sex (male/female)	40/37	78/49	0.192
Multiple birth	21	21	0.075
Apgar score			
1-min[a]	7 (1–9)	5 (0–9)	<0.001
5-min[a]	8 (4–10)	7 (1–9)	<0.001
Gestational age (weeks)[a]	32 (28–34)	27 (22–33)	<0.001
Birth weight (g)[a]	1596 (692–2400)	944 (378–2168)	<0.001
Mode of delivery (CS/TV)	44/34	70/57	0.884
RDS	14	63	<0.001
Oxygen dependency at 36 weeks	1	46	<0.001
BPD (no/mild/moderate/severe)	76/0/1/0	56/25/31/15	<0.001
Blood transfusion (0/1–4/5–)	74/3/0	80/30/17	<0.001
SGA	21	30	0.618
DEX therapy	0	25	<0.001
PDA therapy	3	33	<0.001
Surgical operation	1	16	0.003
Sepsis	1	14	0.011
Bacterial infection	10	47	<0.001
IVH (stage 0/1–2/3–4)	62/15/0	83/36/8	0.082
PVL	4	6	1.000
Duration of IMV (days) (0/1–6/7–27/28–)	52/23/1/1	26/40/23/38	<0.001
Duration of oxygen therapy (days) (0/1–27/28–99/100–)	8/68/1/0	3/53/46/25	<0.001

Abbreviations: BPD, bronchopulmonary dysplasia; CS, caesarean section; DEX, dexamethasone; IMV, intermittent mandatory ventilation; IVH, intraventricular hemorrhage; PDA, patent ductus arteriosus; PVL, periventricular leukomalacia; RDS, respiratory distress syndrome; ROP, retinopathy of prematurity; SGA, small-for-gestational age; TV, transvaginal delivery.
[a]Median and ranges in parentheses.

Statistics

Continuous data were compared by Wilcoxon-signed rank-sum test and categorical data were compared using Fisher's exact test among the groups. Distribution of allele and genotype frequencies between the groups was compared using Pearson's χ^2-test or logistic regression analysis. Multiple logistic regression analysis was conducted to mutually adjust for explanatory variables. Non-severe ROP for gestational age, advanced ROP ($\geqslant$ stage 3) and the development of ROP ($\geqslant$ stage 1) were used as the dependent variables. The explanatory variables were each gene polymorphism, birth weight, gestational age, multiple birth, Apgar score (5-min), mode of delivery, duration of intermittent mechanical ventilation, duration of oxygen therapy, respiratory distress syndrome, oxygen dependency at 36 weeks of post-conceptual age, blood transfusion, small-for-gestational age infants, dexamethasone therapy, patent ductus arteriosus operation,

sepsis, intraventricular hemorrhage and periventricular leukomalacia. Durations of intermittent mechanical ventilation and oxygen therapy were included in the model as dummy variables (intermittent mechanical ventilation, 0, 1 to 6, 7 to 27 and 28–; oxygen therapy, 0, 1 to 27, 28 to 99 and 100–). The multiple logistic regression model was selected by the stepwise model selection method, with a significance level of 0.05 for removing from and 0.01 for addition to the model. All statistical analyses were performed with Stata 10.1 (Stata Corporation, College Station, TX, USA).

Results

VEGF and VEGF-receptor gene polymorphisms

The genotype and allele frequency of VEGF g.−634G>C, VEGF g. + 13553C>T, KDR g. + 4422(AC)11 to 14 and Flt-1 c. + 6724(TG)13 to 23 dinucleotide repeats did not differ between all ROP, advanced ROP (⩾ stage 3), or photocoagulation-required ROP and non-ROP patients. The frequency of each polymorphism in ROP patients was then compared according to the relative severity (Table 2). The genotype distribution of VEGF g. + 13553 C>T polymorphisms differed among the groups of severity (Table 2b, $P = 0.030$). The frequency of TT genotype was higher in non-severe ROP infants than observed in others (moderate and severe ROP infants) ($P = 0.006$), but was not different between severe ROP infants and others (non-severe and severe ROP infants). The frequencies of VEGF g.−634 G>C, KDR g. + 4422(AC) 11 to 14, and Flt-1 c.−6724 (TG) polymorphisms did not differ among the groups of relative severity (Table 2a, c and d).

The effect of gene polymorphism on the relative severity of ROP was assessed by logistic regression analysis (Table 3). The TT genotype of VEGF g. + 13553 C>T showed a higher odds ratio for non-severe ROP than the CC genotype ($P = 0.006$). The presence of C allele had a significantly lower odds ratio for non-severe ROP subjects ($P = 0.006$). No other gene polymorphisms were associated with the relative severity of ROP.

Clinical risk factors for the development and severity of ROP

To address the optimal treatment for ROP, multivariate analyses were performed to determine the clinical variables, including gene polymorphisms influencing the risk of ROP (⩾ stage 1) or advanced ROP (⩾ stage 3). The multiple regression analysis revealed that low gestational age ($P<0.001$), single birth ($P = 0.012$), small-for-gestational age ($P = 0.019$), longer oxygen therapy ($P = 0.025$) and male gender ($P = 0.040$) were the independent risk factors for the development of ROP, and that low birth weight ($P = 0.002$), times of blood transfusion ($P = 0.001$) and respiratory distress syndrome ($P = 0.004$) were those for advanced ROP among ROP patients (Table 4). When the C allele of VEGF g. + 13553 was added to the model, it was not selected as a significant risk factor for advanced ROP ($P = 0.13$). No polymorphism was selected as the risk factor for the development and severity of ROP.

Table 2 VEGF and VEGF-receptor gene polymorphisms in ROP patients according to gestational age

Genotype	*Non-severe*	*Moderate*	*Severe*	P-*value*
(a) *VEGF g.−634 G>C polymorphism*				
GG	9	22	18	0.290
GC	6	30	24	
CC	2	5	11	
(b) *VEGF g.+13553 C>T polymorphism*				
CC	7	34	31	*0.030*
CT	6	20	21	
TT	4	3	1	
(c) *KDR g.−4422(AC) 11–14 dinucleotide repeat*[a]				
A1A1	13	31	34	0.544
A1A2	3	22	17	
A2A2	1	3	2	
(d) *Flt-1 c.−6724 (TG) 13–23 dinucleotide repeat*[b]				
LL	0	1	5	0.097
SL	4	25	23	
SS	13	31	25	

Abbreviations: ROP, retinopathy of prematurity; VEGF, vascular endothelial growth factor.
[a]PCR product length (bp): A1: 223, A2: 225.
[b]PCR product length (bp) of TG repeats: short (S): 104, 106, 108, 110, long (L): 112, 114, 116, 118, 120, 122, 124, 126, 168.

Table 3 VEGF gene polymorphism associated with prevalence of non-severe ROP for gestational age

	Odds ratio	*95% CI*	P-*value*
VEGF g.−634 G>C			
GG	1	Referent	
GC	0.49	0.16–1.50	0.213
CC	0.56	0.11–2.86	0.482
G allele[a]	1.28	0.27–6.12	0.760
C allele[a]	0.51	0.18–1.42	0.197
VEGF g.+13553 C>T			
CC	1	Referent	
CT	1.36	0.43–4.33	0.604
TT	9.29	1.89–45.54	*0.006*
C allele[a]	0.12	0.03–0.55	*0.006*
T allele[a]	2.06	0.73–5.83	0.171

Abbreviations: ROP, retinopathy of prematurity; VEGF, vascular endothelial growth factor. Logistic regression analysis.
[a]The odds ratio for a presence of the allele.

Table 4 Variables associated with the risk of total or advanced ROP

	Odds ratio	*95% CI*	P-*value*
For the development of ROP (⩾ stage 1)			
Gestational age (1-week gain)	0.48	0.36–0.64	<0.001
Multiple birth	0.19	0.52–0.69	0.012
SGA	3.25	1.21–8.71	0.019
Duration of oxygen therapy (1-day gain)	1.08	1.01–1.16	0.025
Female	0.36	0.14–0.96	0.040
For advanced ROP (⩾ stage 3)[a]			
Birth weight (100 g gain)	0.76	0.64–0.90	0.002
Blood transfusion (1 time gain)	1.32	1.12–1.55	0.001
RDS	4.05	1.57–10.49	0.004

Abbreviations: CI, confidence interval; RDS, respiratory distress syndrome; ROP, retinopathy of prematurity, SGA, small-for-gestational age.
The multiple regression analysis with the model selected by stepwise method was performed.
[a]Patients with ROP were analyzed. The oxygen dependency at 36 weeks was not selected as a variable because of the marginal significance (odds ratio 2.58, 95% CI 0.95–7.02, $P=0.06$).

Discussion

This study revealed a significant association between the TT genotype of VEGF g. + 13553 C>T and non-severe ROP for gestational age. Low birth weight, number of times of blood transfusion, respiratory distress syndrome and oxygen dependency at 36 weeks were indicated as the risk factors for advanced ROP (⩾ stage 3). On the other hand, no polymorphisms were selected as variables influencing the significant risk for the development and severity of ROP by multivariate analyses. The VEGF g. + 13553C>T polymorphism might be involved in the progression of ROP, although it could have less clinical impact on the evolution of ROP than other factors.

Chen and Smith[1] explained that ROP occurs in two phases of the disease process. In the first phase, premature birth leads to a cessation of physiological vascular growth of the retina that would normally occur *in utero*, as well as an inhibition of the ordinary neural development of the retina. In the second phase of ROP, hypoxia induces retinal neovascularization, which is promoted by insulin-like growth factor-1 and VEGF signaling through the activation of p44/42 mitogen-activated protein kinase in a coordinated manner. Renner *et al.*[14] reported that plasma VEGF levels were lower in carriers of the VEGF g. + 13553 T allele than in non-carriers, and suggested that a alternative VEGF protein associated with VEGF g. + 13553 C>T polymorphism might be involved in susceptibility to ROP. In this study, the TT genotype of VEGF g. + 13553 was associated with non-severe ROP. It may raise the possibility that low production of VEGF protein in the TT genotype carriers hampers the progression in the second phase of ROP, although serum VEGF concentrations were not measured in this study. As for the VEGF g.−634 G>C polymorphism, Vannay *et al.*[5] reported that heterozygous and homozygous carriers of the C allele had a higher likelihood of developing ROP requiring treatment even after adjustment for other risk factors of ROP, including gestational age, oxygen therapy and sex. On the other hand, Kwinta *et al.*[17] showed no difference in the frequency of VEGF g.−634 G>C polymorphism between low birth weight infants with and without ROP, and serum VEGF levels in the patients did not depend on the VEGF polymorphism. The frequency of VEGF g.−634 G>C polymorphism did not differ among Japanese preterm infants having various ROP stages. Recently, Dunai *et al.*[6] have shown a negligible contribution of genetic variants to the risk and severity of ROP by a meta-analysis on the published data for VEGF (including g.−634 G>C, but not g. + 13553 C>T polymorphism), insulin-like growth factor-1 receptor (G + 3174A), angiopoietin II, estrogen receptor PvuII Pp and endothelial NO-synthase. These controversial results of genetic polymorphisms might be explained in part by the sample size and/or distinct races of study population. Genotypes of VEGF pathway may explain the unusual progression of advanced ROP.[18] There may be less clinical effect of genetic predisposition on the development of ROP than other treatment factors.

Flt-1 and *KDR* genes encode VEGF receptor-1 and receptor-2, respectively. The major function of the VEGF pathway depends on the signaling through VEGF receptor-2. Pieh *et al.*[19] reported elevated plasma levels of soluble VEGFR-2 and soluble Tie2 in active ROP patients. Kwinta *et al.*[17] concluded that serum concentrations of VEGF and soluble VEGFR-1 on the 10th day of life did not differ significantly among patients with no ROP, ROP not requiring treatment and ROP requiring laser or cryotherapy. We found no significant association between KDR g.−4422 (AC) 11 to 14 or Flt-1 c.−6724 (TG) 13 to 23 dinucleotide repeat polymorphism, and development of severe ROP. Genetic variations of VEGF receptor might have negligible effects on the development of ROP in Japanese preterm infants.

Supplemental oxygen therapy for premature infants is one of the most important factors in developing ROP. As for the treatment-related risk factor, duration of oxygen therapy was indicated for the onset of ROP, while oxygen dependency at 36 weeks, blood transfusions and respiratory distress syndrome were selected for the development of ⩾ stage 3 ROP (Table 4). Premature infants having prolonged ventilator support are at high risk of developing severe ROP requiring treatment.[12] High oxygen concentration of blood directly influenced the retinal angiogenesis in an animal model of ROP and this state is known as oxygen-induced retinopathy.[20] On the other hand, optimal supplementation of oxygen might have an inhibitory effect on the progression of ROP because of suppressing the overexpression of VEGF in the second stage of ROP.[1] These findings may raise the need of optimal oxygen therapy that could overcome the individual VEGF responses and reduce the risk of both ocular and pulmonary

complications in premature infants. In conclusion, the genetic polymorphisms in VEGF and its receptor system are less or not sensitive predictors of ROP compared with other clinical parameters.

Conflict of interest

The authors declare no conflict of interest.

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SHORT REPORT

Hepatitis-associated aplastic anemia during a primary infection of genotype 1a torque teno virus

Masataka Ishimura · Shouichi Ohga · Masako Ichiyama · Koichi Kusuhara · Hidetoshi Takada · Toshiro Hara · Masaharu Takahashi · Hiroaki Okamoto

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Abstract A 12-year-old Japanese boy suffered from severe acute hepatitis and pancytopenia. The patient underwent successful bone marrow transplantation from an HLA-identical sister. Torque teno virus (TTV) DNA of genotype 1a and IgM-class antibody against the virus were detected in sera at the onset of hepatitis. TTV/1a DNA and anti-TTV/1a IgM antibody levels were undetectable on the 16th and 46th days after the onset of illness, respectively. Anti-TTV/1a IgG antibody was positive throughout the observation period. Sequential viral load and anti-TTV/1a IgM antibody suggested a primary infection of TTV/1a. Genomic sequence of the virus coincided with that of the original strain first isolated from human. TTV DNA was quantified at 130 copies in 10^5 bone marrow mononuclear cells, which suggested that infection of hematopoietic cells might be the cause of aplasia. This is the first report of TTV hepatitis-associated aplastic anemia assessed by the anti-TTV antibodies and viral load in peripheral blood and bone marrow.

Keywords Hepatitis-associated aplastic anemia · TTV · Genotype 1a · TTV antibody

M. Ishimura · S. Ohga (✉) · M. Ichiyama · K. Kusuhara · H. Takada · T. Hara
Department of Pediatrics, Graduate School of Medical Sciences, Kyushu University,
3-1-1 Maidashi, Higashi-ku,
Fukuoka 812-8582, Japan
e-mail: ohgas@pediatr.med.kyushu-u.ac.jp

M. Takahashi · H. Okamoto
Division of Virology, Department of Infection and Immunity, School of Medicine, Jichi Medical University,
Tochigi, Shimotsuke, Japan

Abbreviations

AA	Aplastic anemia
AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
BM	Bone marrow
BMT	Bone marrow transplantation
EBV	Epstein–Barr virus
HAA	Hepatitis-associated aplastic anemia
MNC	Mononuclear cells
PCR	Polymerase chain reaction
PB	Peripheral blood
rr	Reference range
SAA	Severe aplastic anemia
TTV	Torque teno virus
TB	Total bilirubin

Introduction

Hepatitis-associated aplastic anemia (HAA) is a rare variant of acquired aplastic anemia (AA) [2]. The pathogenesis of HAA is believed to be associated with virus infections with subsequent $CD8^+$ T-cell-mediated injury directed to unidentified endogenous antigens expressed by hematopoietic stem cells [27]. No causative agent has been identified for the majority of cases [22], despite the reported cases associated with hepatitis A, B, C, and G, parvovirus B19, Epstein–Barr virus (EBV), and echovirus [1, 5, 8, 12, 13, 21, 28].

Torque teno virus (TTV), currently classified into the genus *Anellovirus*, was first isolated from a peripheral blood sample of a patient with post-transfusion non-A to non-E hepatitis [6, 15]. The virus has a single-stranded, circular DNA of approximately 3.8 kilobases and replicates not only in the liver but also in multiple tissues including

bone marrow (BM) [16, 18]. The high prevalence of TTV carrier in healthy adults has led to doubt regarding its role as an etiologic agent of hepatitis. No established screening for the virus-specific antibodies against many TTV variants has hampered in clarifying the pathological role of primary infection of each strain. An association of HAA with TTV has been suggested in two cases using only viral DNA screening without serology tests [10, 14].

We report herewith a 12-year-old boy who suffered from severe acute hepatitis and presented with a rapid progression of very severe AA during a primary infection of TTV/1a. This is the first identified case of TTV-HAA assessed by the anti-TTV antibodies and viral load in blood and BM.

Methods

Serum and mononuclear cell (MNC) samples were stored at −30°C and −80°C, respectively. TTV DNA was determined by polymerase chain reaction (PCR) using primers targeting N22 region [19], which detects TTV of genotypes 1a, 1b, and 2–6 classifiable in group 1 [17]. TTV DNA was quantified by real-time PCR (UTR PCR) capable of detecting all genotypes of TTV, using primers NG473-NG352, and a labeled probe NG369-P [5′-(Fam)-AGT CAAGGGGCAATTCGGGCTCGGGA-(Tamra)-3′] [24]; both of them were derived from the conserved sequences of the TTV genome. TTV genotype was determined by direct sequencing of the amplification products of the N22 PCR [19] and analyzed using Genetyx-Mac 12.2.7 (Genetyx Corp., Tokyo, Japan). Anti-TTV/1a IgG and IgM class antibodies were determined as reported previously [26].

Case report

A 12-year-old Japanese boy was hospitalized because of fever and malaise lasting a week and high transaminase levels [aspartate aminotransferase (AST), 1,828 U/L; alanine aminotransferase (ALT), 2,312 U/L]. Five days later, the patient was transferred to us because of precipitous jaundice [total bilirubin (TB), 28 mg/dl], leukocytopenia, and thrombocytopenia (WBC, 1.32×10^9/L; Hb, 12.8 g/dL; platelet, 50×10^9/L; Fig. 1). His past history and family history were not informative. On admission, he was alert with jaundice and had a coalescent maculopapular eruption on the trunk and extremities (Fig. 2). Body temperature was 37.4°C. Liver was palpated 10 cm under the costal margin. No spleen or lymph nodes were palpable. Peripheral blood (PB) counts showed 1.95×10^9/L WBC with 36.0% neutrophils, 53.8% lymphocytes, 9.7% eosinophils, and 0.5% monocytes, $4{,}590\times10^9$/L RBC, 12.8 g/dL Hb, 37.1% hematocrit, 3‰ reticulocyte, and a platelet count of 50×10^9/L. Blood chemistries revealed high levels of AST [827 U/L; reference range (rr), 13–33], ALT (1,210 U/L; rr, 6–30), TB (32.8 mg/dl; rr, 0.3–1.2), direct bilirubin (24.7 mg/dL; rr, 0.0–0.3), ferritin (1,132 ng/mL; rr, 39.9–465.0) and alpha fetoprotein (131.2 ng/mL; rr, <6.2), and

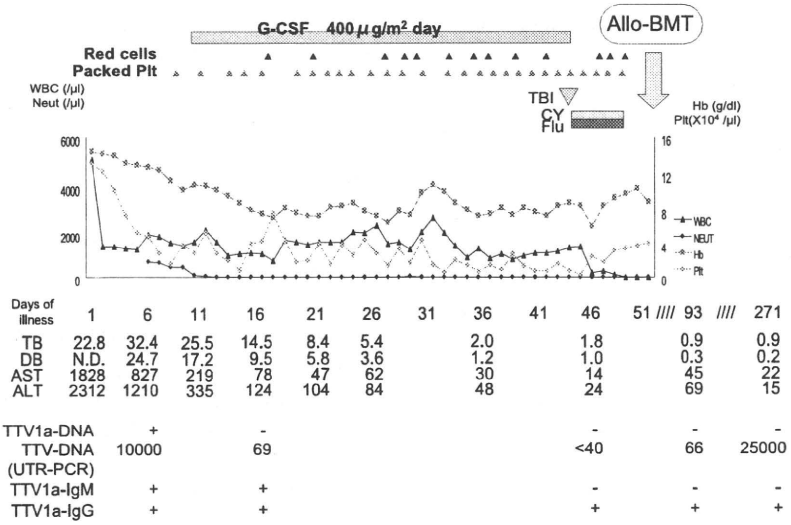

Fig. 1 Changes in the transaminase levels, hemogram, and torque teno virus (TTV) parameters during the clinical course of hepatitis-associated very severe aplastic anemia. Sequential pattern of TTV/1a-DNA and anti-TTV/1a specific antibody titers determined a primary infection of TTV/1a. The viral loads re-ascended after bone marrow transplantation assessed by the real-time UTR-PCR, which indicated a new infection of the other TTV genotypes most likely via multiple transfusions. *BMT* bone marrow transplantation, *CY* cyclophosphamide, *DB* direct bilirubin, *Flu* fludarabin, *G-CSF* granulocyte colony-stimulating factor, *PCR* polymerase chain reaction, *TB* total bilirubin, *Plt* platelet, *TBI* total body irradiation, *TTV* torque teno virus

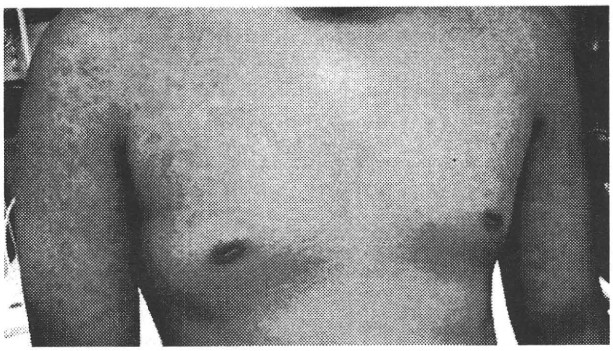

Fig. 2 A coalescent maculopapular eruption on the trunk and extremities on admission

low levels of total protein (6.5 g/dL; rr, 6.7–8.3) and albumin (3.5 g/dL; rr, 4.0–5.0). C-reactive protein concentration was 0.09 mg/dL (rr, <0.1). Coagulation studies showed low hepaplastin test (42%; rr, 60–120), prolonged prothrombin time (15.3 s; rr, 10.0–13.5), normal partial thromboplastin time (37.3 s; rr, 26.0–41.0). There was no evidence of Wilson's disease, drug intoxication, autoimmune hepatitis, or infections with hepatitis A, B, C, and E viruses, EBV, cytomegalovirus, herpes simplex virus, human herpesviruses 6 and 7, parvovirus B19, and mycoplasma. PB HLA-DR$^+$ T cells were increased (10.1%) with a normal CD4/CD8 ratio (1.82). The extremely hypocellular BM showed no morphologic/cytogenetic abnormality. Chromosome instability test was normal. A contrast-enhanced computed tomography of the abdomen showed swelling liver with periportal edema, indicating the diagnosis of acute hepatitis.

Gradual improvement of liver dysfunction required no liver transplantation; however, cytopenias were progressive (Fig. 1). Neutrophil counts became zero within 5 days after admission, and *Pseudomonas aeruginosa* sepsis occurred. He underwent successful BM transplantation (BMT) from an HLA-identical sister. He has been alive and well with complete hematological remission and normal transaminase levels for 27 months after BMT. Serum TTV DNA and anti-TTV-specific antibodies were sequentially analyzed (Fig. 1). The serum sample on admission was positive for both TTV/1a DNA and IgM/IgG-class anti-TTV/1a antibodies. TTV/1a DNA and IgM-class anti-TTV/1a became undetectable on 16th and 46th days after the onset of the illness, respectively, but anti-TTV/1a IgG antibody continued to be positive up through the observation period. TTV DNA load detectable by UTR PCR decreased from 10,000 to 69 copies per milliliter until the first transfusion and was then undetectable. The re-ascended copy number (25,000 copies per milliliter) indicated an infection of the genotype(s) of TTV via repeated transfusions. The complete sequence of the TTV DNA was the same as that of N22 clone, the first isolated human TTV [14]. TTV DNA was quantified at 1,200 copies/10^5 PB MNCs on admission (day 6 of illness) and 130 copies/10^5 MNCs obtained from hypoplastic BM at the diagnosis of AA (day 10 of illness).

Discussion

This is the first report of HAA during a primary infection of TTV. The isolated TTV/1a showed the same sequence of original strain in a patient with post-transfusion hepatitis [14]. Currently, TTV is not categorized as a hepatitis virus because healthy adults could also carry several genotypes. Two reported cases suggesting an association of TTV with HAA did not preclude the possibility of a carrier state [10, 14]. On the other hand, the pathogenicity of each genotype in primary infection remains unclear. Although TTV mostly elicits subclinical infections, some isolates/genotypes may have a pathological role in individuals with liver diseases [7], and neonatal hepatitis could occur in the association of TTV/1a primary infection [17, 23]. In the current patient, a possibility of chance association or reactivation of TTV/1a cannot be excluded. However, the sequential data of anti-TTV/1a antibodies and TTV loads indicated a primary infection of TTV/1a and further suggested that an infection of other genotype(s) could also occur via transfusions after the disappearance of circulating TTV/1a. The time course of liver dysfunction and subsequent cytopenias was in concert with the primary infection of TTV/1a but not with the infection of other genotype(s) (Fig. 1). Moreover, neither hepatitis nor recurrent TTV/1a infection occurred in immunosuppressive state throughout the period before and after BMT. Immunosuppressive therapy for HAA does not exacerbate the preceding hepatitis [20].

The major concern is the mechanism of acute BM failure associated with TTV/1a. In this patient, cytopenias started at the peak transaminase levels, and activated CD8$^+$ T cells were not extremely increased. We first suspected acute liver failure due to the hepato- and hemato-toxic drugs such as organic solvent. However, fever and maculopapular eruptions were suggestive of viral infections. The clinical features might not be atypical for HAA because 30% of patients showed anemia within a week of the onset of hepatitis, and not all patients showed increased activated T cells [2, 11]. TTV/1a replicates not only in hepatocytes but also PB MNC lines [3]. TTV mRNAs were detected in the BM cells obtained from an infected individual [18]. The replicative intermediate forms of TTV DNA are found in BM cells but not in PB MNCs [16]. The effect of TTV/1a on the growth of hematopoietic cells might be more critical than that of the other genotypes [25]. In BM cells, TTV may prefer the erythroid lineage cells because the promoter activity of TTV was highest in K562 cells (erythroid origin) than other cell lines including HepG2 and Huh7 cells [9].