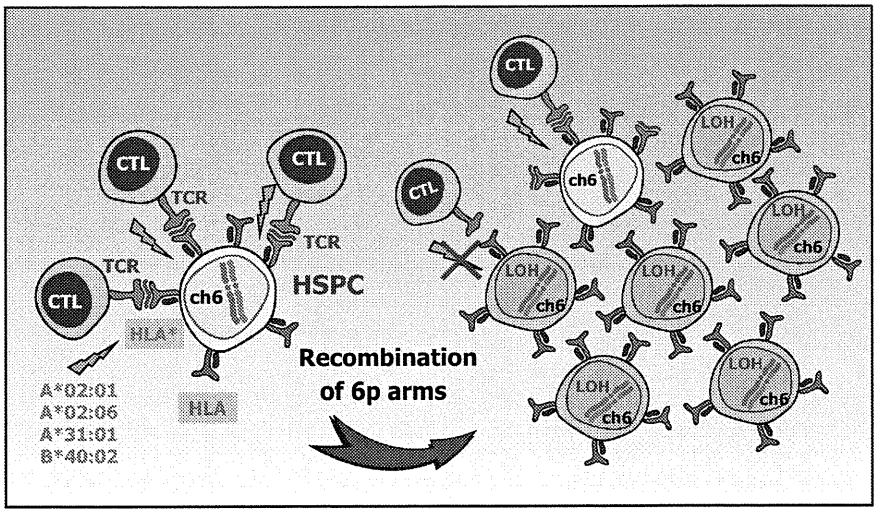

Figure 5. A proposed mechanism for escape hematopoiesis in 6pLOH(+) AA. In AA, the targets of CTLs are the HSPCs that present some auto-antigen through particular class I HLA molecules, including HLA-A*02:01, A*02:06, A*31:01, and B*40:02. In the presence of these autoimmune insults, the HSPCs that lose their expression of the antigen-presenting HLA molecule as a result of CNN-LOH in 6p would acquire a growth advantage over other HSPCs expressing the relevant HLA, leading to clonal outgrowth of the 6pLOH(+) progenies.

associated with the development of AA in Japanese patients in case-control studies using the large JMDP registry.

The conspicuous bias of the missing HLA alleles in 6pLOH to particular HLA types and the significant association of AA with those HLA types strongly suggest that the recurrent 6pLOH in AA is a phenomenon tightly related to the pathogenesis of AA rather than mere secondary event during the course of AA. Based on these observations, it is well reasoned that, in 6pLOH(+) AA cases, the autoimmunity to HSPCs is mediated by the CTLs that target the antigens presented via specific class I HLA molecules and that the 6pLOH(+) cells found in AA could be explained as escape hematopoiesis that survives the autoimmune insult by genetically deleting the relevant HLA species that are required for antigen presentation (Figure 5). These scenarios are further supported by the recent reports showing that the CNN-LOH in 6p provides a common mechanism of leukemic relapse after HLA haploidentical stem cell transplantations, in which leukemic cells that lost the mismatched HLA haplotype through CNN-LOH in 6p are thought to escape the immunologic surveillance of the engrafted donor T cells.[25,26] Importantly, it was experimentally demonstrated by immunologic assays that the 6pLOH(+) leukemic cells actually escaped GVL by CTLs, whereas 6pLOH(−) leukemic cells were effectively killed by the same CTLs. Although the immunologic targets of CTLs are different between relapse after haploidentical transplants (mismatched HLAs themselves) and AA (still unknown autoantigens presented on missing HLAs), the prominent similarities found in both cases further support that CNN-LOH in 6p confers an escape mechanism from autoreactive CTLs in AA.

In light of the above considerations, the chronologic behavior of the 6pLOH(+) components in PB is also interesting and worth discussing. Despite the assumption that 6pLOH is an effective escape mechanism from CTLs, the 6pLOH(+) stem cells were unable to repopulate the BM to cure AA, unless effective IST was applied (supplemental Figure 6). This is most probably explained by the presence of inflammatory cytokines, such as IFN-γ and TNF-α, which have also been shown to play an important role in the BM failure in AA and are thought to be responsible for the continued prevention of the 6pLOH(+) stem cells from fully expanding and reconstituting the BM (supplemental Figure 9A-B).[27,28]

When the autoimmune insults are removed after IST, no further injury of normal stem cells would occur. However, this does not necessarily mean the surviving normal stem cells can eventually outnumber the 6pLOH(+) stem cells over time. Note that, once the autoimmune insults disappear, nothing could biologically or immunologically discriminate a 6pLOH(+) stem cell from a 6pLOH(−) stem cell (supplemental Figure 9A). In particular, a 6pLOH(+) stem cell and a 6pLOH(−) stem cell will produce the same number of progeny on average and feed the same number of mature blood cells. As a consequence, once established, the predominance of 6pLOH(+) stem cells over 6pLOH(−) stem cells should be maintained, after the severely reduced hematopoietic stem cell pool has been re-expanded with removal of the inciting autoimmunity. It is also of note that the recovery of myeloid components after IST, which are affected more strongly by 6pLOH than lymphoid cells, contributes to an apparent increase in 6pLOH components in the SNP array analysis in PB (supplemental Figure 6A).

One of the most significant findings in the current study is the identification of the HLA alleles that are over-represented in the Japanese AA populations, including HLA-A*31:01, B*40:02, A*02:01, and A*02:06. All of these HLA alleles belong to class I MHCs and thus are thought to be involved in the antigen presentation to CTLs. This provides another prominent example, in which specific HLA types play a critical role in the development of a human disease, and the information about these particular HLA types provides a solid basis on which we can ultimately isolate the relevant antigens responsible for the development of AA. Of particular note, there was a previous report indicating that HLA-B*40:02 and A*02:06 were over-represented in PNH as well as AA, although the study size was much smaller than the current study.[29] Combined with our study, these findings support the hypothesis that AA and PNH are the different outcomes of the same immunologic insult[5,30] and may also provide the genetic basis of the high prevalence of AA and PNH in East Asia.[31,32]

In some AA cases, hematopoiesis could be maintained over years by the progenitors that escaped and survived the inciting autoimmune insult by deleting the target HLA through CNN-LOH in 6p. Given that the 6pLOH was detected in only 13% of our series, it is probable that other escape mechanisms may also operate to maintain hematopoiesis in AA. Indeed, clonality was clearly demonstrated in 20% of the 6pLOH(−) cases in the human androgen receptor assay study (supplemental Figure 8). In addition, our SNP array analysis also revealed a variety of clonal abnormalities in AA cases (Figure 1), although it is still open to question

whether these abnormalities actually represent the mechanism of escape hematopoiesis or were related to some neoplastic process. Further studies on the genetic basis of the escape mechanisms would contribute to our understanding of the molecular pathogenesis of AA.

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Authorship

Contribution: S. Ohtake, S. Ogawa, and S.N. developed the concept of the study and supervised the project; T.K., S. Ohtake, and S.N. designed the experiments; T.K., A.S.-O., Y. Sato, Y. Mori, M.K., M.S., K.H., and Y. Sasaki performed the experiments and analyzed the data; K.K. performed high-resolution HLA typing; S.M. and Y. Morishima provided the information of JMDP donor-recipient pairs (JMDP dataset); T.K., A.S.-O., S. Ogawa, and S.N. wrote the paper; and all authors approved the final version of the manuscript.

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Reduced-intensity conditioning for alternative donor hematopoietic stem cell transplantation in patients with dyskeratosis congenita

Nishio N, Takahashi Y, Ohashi H, Doisaki S, Muramatsu H, Hama A, Shimada A, Yagasaki H, Kojima S. Reduced-intensity conditioning for alternative donor hematopoietic stem cell transplantation in patients with dyskeratosis congenita.
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Abstract: DC is an inherited bone marrow failure syndrome mainly characterized by nail dystrophy, abnormal skin pigmentation, and oral leukoplakia. Bone marrow failure is the most common cause of death in patients with DC. Because previous results of HSCT with a myeloablative regimen were disappointing, we used a reduced-intensity conditioning regimen for two patients with classic DC, and one patient with cryptic DC who harbored the *TERT* mutation. Graft sources included two mismatched-related bone marrow (BM) donors and one unrelated BM donor. Successful engraftment was achieved with few regimen-related toxicities in all patients. They were alive 10, 66, and 72 months after transplantation, respectively. Long-term follow-up is crucial to determine the late effects of our conditioning regimen.

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Key words: dyskeratosis congenita – non-myeloablative hematopoietic stem cell transplantation

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DC is an inherited multisystem bone marrow failure syndrome characterized by nail dystrophy, abnormal skin pigmentation, oral leukoplakia, and cancer predisposition. Patients with DC have very short germ-line telomeres compared with normal individuals because of a defect of telomere maintenance. Until now, mutations in six genes (*DKC1*, *TERC*, *TERT*, *NOP10*, *NHP2*, and *TINF2*) involved in telomere maintenance have been identified in patients with DC (1).

Abbreviations: ATG, anti-thymocyte globulin; CMV, cytomegalovirus; DC, dyskeratosis congenita; EBV, Epstein–Barr virus; GVHD, graft-versus-host disease; HSCT, hematopoietic stem cell transplantation; IST, immunosuppressive therapy; RIST, reduced-intensity stem cell transplantation; TBI, total body irradiation.

Bone marrow failure develops in 80–90% of patients with DC and is the most common cause of death, up to 60–70% (2, 3). Although androgen has been used to improve cytopenia since the 1960s, allogeneic HSCT is the only curative treatment for bone marrow failure in patients with DC. However, the outcome in previous reports has been disappointing because of unacceptable transplant-related toxicities such as severe pulmonary/liver complications especially in transplant using a myeloablative conditioning regimen or transplants from an alternative donor (3, 4).

To avoid transplant-related complications, RIST using a non-myeloablative conditioning regimen has been recently used in patients with DC, and encouraging short-term survival has been achieved. Reducing the intensity of

conditioning results in less tissue damage and decreased inflammatory cytokine release compared with myeloablative transplantation (5). However, until now, there have been only a few reports of non-myeloablative transplants, especially from an alternative donor. Here, we report our encouraging results of RIST from an alternative donor using a fludarabine-based conditioning regimen and *in vivo* T-cell depletion by ATG in three patients with DC.

Patients and methods

Case 1

Patient 1 was a 21-yr-old man with classical DC with nail dystrophy, abnormal skin pigmentation, oral leukoplakia, and bone marrow failure. He had no family history of physical or hematologic abnormalities. Nail changes began to develop in early childhood. He suffered from cytopenia and was diagnosed with aplastic anemia at the age of 9. At age 18, he was referred to our hospital and was diagnosed as having DC. He had very short telomeres – i.e., less than the first percentile for his age – although mutation analysis did not identify any mutations in *DKC1*, *TERC*, *TERT*, *NOP10*, or *TINF2*. As pancytopenia progressed, we planned HSCT from a sister who was mismatched at HLA DRB1 allele. He did not undergo HSCT before. Conditioning regimen included cyclophosphamide 750 mg/m^2/day, fludarabine 25 mg/m^2/day, and rabbit-ATG (Thymoglobulin; Genzyme, Cambridge, MA, USA) 2.5 mg/kg/day, all from days −5 to −2, and total lymphoid irradiation 3 Gy (1 fraction) on day −1. GVHD prophylaxis comprised tacrolimus (intravenous infusion of 0.02 mg/kg/day starting on day −1, with dose adjustments to maintain blood levels of 5–15 ng/dL) and short-term methotrexate (15 mg/m^2 on day +1 and 10 mg/m^2 on days +3, +6, and +11). The administration route of tacrolimus was switched to oral after patients recovered from gastrointestinal toxicity.

Case 2

Patient 2 was a nine-yr-old girl with aplastic anemia without any physical abnormalities. At first, she was diagnosed with acquired aplastic anemia of unknown cause. However, she was identified as having a heterozygous *TERT* mutation (T726M) and very short telomeres in our retrospective study of mutation screening for telomere-related genes. She was born to healthy non-consanguineous parents and had no family history of physical or hematologic abnormalities. Subsequent screening of her family members revealed that her father had the same heterozygous *TERT* mutation (6). She was diagnosed with very severe aplastic anemia at the age of 8. She received IST with horse-ATG (Lymphoglobulin; Genzyme) 15 mg/kg/day intravenously for five days and cyclosporine because she had no HLA-matched family donor. However, the response to IST was poor, and she was still transfusion-dependent for six months after treatment. At first, she underwent HSCT from an HLA DRB1 one-allele-mismatched unrelated donor. The first conditioning regimen included cyclophosphamide 50 mg/kg/day for four days, TBI 5 Gy (two fractions), and rabbit-ATG (Thymoglobulin; Genzyme) 2.5 mg/kg for four days. Patient failed to engraft and had no autologous recovery of her bone marrow. She underwent a second transplant from an HLA B and DRB1 alleles-mismatched mother, 48 days post-transplant as salvage therapy. Conditioning regimen included fludarabine 30 mg/m^2/day and ATG 2.5 mg/kg/day from days −5 to −2, and melphalan 60 mg/m^2/day on days −2 and −1. GVHD prophylaxis was the same as for case 1.

Case 3

Patient 3 was an 18-yr-old man with classical DC with nail dystrophy, abnormal skin pigmentation, oral leukoplakia, and bone marrow failure. He had very short telomeres, and mutation analysis showed *DKC1* mutation. Nail changes began in early childhood, and pancytopenia was noted at age 13. Because pancytopenia progressed, we planned HSCT from an HLA 6/6 alleles-matched unrelated donor. He did not undergo HSCT before. Conditioning regimen included cyclophosphamide 750 mg/m^2/day, fludarabine 25 mg/m^2/day, and rabbit-ATG 2.5 mg/kg/day, all from days −5 to −2, and TBI 3 Gy (one fraction) on day −1. GVHD prophylaxis was the same as for cases 1 and 2.

Table 1 shows patient and disease characteristics. Pre-transplant cardiac, lung, or liver dysfunction was not observed in any patient except for slight elevation of liver transaminase levels in patient 2. Bone marrow examination

Table 1. Patient and disease characteristics

Patient no.	Sex	Age at diagnosis of DC	Mutation	Clinical triad	Other synptoms	Pre-transplant hematological data			Number of pre-transplant transfusions		Cytogenetics
						ANC (×10^9/L)	Hb (g/dL)	PLT (×10^9/L)	RBC	PLT	
1	Male	18	Not detected	Nail, skin, oral	Cerebellar hypoplasia, growth retardation	0.9	5.7	16	25	2	46, XY
2	Female	9	*TERT*	None	None	0.3	6	0.9	40	90	46, XX
3	Male	15	*DKC1*	Nail, skin, oral	None	0.84	7.7	19	0	2	46, XY

ANC, absolute neutrophil count; Hb, hemoglobin; PLT, platelet; RBC, red blood cell.

Table 2. Pre-transplant characteristics of donors and patients

Patient no.	Age at transplant	Donor	Donor sex	Donor age	ABO incompatibility	Source	HLA match	Mismatch locus
1	21	Sister	Female	24	Compatible	BM	5/6	DR
2	9	Mother	Female	36	Major	BM + PBSC	4/6	B, DR
3	18	UD	Female	37	Compatible	BM	6/6	–

UD, unrelated donor; BM, bone marrow; PBSC, peripheral blood stem cell.

revealed severe hypocellularity and normal karyotypes in all three patients. Table 2 shows pretransplant characteristics of donors and patients.

Supportive care

All patients received trimethoprim-sulfamethoxazole orally or inhaled pentamidine as prophylaxis against *Pneumocystis jiroveci.* Patients received standard doses of oral amphotericin B and acyclovir as fungal and viral prophylaxis. Patients received pre-emptive therapy with ganciclovir when CMV antigenemia became positive. Weekly viral studies for CMV, EBV and human herpesvirus 6 were obtained until day 90 post-transplant (7). Granulocyte colony-stimulating factor was started from day 5 to neutrophil engraftment. Acute and chronic GVHD was diagnosed and graded according to established criteria (8, 9).

Results

Transplant outcomes are shown in Table 3. Engraftment day was defined as the first of three consecutive days in which the patient had an absolute neutrophil count greater than 0.5×10^9/L. Neutrophil engraftment was achieved in all patients, although the days of platelet recovery were delayed. Analysis of short tandem repeats or fluorescent *in situ* hybridization of sex chromosomes revealed that all patients achieved >95% donor chimerism by day 100.

Engraftment syndrome developed in patient 3 and responded well to steroid therapy (10, 11). Acute GVHD did not occur in any patient, while chronic GVHD of the skin occurred in patient 3 and responded to tacrolimus therapy. Patients 1 and 2 discontinued their treatment with immunosuppressive drugs at 18 and 14 months, respectively, following transplant.

Increases in the EBV genome load were observed in patients 1 and 3. The dose of tacrolimus was decreased in patient 1, and one course of rituximab was administered in patient 3. As a result, EBV genome load decreased in both patients. Positive CMV antigenemia was seen only in patient 3. Preemptive therapy with ganciclovir was administered until the test for CMV antigenemia became negative. He did not progress to CMV disease.

To date, all three patients are alive with a follow-up of 10, 66, and 72 months, respectively. No patients have developed pulmonary or liver complications or malignancies.

Discussion

In our case reports, we report the outcome of two patients with classical DC and one patient with aplastic anemia harboring the *TERT* mutation to assess the feasibility and efficacy of a fludarabine-based non-myeloablative regimen. Our regimens are promising, as all three patients achieved complete chimerism and hematologic recovery without severe transplant-related toxicities.

Previously, results of HSCT using a myeloablative regimen for patients with DC were disappointing mainly because of pulmonary/liver complications and GVHD (12–19). Until recently, there were no survivors who received unrelated sources of stem cells (3). A high transplant-related mortality rate is considered

Table 3. Outcomes of transplantation

	Cell dose		Engraftment		GVHD				
Patient no.	NCC ($\times 10^8$)	CD34 ($\times 10^6$)	ANC (>500 μL)	PLT (>20 $\times 10^9$/L)	Acute	Chronic	Complication	Follow-up	Outcome
1	3.45	1.73	16	31	No	No	EBV reactivation	5 yr 6 months	Alive
2	9.6	2.6	23	123	No	No	DM, enteritis	6 yr	Alive
3	0.81	N.E	19	111	No	Skin	Sepsis, engraftment syndrome, CMV antigenemia, EBV reactivation	10 months	Alive

NCC, nuclear cell count; ANC, absolute neutrophil count; PLT, platelet; N.E, not evaluated.

to be associated with impaired restorative ability of tissue damage because of defective telomere maintenance. To avoid these complications, reduced-intensity regimens have been recently used and have achieved engraftment with fewer complications in both related and unrelated settings (16, 20–24). Most recently, Dietz et al. reported encouraging results of six patients with DC who underwent HSCT using fludarabine-based non-myeloablative regimens (26). Their non-myeloablative regimen consisted of cyclophosphamide 50 mg/kg for one day, fludarabine 40 mg/m^2 for five days, and TBI 2 Gy and alemtuzumab 0.2 mg/kg for five days. Engraftment was achieved in five of six patients. Four patients are alive, three of whom were recipients of unrelated grafts. Our regimen is similar to theirs, including cyclophosphamide, fludarabine, low-dose irradiation, and ATG instead of alemtuzumab. The results of HSCT from an alternative donor for DC are shown in Table 4.

It is still unclear whether HSCT can prolong the overall survival of patients with DC. Dietz et al. combined 18 cases who had undergone RIST in the literature with their six cases and calculated an overall survival rate of 65%, which was similar to another historical cohort that included both myeloablative and non-myeloablative transplants reported by Alter et al. (4). However, the follow-up periods in non-myeloablative transplants seem to be shorter than in myeloablative transplants. Although bone marrow failure is the most common cause of death in patients with DC, pulmonary fibrosis is another common cause of death (27). Alter et al. reviewed 65 patients who had received HSCT until 2008 (4). According to the review, nine of 30 deaths after HSCT were because of pulmonary fibrosis, suggesting that the high rate of this lung complication might originate from the natural history of DC. A prospective long-term follow-up study is necessary to clarify whether HSCT procedures, including conditioning agents and allogeneic immune responses to recipient's organ such as the lungs and liver, affect the natural course of DC.

Fludarabine is a potent immunosuppressive and less myeloablative agent, which has been used successfully in RIST for aplastic anemia (28) and other bone marrow failure syndromes

Table 4. Summary of HSCT from an alternative donor for dyskeratosis congenita

Patient	Age/sex	Donor source	HLA	Conditioning regimen	Outcome	Complication	References
1	23/M	MUD BM	6/6	CY 120 mg/kg and TBI 12 Gy	Death	Disseminated candidasis	Langston et al. (16)
2	20/M	MUD BM	6/6	CY 120 mg/kg and TBI 12 Gy	Death	Disseminated candidasis	Langston et al. (16)
3	29/M	MUD BM	6/6	CY 200 mg/kg and TBI 6 Gy	Death	Rejection Died of respiratory failure after 2nd BMT	Dokal et al. (17)
4	3/M	MUD	6/6	CY 120 mg/kg, Flu 180 mg/m^2 and ATG 160 mg/kg	Alive >15 months		Dror et al. (24)
5	8/F	MUD	6/6	CY 120 mg/kg, Flu 180 mg/m^2 and ATG 160 mg/kg	Alive >16 months	EBV reactivation	Dror et al. (24)
6	15/M	MUD	6/6	CY 120 mg/kg, Flu 180 mg/m^2 and ATG 160 mg/kg	Death	Cardio-respiratory arrest on day 0 Diffuse capillaritis	Brazzola et al. (25)
7	24/M	MMUD dUCB	4/6 4/6	CY 50 mg/kg, Flu 200 mg/m^2, TBI 2 Gy and Alem 1 mg/kg	Death	Sepsis outside of hospital	Dietz et al. (26)
8	5/F	MUD BM	6/6	CY 50 mg/kg, Flu 200 mg/m^2, TBI 2 Gy and Alem 1 mg/kg	Alive >40 months		Dietz et al. (26)
9	2/M	MUD BM	8/8	CY 50 mg/kg, Flu 200 mg/m^2, TBI 2 Gy and Alem 1 mg/kg	Death	Adenoviral sepsis	Dietz et al. (26)
10	18/F	MMUD dUCB	4/6 5/6	CY 50 mg/kg, Flu 200 mg/m^2, TBI 2 Gy and Alem 1 mg/kg	Alive >12 months	Acute GVHD grade IV (gut)	Dietz et al. (26)
11	25/M	MMUD dUCB	4/6 5/6	CY 50 mg/kg, Flu 200 mg/m^2, TBI 2 Gy and Alem 1 mg/kg	Alive >12 months		Dietz et al. (26)
12	21/M	MMRD BM	5/6	CY 3 g/m^2, Flu 100 mg/m^2, TLI 3 Gy and ATG 10 mg/kg	Alive >5 yr	EBV reactivation	This report
13	9/F	MMRD BM+PBSC	4/6	MEL 120 mg/m^2, Flu 120 mg/m^2 and ATG 10 mg/kg	Alive >6 yr	DM, enteritis	This report
14	18/M	MUD	6/6	CY 3 g/m^2, Flu 100 mg/m^2, TBI 3 Gy and ATG 10 mg/kg	Alive >10 months	Sepsis, engraftment syndrome, EBV reactivation	This report

MUD, matched unrelated donor; MMUD, mismatched unrelated donor; MMRD, mismatched related donor; BM, bone marrow; PBSC, peripheral blood stem cell; dUCB, double unrelated cord blood; CY, cyclophosphamide; Flu, fludarabine; Alem, alemtuzumab; MEL, melphalan; BMT, bone marrow transplantation; DM, diabetes mellitus.

such as Fanconi anemia (29), Shwachman-Diamond syndrome (30), and Diamond-Blackfan anemia (31). In this study, fludarabine seemed to be well tolerated in patients with DC who achieved engraftment even after transplant from an alternative donor.

Reduction in the dose of cyclophosphamide may contribute to a decrease in transplant-related toxicity. We administered cyclophosphamide at a total dose of 3000 mg/m^2, which was a tolerable dose for our patients. In several reports, the total dose was reduced to 40–50 mg/kg with durable engraftment. However, one patient who received 50 mg/kg cyclophosphamide and an unrelated double-cord graft (one set of 4/6 and 4/6 HLA match) developed primary graft failure (26). The appropriate dose of cyclophosphamide remains undetermined.

The dose of irradiation is another important issue to achieve engraftment without increasing toxicities. Because patients with DC possess chromosomal instability, they are suspected to show increased radiosensitivity. In fact, a full-dose TBI regimen resulted in unacceptable toxicities in previous reports (16). From our experience as well as other reports, inclusion of low-dose TBI may contribute to achieve durable engraftment without undesirable complications.

Dietz et al. tried to provide a natural pulmonary compensation by delivering irradiation side-to-side, instead of anterior-to-posterior, with the patient in a seated position and the arms resting at the side of the thoracic cage (26). In our institute, patients are in a supine position with the arms at the side of the thoracic cage during TBI, which is delivered side-to-side. Our method also can provide for pulmonary compensation. In addition to the dose of irradiation, the method of irradiation may be important to assess the true effects on lungs in patients with DC.

GVHD prophylaxis is another important issue for successful transplant from an alternative donor. *In vivo* T-cell depletion can reduce the risk of GVHD in HSCT for bone marrow failure syndrome (32). Our conditioning regimen included rabbit-ATG for the purpose of *in vivo* T-cell depletion to prevent severe acute GVHD. Acute GVHD did not occur in any patient in our series, even in the patient who received both bone marrow and peripheral blood from an HLA haploidentical donor. Finke et al. reported the outcome of patients with hematologic malignancies who underwent HSCT from unrelated donors using a regimen containing rabbit-ATG (33). The cumulative incidence of grade II-IV acute GVHD and chronic GVHD for HLA-mismatched transplantation was 20% and 44%, respectively, which were equal to 21% and 43% for HLA-matched transplants. The authors concluded that a single-antigen mismatch might not compromise the outcome after HSCT from an unrelated donor when ATG is used in addition to standard GVHD prophylaxis.

In conclusion, our study indicated that RIST can provide successful engraftment with few complications in patients with DC, even in transplants from an alternative donor. Long-term follow-up is crucial to monitor the late effects of conditioning agents and allogeneic immune responses to the recipient's organs, such as the lungs and liver. Given these encouraging results, we believe that RIST should be explored further.

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Decreased Serum Testosterone Levels in Long-Term Adult Survivors with Fatty Liver after Childhood Stem Cell Transplantation

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Fatty liver and male gonadal dysfunction are potential late effects of therapy in adult survivors treated with stem cell transplantation (SCT) in childhood. Obesity and metabolic syndrome also are associated with low serum testosterone levels in the general population. However, the relationship between the degree of fatty liver and changes in serum testosterone levels in adult survivors has not been fully studied. We reviewed the clinical records of 34 male patients who received allogeneic SCT in childhood or adolescence. The median age at SCT was 10.0 years, and the median follow-up after SCT was 15.9 years. All but one patient showed no tendency toward overweight/obesity during the follow-up period. Fatty liver was diagnosed by ultrasound in 15 patients at 4 to 20 years after SCT. Patients who received cranial radiation therapy before SCT were more likely to develop fatty liver and insulin resistance. Moreover, fatty liver was statistically associated with decreased serum testosterone levels, whereas nonfatty liver was not (median, 527 ng/dL [range, 168-944 ng/dL] versus 302 ng/dL [165-698 ng/dL]; $P < .0001$). Changes in testosterone levels after SCT are affected not only by primary gonadal dysfunction but also by subsequent development or exacerbation of fatty liver.

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KEY WORDS: Insulin resistance, Gonadal function, Cranial radiation therapy, Childhood cancer survivor, Obesity

INTRODUCTION

Continuing advances in the management of childhood malignancies are producing a rapidly enlarging group of childhood cancer survivors [1-3]. With survival rates currently at 70% to 80%, an estimated one in 700 of the young adult population in Japan is a long-term survivor of childhood cancer [4,5]. Children and adult survivors commonly experience morbidity, generally related to the treatment they received to cure their cancer rather than to the cancer itself. Treatment-related morbidity is extraordinarily diverse. Childhood cancer survivors are at significant risk for developing late effects after successful cancer treatment during childhood. In particular, endocrine system disorders are common, affecting up to 40% of childhood cancer survivors and include gonadal dysfunction, metabolic disorders, thyroid dysfunction, and growth impairment [6-11].

Fatty liver is associated with metabolic abnormalities characterized by obesity [12], type 2 diabetes mellitus (DM) [13], dyslipidemia [14], and hypertension [15], each of which also carries a cardiovascular disease risk. Moreover, fatty liver is increasingly recognized as a major cause of liver-related morbidity and mortality in the general population because of its potential to progress to cirrhosis and liver failure [16]. Numerous epidemiologic investigations in the general population have established associations between low serum testosterone level and obesity and metabolic syndrome [17-19]; however, the mechanism underlying these conditions is incompletely understood. In particular, whether low serum testosterone causes metabolic syndrome or vice versa has not been clarified. We hypothesized that fatty liver with insulin resistance may be considered as the cause of reduced testosterone levels in adult childhood cancer survivors.

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Although metabolic syndrome and gonadal dysfunction in adult survivors have been well documented, a longitudinal study investigating the relationship between the degree of fatty liver and changes in gonadal function in survivors of childhood stem cell transplantation (SCT) has not yet been reported, and the mechanism of these conditions has not been completely elucidated. To test our hypothesis, we performed a longitudinal retrospective study of adult male survivors of childhood cancer treated with SCT to investigate the relationship between fatty liver and gonadal function.

METHODS

Patients

We reviewed the clinical records of 98 male patients who underwent SCT at Tokai University Hospital between 1982 and 1997 and had annual follow-up. Inclusion criteria were survival for at least 5 years after SCT, age at least 18 years at the last evaluation, and no past history of or laboratory findings suggesting liver dysfunction or endocrine or metabolic abnormalities before SCT. Thirty-four adult survivors met these criteria and were included in this analysis. In these 34 patients, the median age at the time of SCT was 10.0 years, the median age at the last evaluation was 25.1 years, and the median duration of follow-up after SCT was 16.3 years (Table 1). To examine the risk factors for developing fatty liver, the study population was categorized into four groups according to age at SCT, primary disease, presence of fatty liver, doses of radiation to the brain and testes, and conditioning regimen received: the cranial radiation therapy (CRT) plus total body irradiation (TBI) group, the TBI group, the thoracoabdominal irradiation (TAI) group, and the chemotherapy (Chemo) group. Patient characteristics are summarized in Table 1. Written informed consent was obtained from all patients and/or their parents. This study was approved by Tokai University's Clinical Research Review Committee.

Transplantation Procedure

In addition to conventional chemotherapy, three patients with acute lymphoblastic leukemia (ALL) and one patient with non-Hodgkin lymphoma (NHL) received prophylactic CRT (one with 12 Gy and three with 18 Gy) at 1 to 5 years before SCT. In 28 patients, conditioning regimens consisted of irradiation with or without cyclophosphamide (CY) and/or other drugs; 8 to 12 Gy of TBI was given in four to six fractions for patients with malignant diseases, and 6 to 8 Gy of TAI was given in three or four fractions for patients with nonmalignant diseases. The testes were shielded from radiation during the TAI procedures. The remaining six patients received conditioning without irradiation. Prophylaxis against graft-versus-host disease (GVHD) varied during the time period, with methotrexate, cyclosporine, or a combination of the two drugs used.

Anthropometric Measures of Body Composition

All patients had achieved their final height at the last evaluation. Body mass index (BMI) was calculated as weight in kilograms divided by the height in meters squared (kg/m^2). Patients with a BMI $\geq$25 kg/m^2 were classified as overweight/obese, and those with a BMI $\leq$18.5 kg/m^2 were classified as underweight in accordance with World Health Organization criteria [20]. Waist circumference (WC) was measured at the level of the superior iliac crest. Abdominal adiposity was defined as a WC-to-height ratio >0.5 [21-23]. Bioelectronic impedance analysis was performed to measure body fat by (InnerScan; Tanita, Tokyo, Japan).

Evaluation of Fatty Liver

Fatty liver was evaluated by a total of 303 longitudinal ultrasound examinations performed in the 34 patients during the follow-up period. Up to 1994, Hitachi EUB340 (Hitachi, Tokyo, Japan) and Yokogawa RT2800 and RT3000 (GE Yokogawa Medical Systems, Tokyo, Japan) ultrasound systems were used to evaluate fatty liver. After 1995, this evaluation was done using an Aloka SSD 650CL system (Aloka, Tokyo, Japan). Of the four criteria used for the diagnosis of fatty liver—hepatorenal echo contrast (HRE), liver brightness (LB), deep attenuation (DA), and vascular blurring (VB)—the first two were used as definitive criteria, and the last two were taken into account as needed [24]. The degree of fatty liver was classified as follows: severe, both HRE and LB positive; moderate, either HRE or LB positive and/or either DA or VB positive; or mild, HRE, LB, DA, and VB negative. In all cases, two gastroenterology specialists separately confirmed the diagnosis without access to detailed information about the patient's background. Abdominal computed tomography was performed in 23 patients, and the results were coordinated with fatty liver diagnosed by ultrasound, as described in detail elsewhere [9].

Evaluation of Glucose and Lipid Metabolism Profiles

An overnight fasting blood sample was obtained from all patients for the measurement of plasma glucose, plasma insulin, serum triglycerides (TG), serum total cholesterol (TC), serum high-density lipoprotein cholesterol (HDL-C), serum low-density lipoprotein-cholesterol (LDL-C), and serum-free fatty acid. To convert our data to SI units, multiply plasma glucose by 0.0555 (result in mmol/L), plasma insulin by

Table 1. Patient Characteristics

	All (n = 34)	Fatty Liver (n = 15)	Non-Fatty Liver (n = 19)	*P* Value[a]
Age at SCT, years, median (range)	10.0 (0.7-15.8)	8.8 (0.7-15.5)	10.5 (0.9-15.8)	.627
Age at last evaluation, years, median (range)	25.1 (18.0-36.0)	24.8 (18.0-36.0)	25.3 (18.0-33.2)	.945
Dollow-up duration after SCT, years, median (range)	16.3 (6.7-27.7)	16.0 (6.7-27.7)	16.8 (7.6-24.8)	1
Primary disease, n				
Malignant disease				
Acute lymphoblastic leukemia	8	5	3	
Acute myelogenous leukemia	6	3	3	
Chronic myelogenous leukemia	3	2	1	
Non-Hodgkin lymphoma	4	1	3	
Nonmalignant disease				
Aplastic anemia	8	2	6	
Other	5	2	3	
Cranial radiation before SCT, n	4	3	1	
Conditioning regimen for SCT, n				
TBI + cyclophosphamide + other drugs	20	9	11	
Thoracoabdominal irradiation + cyclophosphamide + other drugs	8	3	5	
Chemotherapy	6	3	3	
Radiation therapy, n				
Brain 0 Gy	14	6	8	
Brain ≥8 Gy	20	9	11	
Testes 0 Gy	14	6	8	
Testes 8-12 Gy	20	9	11	

[a]For comparison of fatty liver and non-fatty liver.

6.945 (pmol/L), serum TG by 0.0113 (mmol/L), and serum TC, serum HDL-C, and serum LDL-C by 0.0259 (mmol/L).

Insulin resistance was estimated by the homeostatic model assessment of insulin resistance (HOMA-IR) using the formula fasting plasma insulin (mU/L) × plasma glucose (mg/dL)/405, with a value ≥2.5 indicating insulin resistance. The oral glucose tolerance test (OGTT) was performed in 25 patients for evaluation of glucose and insulin metabolism. For the OGTT, the patient was given glucose at 1.75 g/kg (maximum, 75 g) after a 12-h overnight fast, and samples for measurement of plasma glucose and plasma insulin were drawn at baseline and every 30 minutes for up to 120 minutes. Hyperinsulinemia was defined as a fasting plasma insulin value of ≥13 mU/L or a peak plasma insulin level ≥150 mU/L on the OGTT. Definitions of DM and impaired glucose tolerance were based on Japan Diabetes Society criteria. The presence of either type 1 or type 2 DM was diagnosed by a fasting plasma glucose level ≥126 mg/dL and/or a plasma glucose level ≥200 mg/dL at 2 h after the glucose load. A random plasma glucose value >200 mg/dL was also considered to indicate DM. A fasting plasma glucose level <110 mg/dL and a 2-h plasma glucose level <140 mg/dL were considered normal values. Impaired glucose tolerance was diagnosed in patients with values that were not normal but that did not indicate DM.

Evaluation of Metabolic Syndrome

Metabolic syndrome was defined according to the criteria published by a committee convened to establish the definition and diagnostic criteria of metabolic syndrome in Japan [25]: central obesity (WC ≥85 cm in males) and the presence of at least two of the following factors—serum TG ≥150 mg/dL and/or HDL-C <40 mg/dL, systolic blood pressure ≥130 mm Hg and/or diastolic blood pressure ≥85 mm Hg, and fasting plasma glucose ≥110 mg/dL.

Evaluation of Endocrine Function

Onset of puberty was defined as a testicular volume ≥4 mL [26]. Testicular volume was measured with an orchidometer, as described by Prader [27]. Testicular Leydig cell function and germinal epithelium damage were evaluated by measurement of basal serum luteinizing hormone (LH) level, basal serum follicle-stimulating hormone (FSH) level, and serum testosterone level before SCT and annually during the follow-up period. The normal basal serum LH and FSH levels at our institutions were <9 mIU/mL and <14 mIU/mL, respectively, and the normal range of serum testosterone of the patients in their 20s and 30s was 131-871 ng/dL. Partial Leydig cell dysfunction and partial germinal epithelium damage were defined by either increased basal LH level (≥15 IU/mL) or increased basal FSH level (≥20 IU/mL) with a normal testosterone level. To convert to SI units, serum testosterone values were multiplied by 0.0347 (results in nmol/L). Plasma sex hormone-binding globulin (SHBG) levels and free testosterone measurements were performed by Special Reference Laboratories (Tachikawa, Japan). All analyses except plasma SHBG and free testosterone were performed in our hospital's routine clinical laboratory. Blood draws for

endocrine tests were performed with the patient in a morning fasting state, to avoid diurnal hormonal variation.

Statistical Analysis

Because the data had a skewed distribution, values are presented as median and range. Differences in anthropometric and laboratory variables among groups were analyzed using the Kruskal-Wallis test with Dunn's multiple comparison test. The Mann-Whitney test was used to compare differences between groups. Kaplan-Meier survival curves were constructed to assess the probability of fatty liver, and the log-rank test was used to compare survival curves. All statistical analyses were performed with the statistical package GraphPad Prism 5 for Mac OS X (GraphPad Software, La Jolla, CA). A P value $<.05$ was considered statistically significant.

RESULTS

Anthropometrics

Among the 34 patients, only one (unique patient number [UPN] 105), who had received chemotherapy only, had a BMI >25 kg/m^2 (26.2 kg/m^2) at the last evaluation. However, 11 patients had a BMI <18.5 kg/m^2 (one patient in the CRT+TBI group, seven patients in the TBI group, two patients in the TAI group, and one patient in the Chemo group). No patient satisfied the criteria for metabolic syndrome, although three patients had a WC >85 cm (one with a WC of 86.7 cm in the CRT+TBI group, one with a WC of 90.0 cm in the TAI group, and one with a WC of 92.2 cm in the Chemo group).

Fatty Liver

All 34 patients exhibited liver function within the normal range during the follow-up period, although one patient who received TBI (UPN 118) showed a transient increase in transaminase levels after SCT, and two patients had a positive hepatitis C virus-RNA test result (UPNs 64 and 100 in the TAI group). Information on daily alcohol consumption was obtained from all patients by self-report. The majority of the patients were nondrinkers or drank only minimally. Fatty liver was diagnosed in 15 patients (44%) by ultrasound during the follow-up period (Figure 1). The patients with fatty liver showed no tendency toward overweight/obesity during the follow-up period, however; the mean BMI in these patients was 18.8 kg/m^2 (range, 15.9-23.0 kg/m^2) at the last evaluation (Table 1). No relationships between the development of fatty liver and age at SCT, primary disease, or GVHD were observed. Concerning the mode of irradiation, the prevalence of fatty liver was higher in the CRT+TBI group compared with the TBI, TAI, and Chemo groups (75%, 38%, 38%, and 50%, respectively), although the difference among groups was not significant because of the small number of patients in each group ($P = .070$; log-rank test) (Figure 1). In five patients (UPN 91 in the CRT+TBI group, UPNs 1 and 11 in the TBI group, UPN 215 in the TAI group, and UPN 255 in the Chemo group), fatty liver improved with exercise and dietary regimens provided by physicians and dieticians during the follow-up period (Figure 1).

Evaluation of Lipid and Glucose Metabolism

Studies of lipid and glucose metabolism were performed to investigate the mechanism of development of fatty liver in patients who underwent SCT. Median TG and HDL-C levels were statistically significantly different between patients with fatty liver and those without fatty liver (Table 2). Moreover, the presence of fatty liver was significantly associated with increased insulin resistance as determined by HOMA-IR score ($P = .032$).

Evaluation of Endocrine Function

Puberty started spontaneously in all patients according to increases in testicular volume (≥4 mL), and all patients had developed adult genitalia (Tanner stage V) at the last evaluation. Serum testosterone level reached the adult range at some point between adolescence and adulthood after SCT in all patients (data not shown). To clarify the relationship between testicular irradiation and serum testosterone levels, patients were divided into two groups, the testicular irradiation group and the no testicular irradiation group. The median serum testosterone level was not significantly different between the two groups (448 ng/dL in the testicular irradiation group versus 468 ng/dL in the no testicular irradiation group), but serum LH and FSH levels were significantly higher in the testicular irradiation group (9.6 mIU/mL and 33.0 mIU/mL versus 5.9 mIU/mL and 14.3 mIU/mL; $P < .0001$) (Figure 2A-C). These findings indicate that testicular irradiation did not affect testosterone production. On the other hand, the presence of fatty liver was statistically associated with decreased serum testosterone levels (median, 527 ng/dL [range, 168-944 ng/dL] in patients with fatty liver versus 302 ng/dL [165-698 ng/dL] in those without fatty liver; $P < .0001$). Moreover, severe fatty liver tended to be associated with lower median serum testosterone levels compared with moderate, mild, and nonfatty liver (273 ng/dL, 333 ng/dL, 345 ng/dL, and 530 ng/dL, respectively; $P < .0001$) (Figure 2D-F). Decreased serum testosterone levels were associated with the development and progression of fatty liver, and recovery of serum testosterone levels was observed after resolution of fatty liver by exercise and diet in three patients (UPNs 011, 044,

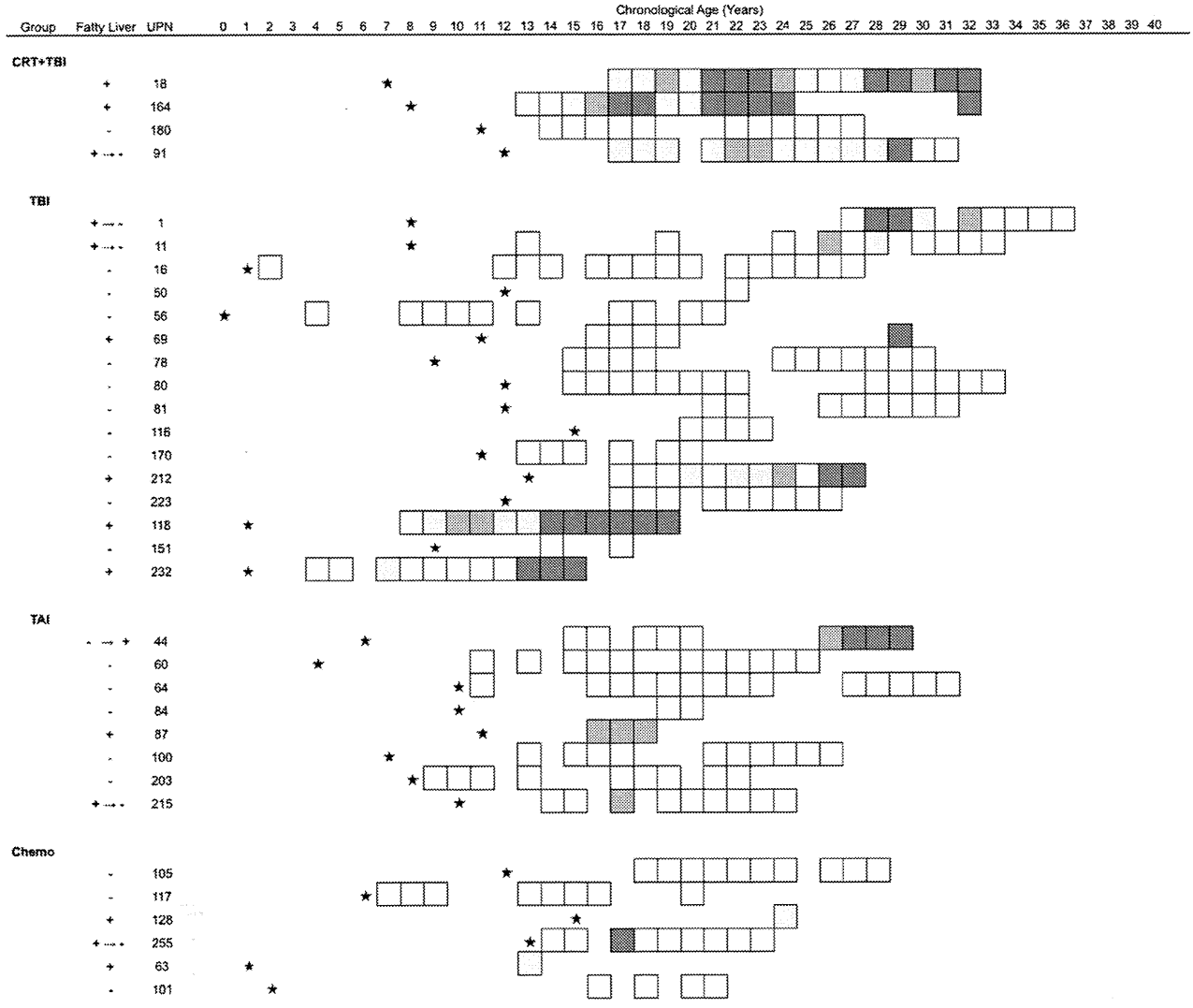

Figure 1. Changes in fatty liver in adult survivors of childhood SCT. The degree of fatty liver is classified as follows: □, absence; ▒, mild; ▓, moderate; ■, severe; +, existence; + → -, improved; ★, SCT.

and 212; Figure 3). Plasma SHBG levels were negatively correlated with HOMA-IR score (Spearman's rank correlation: r = -0.216; P = .375), but were positively correlated with serum testosterone levels (r = 0.498; P = .030).

DISCUSSION

This is the first study of adult survivors of childhood cancer treated with SCT to indicate a relationship between the degree of fatty liver and changes in serum testosterone levels after SCT. Our novel finding is that severe fatty liver presenting with insulin resistance is associated with decreased serum testosterone levels during the follow-up period after SCT. The incidence of fatty liver with decreased serum testosterone level was not associated with either overweight/obesity or metabolic syndrome in these patients who received SCT. These findings highlight the increased health risks in adult survivors of childhood SCT.

Fatty liver is increasingly recognized as a major cause of liver-related morbidity and mortality because of its potential to progress to cirrhosis and liver failure. In a previous study, we found an 18% to 27% prevalence of fatty liver in Japanese male subjects in their 20s and 30s [24]. However, in the current study, the prevalence of fatty liver was 44% in male adult survivors who were not overweight/obese during the follow-up period. These results indicate that SCT may affect the prevalence of fatty liver in adult survivors, and that these survivors are more likely to develop fatty liver compared with the general Japanese population. Moreover, the prevalence of fatty liver was greater in patients who received 24 to 30 Gy to the brain compared with those who received 8 to 12 Gy or 0 Gy. This suggests that radiation therapy to the brain may be a contributing factor to the development of fatty liver in adults who underwent SCT during childhood.

Table 2. Anthropometric Measurements, Liver Dysfunction Data, and Lipid and Glucose Metabolism Data in Adult Survivors of Childhood SCT

	All (n = 34)	Fatty Liver (n = 15)	Non-Fatty Liver (n = 19)	*P* Value[a]
Anthropometric measures				
Height, cm	159.1 (139.2-172.4)	158.5 (139.2-172.4)	159.7 (145.1-172.0)	.716
Weight, kg	47.5 (31.3-72.8)	46.1 (37.6-67.1)	49.2 (31.3-72.8)	.500
Waist circumference, cm	70.3 (53.0-92.2)	68.8 (61.5-82.0)	71.9 (53.0-92.2)	.728
BMI, kg/m^2	19.3 (13.8-26.2)	18.8 (15.9-23.0)	20.2 (13.8-26.2)	.659
WC-to-height ratio	0.4 (0.4-0.6)	0.5 (0.4-0.5)	0.4 (0.4-0.6)	.977
Body fat, %				
Liver dysfunction				
Aspartate aminotransferase, U/L	27 (15-74)	36 (20-74)	27 (15-68)	.183
Alanine aminotransferase, U/L	27 (13-183)	32 (15-183)	23 (13-99)	.167
Gamma-glutamyl transpeptidase, U/L	34 (14-224)	92 (14-224)	29 (19-175)	.042
Lipid and glucose metabolism				
TG, mg/dL	107 (35-409)	113 (67-409)	91 (35-185)	.038
TC, mg/dL	203 (148-294)	195 (157-294)	207 (148-250)	.851
HDL-C, mg/dL	63 (36-114)	47 (36-114)	67 (53-98)	.046
LDL-C, mg/dL	128 (71-892)	135 (86-892)	127 (71-676)	.805
Free fatty acid, mEq/L	0.60 (0.2-1.3)	0.60 (0.2-1.3)	0.50 (0.2-1.3)	.351
HOMA-IR	1.9 (0.5-9.4)	3.5 (0.9-9.4)	1.6 (0.5-6.3)	.032

Data are presented as median (range). To convert to SI units, multiply serum TG by 0.0113 (mmol/L) and multiply serum TC, serum HDL-C, and serum LDL-C by 0.0259 (mmol/L).
[a]For comparison of fatty liver and non-fatty liver.

The present study included an insufficient number of patients on which to base any definitive conclusions, however, and additional prospective and multicenter studies in larger populations are needed to investigate the mechanism of this effect.

Many previous studies have reported low plasma testosterone levels in men with metabolic syndrome or type 2 DM [28,29]. Visceral obesity suppresses testosterone production, and low plasma testosterone levels induce visceral obesity and insulin resistance. Kapoor et al [30] and others [31,32] reported randomized double-blind, placebo-controlled studies of the effect of testosterone replacement therapy on correction of metabolic syndrome markers and inflammation in hypogonadal men. They found significant improvements in weight, BMI, WC, and insulin level in the testosterone treatment group compared with the placebo group, and concluded that the decrease

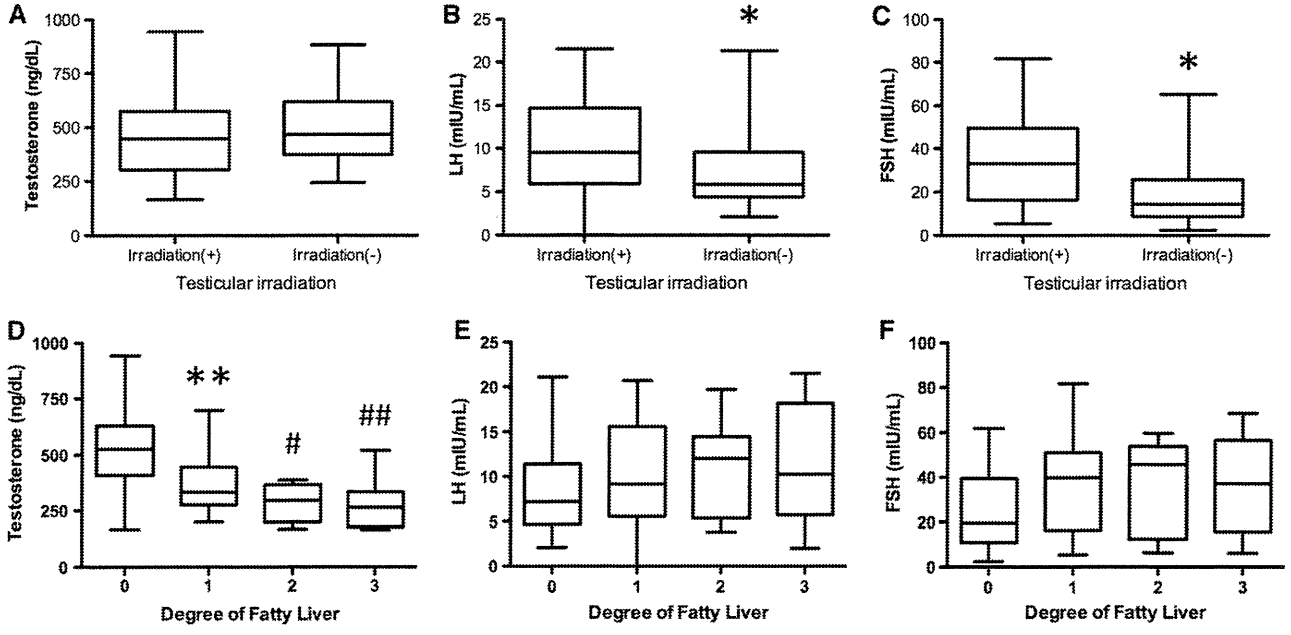

Figure 2. Comparisons of serum testosterone levels (A and D), basal serum LH levels (B and E), and basal serum FSH levels (C and F). The box contains the middle 50th percentile of the value. The lower and upper boundaries of the box indicate the 25th and 75th percentiles, respectively. The bottom and top of the bar show maximum and minimum values, respectively. The degree of fatty liver was classified as follows: 0, absence; 1, mild; 2, moderate; or 3, severe. Significant differences are shown between *testicular irradiation versus no testicular irradiation ($P < .0001$), **mild fatty liver versus nonfatty liver ($P < .001$), #moderate fatty liver versus nonfatty liver ($P < .001$), and ##severe fatty liver versus nonfatty liver ($P < .001$).

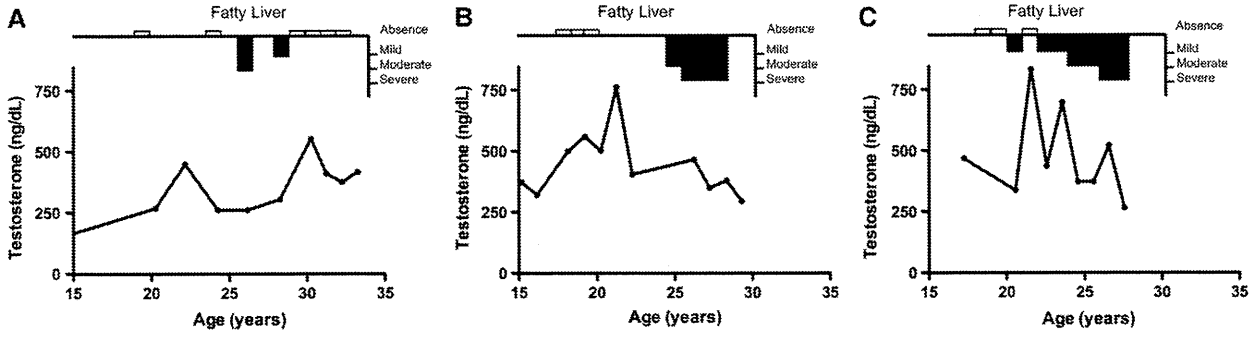

Figure 3. Typical relationship between changes in serum testosterone levels and degree of fatty liver in adult survivors of childhood stem cell transplantation: (A) UPN 011, (B) UPN 044, (C) UPN 212.

in testosterone levels with aging was one of the causes of metabolic syndrome.

Little is known about whether the metabolic syndrome itself may predict the development of hypogonadism, however. One study reported that men with metabolic syndrome had a 2.6-fold increased risk of developing hypogonadism after 11 years [33]. Thus, prevention of abdominal obesity and accompanying metabolic syndrome may reduce the risk of hypogonadism in men. In other studies, weight loss and weight maintenance in men with mainly generalized obesity or abdominal obesity and metabolic syndrome brought about sustained increases in free testosterone levels [34,35]. The prevalence of hypogonadism, as defined by total testosterone levels, decreased from 48% at baseline to 21% after weight loss and 12 months of successful weight maintenance. Weight loss and weight maintenance increased testosterone levels in men with metabolic syndrome after improvement of fatty liver.

This finding of an inverse association between metabolic abnormalities, including insulin resistance, and serum testosterone level is in good agreement with the results of the present study. Fatty liver was more prevalent in patients who received radiation to the brain before undergoing SCT than in those who did not. Although testicular irradiation contributed to damage to the testicular germinal epithelium, testosterone production was maintained during the follow-up period even in patients who received radiation to the testes. Moreover, the degree of fatty liver in patients with insulin resistance was associated with the degree of decreased testosterone secretion. Appropriate exercise and dietary regimens during the follow-up period improved not only insulin resistance, but also fatty liver, and recovery of testosterone secretion followed. Based on these findings, the development and progression of fatty liver in adult survivors of childhood cancer treated with SCT can be considered the cause of, rather than the result of, reduced testosterone levels.

A previous cross-sectional clinical study found an inverse correlation between serum testosterone level and fasting insulin level [36]. Men with insulin resistance, such as those with obesity and those with type 2 DM, have significantly lower testosterone levels than age-matched normal-weight and nondiabetic controls. The mechanism underlying the low testosterone levels associated with insulin resistance in men has not been clarified. Recently, Pitteloud et al [37] reported a positive correlation between testosterone secretion and insulin sensitivity in men. They found a clear association between increased insulin resistance and decreased Leydig cell testosterone secretion in men evaluated by the hyperinsulinemic-euglycemic clamp test. Their findings suggest that the decreased serum testosterone levels in adult survivors of childhood cancer treated with SCT might be explained not only by primary gonadal dysfunction due to the effect of irradiation and/or alkylating agents before SCT but also by the presence of fatty liver and insulin resistance. In addition, several clinical studies have shown significant associations between low SHBG levels and insulin resistance [38] and metabolic syndrome [39]. Shin et al [40] clarified the association between serum SHBG levels and nonalcoholic fatty liver disease in patients with type 2 DM. SHBG levels were lower in patients with high-grade nonalcoholic fatty liver disease than in those without it, and SHBG level was negatively correlated with HOMA-IR score. In the present study, although plasma SHBG level was positively correlated with serum testosterone level, it was not negatively correlated with HOMA-IR, because of an insufficient number of patients for statistical analysis.

A few limitations of the present study need to be considered when interpreting our findings. This longitudinal retrospective study included a small number of patients who underwent SCT in a single institution. In a future study, we plan to analyze the relationship between testosterone level and degree of fatty liver both in patients who underwent SCT and in patients who received chemotherapy only. Our data require confirmation in a large group of patients.

In conclusion, we found significantly higher prevalences of fatty liver and insulin resistance with

decreased serum testosterone levels in our patients who underwent SCT to treat childhood cancer than in those who did not. Even those patients who were not overweight or obese were likely to develop fatty liver after SCT. Furthermore, the degree of fatty liver was associated with the degree of reduced serum testosterone level. The incidence of late effects after SCT in our study population increased with follow-up time and did not appear to plateau. We strongly recommend close monitoring of metabolic and endocrine function in adult survivors of childhood SCT in an effort to improve their quality of life.

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High Incidence of Radiation-Induced Cavernous Hemangioma in Long-Term Survivors Who Underwent Hematopoietic Stem Cell Transplantation with Radiation Therapy during Childhood or Adolescence

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Radiation-induced cavernous hemangioma (RICH) is a late complication of cerebral radiation therapy. Long-term survivors of hematopoietic stem cell transplantation (HSCT) who underwent radiation therapy could be at increased risk for RICH. We investigated records of 68 patients who underwent HSCT during childhood or adolescence and were assessed by magnetic resonance imaging (MRI), including T2*-weighted imaging of the brain, annually for 5 years over a range of 6 to 29 years after HSCT. We developed a scoring and grading system for RICH to monitor the process and the progress of radiologic changes. Among the 68 patients investigated, 28 (41.2%) were diagnosed with CH. All 28 patients had received total body irradiation as a conditioning treatment for HSCT and/or cranial radiation therapy before HSCT as part of the treatment of their primary disease. RICH was diagnosed in none of the patients who did not receive radiation (n = 19), in 46.2% of those who received 6 to 12 Gy (n = 39), and in all of those who received 18 to 36 Gy (n = 10). Total RICH scores were correlated with higher radiation doses. Careful and long-term evaluation with MRI, including T2*-weighted imaging, is necessary for HSCT recipients who received radiation therapy before and/or during HSCT.

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KEY WORDS: Cavernous hemangioma, Radiation, Late effect, T2*-weighted magnetic resonance imaging, Score

INTRODUCTION

Hematopoietic stem cell transplantation (HSCT) has been widely applied to treat a variety of malignant and nonmalignant diseases in childhood. Advances in transplantation practices and supportive care have led to improved outcomes and have resulted in an increasing number of long-term HSCT survivors [1-3]. The overall survival rate after HSCT in childhood is now >50%, and more than 5,000 childhood HSCT recipients are estimated to be currently surviving long-term in Japan [4]. However, childhood and adulthood survivors experience morbidity, which is generally related to the treatment they received to cure their original disease rather than to the disease itself, and treatment-related morbidity is extraordinarily diverse [5]. HSCT survivors are at significant risk of developing late complications after successful transplantation during childhood [6]. In particular, radiation therapy may be a cause of later complications, such as secondary neoplasm [6], growth failure [7], thyroid dysfunction [8], and gonadal dysfunction [9].

Cranial radiation therapy (CRT) has been an integral part of the treatment of patients with cranial tumors, other solid tumors of the head, and disorders with central nervous system involvement, as well as to prevent central nervous system relapse in acute leukemia. Known late complications of radiation therapy include not only endocrine dysfunction, cognitive deterioration, and secondary neoplasm, but also the risk of developing cavernous hemangioma (CH). The prevalence of CH or cavernous malformation was found to be as high as 0.5% in a large prospective cohort from the general population without radiation exposure [10]. Although these malformations were initially thought to be congenital, in 1994, Ciricillo et al [11]

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reported seven patients with intracerebral cavernoma as a possible consequence of radiation, and since then, another 84 cases and reviews have been described [12].

The literature suggests that radiation doses >30 Gy are associated with a shorter latency period and that younger age at the time of radiation exposure is a risk factor for developing radiation-induced cavernous hemangioma (RICH) [13]. However, despite the increasing number of reported cases, the mechanisms of CH formation after radiation are complex, and the impacts of radiation dose, sex, and age at the time of radiation therapy remain poorly understood. A longitudinal study investigating the relationship between HSCT and developing RICH in survivors of childhood HSCT has yet to be published. To evaluate the prevalence of RICH, we performed a longitudinal retrospective study of adult survivors of childhood HSCT at a single institution.

PATIENTS AND METHODS

Patients

We investigated 147 patients who underwent HSCT during childhood or adolescence at Tokai University Hospital between March 1982 and July 2000 and had annual follow-up after HSCT. Inclusion criteria were survival for at least 10 years after HSCT, no previous history of brain disease before HSCT, and annual brain magnetic resonance imaging (MRI) studies between April 1, 2006, and July 31, 2011.

Among the 147 patients, 68 (35 males and 33 females) visited Tokai University Hospital annually throughout the follow-up period and were enrolled in this study. The remaining 79 patients were followed up at Tokai University Hospital initially but then received continuing follow-up at their referring hospitals without T2*-weighted MRI; these patients were excluded from this study.

The median age of the 68 patients at HSCT was 7 years (range, 0-24 years) and that at the last evaluation was 28 years (range, 14-43 years). The median follow-up duration after HSCT was 19 years (range, 10-28 years). Patient characteristics are summarized in Table 1. Written informed consent was obtained from all patients and/or parents. The study was approved by Tokai University's Clinical Research Review Committee.

Transplantation Procedure

In addition to conventional chemotherapy, nine patients with acute lymphoblastic leukemia (ALL) and one patient with non-Hodgkin lymphoma (NHL) received prophylactic CRT (24 Gy in one patient and 18 Gy in nine patients) between 1 year and 9 years before HSCT. In 54 patients, the consitioning regimen comprised irradiation with or without cyclophosphamide and/or other drugs. Between 6 and 12 Gy of total body irradiation (TBI) for malignant diseases was given in 3 to 6 fractions, and 6 to 8 Gy of thoracoabdominal irradiation for nonmalignant diseases was given in three or four fractions. The remaining 14 patients received conditioning without irradiation.

The method of TBI delivery remained constant during the period when those patients underwent HSCT, whereas the method of CRT delivery varied among the institutions in which those patients were treated initially. Prophylaxis against graft-versus-host disease varied during the study period and used methotrexate, cyclosporine, or a combination of the two.

Brain MRI

The radiologic diagnosis of CH was made based on MRI findings, including gradient-echo sequence (T2*-weighted imaging) and contrast MRI with gadolinium chelates, performed annually for 6 to 29 years after HSCT. MRI was done using mainly a 1.5-T and occasionally a 3-T superconducting MRI system. Typical appearance on T2-weighted MRI was a peripheral rim of hypointense hemosiderin surrounding a central core of heterogeneous reticulated signal, often described as "popcorn-like." The hypointense ring may represent repeated subclinical intralesional and perilesional hemorrhages leading to ferritin deposition secondary to erythrocyte breakdown [14]. In particular, T2*-weighted imaging of the brain is highly sensitive for the detection of small CH.

CH Score

The patients were evaluated for CH number, size, and distribution and for annual changes in these parameters. We developed a CH scoring system based on MRI findings to monitor the process and the progress of radiologic changes. CH size <3 mm was defined as score1 (1 point), CH size 3 to 5 mm as score2 (2 points), CH size 6 to 8 mm as score3 (3 points), and CH size ≥9 mm as score4 (4 points) (Figure 1).

CH Grade

The magnitude of CH was graded according to total CH score and the presence of mass effect as grade 0, total score 0; grade I, total score 1-4; grade II, total score 5-9; grade III, total score ≥10; or grade IV, total score ≥5 and mass effect present.

Statistical Analysis

Because the data had a skewed distribution, values are presented as median and range. Differences in eight variables—sex, age at HSCT, age at first radiation therapy, age at latest MRI, age at diagnosis of CH, original disease, radiation dose to the head, and type of transplantation—among groups were analyzed by the Pearson χ^2 test or Fisher exact test. The

Table 1. Patient Characteristics

	Total	Groups According to Total Cranial Radiation Dose		
		A (0 Gy)	B (6-12 Gy)	C (18-36 Gy)
Number of patients	68	19	39	10
Females/males, n	33/35	6/13	21/18	6/4
Primary disease, n				
Malignant diseases				
Acute lymphoblastic leukemia	18	0	9	9
Acute myelogenous leukemia	11	0	11	0
Chronic myelogenous leukemia	5	1	4	0
Juvenile myelomonocytic leukemia	1	0	1	0
Myelodysplastic syndrome	2	0	2	0
Non-Hodgkin lymphoma	4	0	3	1
Neuroblastoma	2	0	2	0
Yolk sac tumor	1	0	1	0
Nonmalignant diseases				
Severe aplastic anemia	10	6	4	0
Others	14	12	2	0
Autologous/allogeneic HSCT, n	7/61	0/19	6/33	1/9
Age at HSCT, years, median (range)	7 (0-24)	5 (1-12)	9 (0-24)	10 (4-19)
Age at first irradiation, years, median (range)	8 (0-24)		9 (0-24)	6 (0-17)
Age at latest MRI, years, median (range)	28 (14-43)	24 (12-33)	29 (16-43)	28.5 (21-37)
Follow-up after HSCT, years, median (range)	19 (10-28)	16 (10-23)	20 (11-28)	18 (16-21)

Mann-Whitney *U* test and the Kruskal-Wallis test were used to compare differences between groups. A Kaplan-Meier survival curve was constructed to assess the probability of CH incidence, and the log-rank test was used to compare probabilities. In multivariate analyses, outcome comparisons were adjusted with Cox proportional hazards models and tested by the likelihood ratio test. All statistical analyses were performed with SPSS version 19. A P value $<.05$ was considered statistically significant.

RESULTS

Incidence of CH

CH was diagnosed in 28 of the 68 patients (41.2%) by MRI during the follow-up period (Table 2). The probability of developing CH at 25 years after HSCT was 61.5% ± 9.1% by the Kaplan-Meier method (Figure 2A). The median age at diagnosis of CH was 27 years (range, 11-40 years). The median age at HSCT was 9.5 years (range, 1-22 years), and the median age at first irradiation treatment in the patients diagnosed with CH was 7.5 years (range, 0-22 years).

Incidence and probability were further analyzed according to several variables to evaluate risk factors for CH (Table 2). Gender, type of transplantation and age at the first irradiation did not give statistically significant differences in frequency or in probability of CH. However, compared with patients with nonmalignant diseases, those with malignant diseases in which HSCT was indicated had a significantly higher frequency of CH (59.1 versus 8.3%; $P < .001$) and

CH score	CH size
1	<3mm
2	3mm≤, <6mm
3	6mm≤, <9mm
4	9mm≤

CH grade	Total CH score
0	0
I	1-4
II	5-9
III	10-
IV	Mass effect and CH score ≥ 5

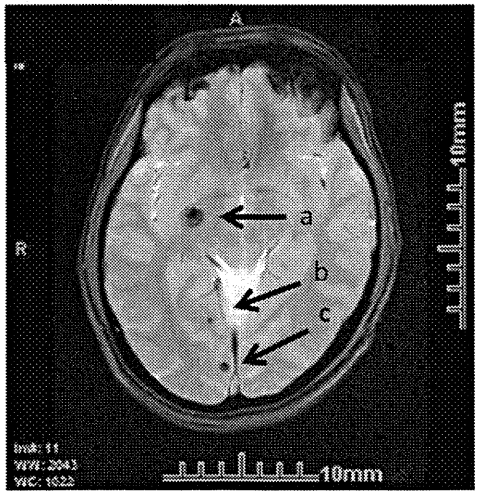

Figure 1. CH scoring and grading system. This example case has a total CH score of 6 [a(score 3) + b(score 1) + c(score 2)] and CH grade II.