respectively. For patients with PS $\geqslant 2$ ($n = 16$), NRM at 2 years was 77% (45–92%), and NRM at 5 years was not evaluable because of lack of patients alive beyond 5 years after transplantation.

OS

OS rates at 2 and 5 years after transplantation were 63% (95% CI, 38–80%) and 63% (38–80%) in related BM, 48% (28–66%) and 43% (23–61%) in related PBSC and 41% (21–59%) and 41% (21–59%) in unrelated BM transplantations, respectively (Figure 3). The OS rate at 2 years after unrelated UCB transplantation was 36% (11–63%), and the OS rate at 5 years after UCB transplantation was not evaluable because of a lack of patients alive beyond 5 years after transplantation (longest follow-up, 48 months). There was no significant difference among stem cell donor sources ($P = 0.15$).

Cox's proportional hazards model was used with all clinical features listed in Table 1, and the final multivariate model is shown in Table 2. After adjustment by PS and frequency of RBC transfusion, which were significant on univariate analysis, donor source was not a significant factor for predicting OS. Instead, PS $\geqslant 2$ predicted a lower OS rate, and RBC transfusion $\geqslant 20$ times before transplantation showed a trend toward a lower OS. We confirmed that there was no significant difference in the frequencies of PS $\geqslant 2$ between patients receiving different stem cell sources (2 of 13 related BM, 6 of 24 related PBSC, 5 of 27 unrelated BM and 3 of 6 unrelated UCB transplantations; $P = 0.30$). Similarly, we confirmed that there was no significant difference in the frequencies of RBC transfusion $\geqslant 20$ times between patients receiving different stem cell sources (2 of 8 related BM, 5 of 18 related PBSC, 8 of 20 unrelated BM and 2 of 5 unrelated UCB transplantations; $P = 0.80$).

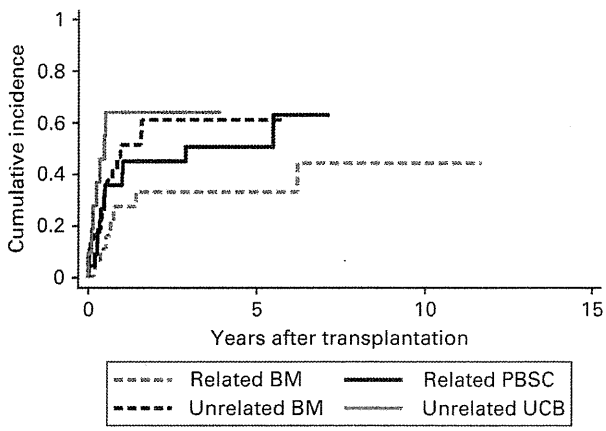

Figure 2. NRM after transplantation in PMF patients. Cumulative incidences of NRM after related BM (gray and dash line), related PBSC (black and solid line), unrelated BM (black and dash line) and unrelated UCB (gray and solid line) transplantations are shown.

Causes of death

The causes of death after transplantation are summarized in Table 3. For patients after related donor transplantation ($n = 23$), the most common cause of death was primary disease ($n = 9$, 39%), followed by infection ($n = 4$, 17%) and organ failure ($n = 3$, 13%). For patients after unrelated donor transplantation ($n = 22$), the most common causes of death were infection ($n = 7$, 32%) and organ failure ($n = 7$, 32%), followed by GVHD ($n = 3$, 14%), and only 1 patient (5%) died of primary disease.

DISCUSSION

The present study confirmed 5-year OS of 63%, 43% and 41% after related BM, related PBSC and unrelated BM transplantations, respectively. These results are comparable to previous reports in which long-term survival rates in patients with PMF or secondary myelofibrosis were 30–67% after transplantation.[10,11,21–26] This is the first report of UCB transplantation for more than 10 patients with PMF, and a 2-year OS of 36% was confirmed.

Several investigators have examined factors to predict outcomes after allogeneic HCT for PMF patients. The largest retrospective study of PMF patients from the CIBMTR demonstrated that Karnofsky score of $<90\%$ and the presence of blasts in peripheral blood, but not donor source, predicted lower disease-free survival of patients who had received BM or PBSC transplantation from related or unrelated donors.[10] Other retrospective studies including both PMF and secondary myelofibrosis demonstrated negative predictors for OS of higher patient age, nonchronic phase disease, RBC transfusion >20 times, increased comorbidity score, intermediate-2 and high scores of the Dynamic IPSS and non-HLA-matched sibling donor.[11,21,24,26,27] In the present study, multivariate analysis demonstrated that PS $\geqslant 2$ predicted a lower OS and that RBC transfusion $\geqslant 20$ times before transplantation showed a trend toward a lower OS (Table 2). Unexpectedly, the stem cell source was not a significant factor for OS. One possibility is that a significant association between stem cell source and OS was not detected because of a lack of statistical power, namely, the small

Table 2. Significant factors in multivariate analyses for nonrelapse mortality and OS after transplantation

	Nonrelapse mortality	P-*value*	*Overall survival*	P-*value*
	HR (95% CI)		*HR (95% CI)*	
Performance status at transplant				
0–1	1.0		1.0	
$\geqslant 2$	3.36 (1.42–7.95)	0.006	2.67 (1.03–6.95)	0.044
Frequency of RBC transfusion[a]				
$\leqslant 9$	NA		1.0	
10–19	NA		0.48 (0.97–2.36)	0.37
$\geqslant 20$	NA		2.42 (0.99–5.93)	0.053
Donor source				
Related BM	1.0		1.0	
Related PBSCs	2.43 (0.73–8.07)	0.15	3.86 (0.81–18.44)	0.091
Unrelated BM	3.58 (1.07–12.01)	0.039	3.13 (0.66–14.79)	0.15
Unrelated umbilical cord blood	2.71 (0.49–14.86)	0.25	3.79 (0.60–23.91)	0.16

Abbreviations: CI = confidence interval; HR = hazard ratio; NA = not applicable.
[a]Frequency of RBC transfusion before transplantation.

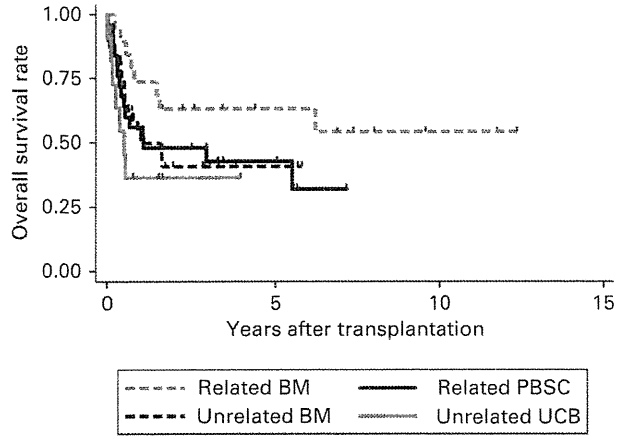

Figure 3. OS rates after transplantation in PMF patients. OS rates after related BM (gray and dash line), related PBSC (black and solid line), unrelated BM (black and dash line) and unrelated UCB (gray and solid line) transplantations are shown.

Table 3. Causes of death

	Related BM, n *(%)*	*Related PBSC*, n *(%)*	*Unrelated BM*, n *(%)*	*Unrelated UCB*, n *(%)*
Primary disease	2 (25)	7 (46)	1 (7)	0
Infection	1 (13)	3 (20)	6 (40)	1 (14)
Interstitial pneumonitis	2 (25)	0	1 (7)	0
ARDS	0	0	1 (7)	0
GVHD	1 (13)	1 (7)	2 (13)	1 (14)
Organ failure	1 (13)	2 (13)	3 (20)	4 (58)
Graft failure	1 (13)	0	0	0
Bleeding	0	1 (7)	1 (7)	0
Other	0	1 (7)	0	1 (14)
Total	8 (100)	15 (100)	15 (100)	7 (100)

Abbreviations: ARDS = acute respiratory distress syndrome; UCB = umbilical cord blood.

number of patients in each group, and the short-term follow-up. In particular, the number of patients with UCB transplantation was very small, and therefore, careful interpretation of these data is required. Further analysis with data including more patients undergoing UCB transplantation is required in order to determine the effect of UCB transplantation on outcomes of PMF patients. Another possibility is that the HCT outcome for PMF patients is more adversely affected by the deterioration in a patient's systemic condition as a consequence of multiple transfusions of blood and so on, rather than by the difference in stem cell sources.

In practice, UCB transplantation may be avoided in the treatment of PMF patients because of delayed engraftment and a higher probability of graft failure.[9] The present study demonstrated that UCB transplantation was significantly associated with a lower probability of hematopoietic recovery in comparison with related BM transplantation (Figure 1). The incidences of neutrophil recovery at 60 days and platelet recovery at 1 year were 82% and 44% for UCB transplantation, respectively. In a recent report of nonmyeloablative UCB transplantation for 14 patients with myelofibrosis, including 1 patient with PMF and 13 patients with secondary myelofibrosis, the incidences of neutrophil recovery at 60 days and platelet recovery at 100 days were 93% and 43%, respectively.[28] Thus, careful management is required for PMF patients, especially in the early period after unrelated UCB transplantation.

NRM was 30–60% (Figure 2), which is higher than in previous studies from large, well-known transplant center(s).[22–24,26,27,29–32] This may be explained by the large number of the participating centers, the heterogeneity of patients' clinical features and the fact that 18% of patients were ⩾60 years in the present study.

Nonmyeloablative preconditioning regimens have advantages of less NRM and a broader applicability in elderly patients and may, therefore, be appropriate for PMF patients. After small studies demonstrated the feasibility of allogeneic HCT with nonmyeloablative preconditioning for myelofibrosis,[33–35] Kröger *et al.*[11] prospectively treated 103 patients with PMF or post essential thrombocythemia and post polycythemia vera myelofibrosis with BU and fludarabine-based nonmyeloablative preconditioning. They reported encouraging 1-year NRM of 16% and 5-year OS of 67%. The Swedish group compared results from 10 patients undergoing nonmyeloablative transplant with 17 patients undergoing myeloablative transplant for secondary myelofibrosis. NRM was lower in the nonmyeloablative group than in the myeloablative group (10% vs 30%). With a median follow-up of 55 months, 9 (90%) of 10 patients undergoing nonmyeloablative transplant and 9 (55%) of 16 patients undergoing myeloablative transplant survived.[36] In contrast, the present study could not find any advantage of nonmyeloablative preconditioning in terms of decreasing NRM or increasing OS (Table 2). Other retrospective studies, including a large study ($n = 289$), also did not find any favorable affect with nonmyeloablative preconditioning.[10,22,24] In retrospective studies, drugs and doses of preconditioning regimens were heterogeneous, which could partly explain the failure to detect an advantage of nonmyeloablative preconditioning. There has been no randomized study to compare the efficacy of nonmyeloablative and myeloablative preconditioning for patients with PMF. The advantage of nonmyeloablative preconditioning for patients with PMF remains in question.

The molecular assessment of the *JAK2* mutation was performed in a very limited number of patients (six cases for pretransplant mutation and four cases for post transplant mutation). Therefore, we were unable to analyze association between the presence of pretransplant *JAK2* mutation and transplant outcomes or between the minimum residual disease and relapse after transplant. However, the present study clearly demonstrated that allogeneic BM and PBSC transplantations provide long-term survival for PMF patients and suggested the feasibility of UCB transplantation for PMF patients. Given the constant improvement in supportive care for transplant patients and the beginning of the use of molecular targeted therapy for myelofibrosis, the NRM and relapse rates may be further decreased. Allogeneic HCT should be considered in the treatment plan for PMF patients. The indications for allogeneic HCT in PMF patients have to be defined in a future study.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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APPENDIX

Institutes participating in this study: Japanese Red Cross Asahikawa Hospital; Hokkaido University Hospital; Sapporo Medical University Hospital; Sapporo Hokuyu Hospital; Akita University Hospital; Iwate Medical University; Tohoku University Hospital; Fukushima Medical University Hospital; Nagaoka Red Cross Hospital; Gunmaken Saiseikai Maebashi Hospital; Tsukuba Memorial Hospital; Chiba University Hospital; Kameda Medical Center; National Defense Medical College Hospital; Saitama Medical Center, Jichi Medical University; Keio University Hospital; Tokyo Metropolitan Cancer and Infectious diseases Center, Komagome Hospital; Toranomon Hospital; National Cancer Center Hospital; Tokyo Women's Medical University Hospital; Institute of Medical Science, University of Tokyo; Nippon Medical School Hospital; Kanagawa Cancer Center; Yokohama City University Medical Center; Nagano Red Cross Hospital; Shinshu University Hospital; Toyama Prefectural Central Hospital; Kurobe City Hospital; Kanazawa University Hospital; Shizuoka General Hospital; Japanese Red Cross Shizuoka Hospital; Hamamatsu University Hospital; Hamamatsu Medical Center; Anjo Kosei Hospital; Fujita Health University Hospital; Japanese Red Cross Nagoya Daiichi Hospital; Japanese Red Cross Nagoya Daini Hospital; Meitetsu Hospital; Nagoya University Hospital; Nara Medical University Hospital; Tenri Hospital; Takanohara Central Hospital; Kyoto University Hospital; Kyoto-Katsura Hospital; Osaka Red Cross Hospital; Osaka Medical Center for Cancer and Cardiovascular Diseases; Takatsuki Red Cross Hospital; Seichokai Fuchu Hospital; Kinki University Hospital; Wakayama Medical University Hospital; Hyogo College of Medicine; Institute of Biomedical Research and Innovation; Kurashiki Central Hospital; Okayama Medical Center; Hiroshima Red Cross Hospital & Atomic-bomb Survivors Hospital; Shimane Prefectural Central Hospital; Yamaguchi University Hospital; Ehime University Hospital; Ehime Prefectural Central Hospital; Kochi Medical School Hospital; Kitakyushu Municipal Medical Center; University of Occupational and Environmental Health; Kyushu Cancer Center; Kyushu Medical Center; Kyushu University Hospital; Kurume University Hospital; Ryukyu University Hospital.

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ORIGINAL ARTICLE

Risk factors and organ involvement of chronic GVHD in Japan

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Few studies have evaluated the risk factors for chronic GVHD and organ involvement associated with different graft types, including unrelated cord blood (U-CB). We retrospectively studied 4818 adult patients who received their first allogeneic transplantation and survived for at least 100 days. The incidence of chronic GVHD at 2 years was 37%. The following factors were associated with the development of chronic GVHD: female donor/male recipient, CMV-Ab seropositivity, matched related peripheral blood grafts vs matched related BM grafts, no *in vivo* T-cell depletion and the occurrence of grade II–IV acute GVHD. Among these factors, the association with acute GVHD occurrence was consistently significant across donor subtypes. The use of U-CB was not associated with chronic GVHD, but was associated with a low incidence of extensive chronic GVHD. Chronic GVHD patients who had received U-CB transplants showed less frequent involvement of the oral cavity (28% vs 55%), eye (12% vs 26%), liver (20% vs 44%), lung (11% vs 25%) and joint (0% vs 6%) than those with matched related BM grafts. In conclusion, we found that U-CB transplants were associated with a low incidence of extensive chronic GVHD and less frequent involvement of the oral cavity, eye, liver, lung and joints.

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Keywords: chronic GVHD; unrelated cord blood; acute GVHD; risk factors

INTRODUCTION

Chronic GVHD is a serious complication that affects the survival and quality of life of long-term survivors after allogeneic hematopoietic SCT.[1–3] Various pre- and post-transplant risk factors associated with chronic GVHD have been identified, mostly in transplantations using BM and PBSC grafts from related or unrelated donors.[2,3] Several studies have reported a history of acute GVHD to be a strong risk factor that is consistently associated with chronic GVHD development.[4–8] Other identified risk factors include the following: female donor and male recipient,[4,6] use of PBSC grafts,[6,9–13] older patient,[4,6–8] older donor,[6,7] transplantation from a mismatched or unrelated donor,[5,6,14] diagnosis of CML[4,7,8] and absence of anti-thymocyte globulin (ATG) use.[15]

The number of unrelated cord blood (U-CB) transplantations performed has rapidly increased during the past decade. However, few studies have compared the incidences and risk factors of chronic GVHD and its organ-specific symptoms in adult patients receiving U-CB and other available grafts, including related or unrelated BM/PBSC grafts.[16,17] Therefore, we conducted a retrospective study using national registry data involving 4818 patients who underwent allogeneic transplantation. This study aimed to evaluate the incidence and risk factors of chronic GVHD, and the prevalence of chronic GVHD organ involvement in patients who received transplantation using various types of graft, including U-CB.

MATERIALS AND METHODS

Data collection

Data for 54 072 patients who had received auto-SCT or allo-SCT by December 31, 2009 were provided by the Transplant Registry Unified Management Program (TRUMP).[18] We included 4993 adult patients who had: (1) received allogeneic transplantation for hematologic malignancies; (2) received their first SCT; (3) used the same questionnaire form involving chronic GVHD organ involvement (skin, oral cavity, eye, liver, lung, joint, intestine/genitals and other manifestations; 2006–2009 for transplantations using BM or PBSC grafts and 2007–2009 for transplantations using U-CB units); (4) achieved neutrophil engraftment; (5) survived for at least 100 days; and (6) received the following: (a) a related BM or PBSC graft (R-BM/PB), (b) an unrelated BM (U-BM) or (c) a single U-CB unit. Donation of peripheral blood by unrelated volunteers was permitted for the first time in Japan in 2011. The following patients were excluded: (1) patients who received *ex vivo* T-cell-depleted grafts ($n = 26$) and (2) patients who lacked data on acute or chronic GVHD ($n = 149$). Thus, 4818 patients were included in this study, which was approved by the TRUMP Data Management Committees and by the institutional review board of the Nagoya University Graduate School of Medicine, where this study was performed.

Histocompatibility

Histocompatibility data for the HLA-A, HLA-B and HLA-DR loci were obtained through reports acquired from the institution where the transplantation was performed or from the cord blood bank. HLA

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matching was assessed using serological data for the HLA-A, HLA-B and HLA-DR loci in R-BM/PB or U-CB transplantations, and using allelic data for the HLA-A, HLA-B and HLA-DRB1 loci in U-BM transplantations.

Statistical analysis

The physicians who performed the transplantations at each center diagnosed and classified acute and chronic GVHD according to traditional criteria.[1,19] The reported type of chronic GVHD was reclassified according to the information on its organ involvement. 'Progressive onset' of chronic GVHD was defined as chronic GVHD transitioned from active acute GVHD, 'quiescent onset' as chronic GVHD after remission of acute GVHD and '*de novo* onset' as chronic GVHD without history or acute GVHD. The intensity of conditioning regimen was classified as myeloablative or reduced intensity on the basis of the Center for International Blood and Marrow Transplant Research report and the information from the questionnaire, as previously described.[20–23] We defined the following as standard-risk diseases: AML and ALL in first or second remission; CML in the first or

Table 1. Patient characteristics

Variable	*R-BM/PB*		*U-BM*		*U-CB*		*P-value*
	n = *1859*	%	n = *2215*	%	n = *744*	%	
Recipient age, years, median (range)	46 (16–74)		47 (16–73)		51 (16–82)		<0.001
Donor age, years, median (range)	43 (10–79)		35 (20–55)[a]		—	—	—
Recipient sex							
Female	789	42	916	41	334	45	0.238
Male	1070	58	1299	59	410	55	
Sex match between recipient and donor							
Match	965	52	1251	56	227	31	<0.001
Male to female	398	21	573	26	109	15	
Female to male	496	27	389	18	131	18	
Missing	0	0	2	0	277	37	
Disease							
AML	799	43	986	45	395	53	0.004
MDS	210	11	276	12	76	10	
CML	60	3	73	3	25	3	
ALL	385	21	439	20	123	17	
ATL	110	6	131	6	29	4	
NHL	206	11	214	10	70	9	
Other diseases	89	5	96	4	26	3	
Disease risk							
Standard	1058	57	1351	61	331	44	<0.001
High	724	39	780	35	390	52	
Missing	77	4	84	4	23	3	
Source of stem cells							
BM	842	45	2215	100	—	—	—
Peripheral blood	1017	55	—	—	—	—	
Cord blood	—	—	—	—	744	100	
HLA compatibility[b]							
Matched	1486	80	1507	68	53	7	<0.001
Mismatched	373	20	708	32	691	93	
Conditioning regimen							
Myeloablative	1202	65	1505	68	436	59	<0.001
Reduced intensity	649	35	696	31	308	41	
Missing	8	1	14	1	0	0	
GVHD prophylaxis							
CsA based	1367	74	469	21	311	42	<0.001
Tac based	449	24	1737	78	425	57	
Others/missing	43	2	9	1	8	1	
Use of in vivo *T-cell depletion*							
No	1741	94	2143	97	730	98	<0.001
Yes	118	6	72	3	14	2	
CMV Ab (recipient and donor)							
Both negative	127	7	150	7	151	20	<0.001
Either positive	1561	84	2003	90	535	72	
Unknown	171	9	62	3	58	8	
Acute GVHD							
Grade II–IV	665	36	897	41	338	45	<0.001
Grade III–IV	217	12	236	11	81	11	0.578
Follow-up of survivors (years), median (range)	2.0 (0.3–4.7)		1.9 (0.3–4.8)		1.7 (0.3–3.9)		<0.001

Abbreviations: ATL = adult T-cell leukemia; MDS = myelodysplastic syndrome; NHL = non-Hodgkin's lymphoma; R-BM/PB = related BM or PBSC; Tac = tacrolimus; U-BM = unrelated BM; U-CB = unrelated cord blood. [a]Data are missing in 20 patients [b]HLA matching was assessed by serological data for HLA-A, HLA-B and HLA-DR loci in transplantation using R-BM/PB or U-CB grafts, whereas it was assessed by allelic data for HLA-A, HLA-B and HLA-DRB1 loci in transplantation using U-BM grafts.

second chronic phase or in the accelerated phase; myelodysplastic syndrome (MDS) with refractory anemia or refractory anemia with ringed sideroblasts; adult T-cell leukemia (ATL) in CR; and Hodgkin's or non-Hodgkin's lymphoma (NHL) in CR or PR. Others were defined as high-risk diseases.

The probability of developing chronic GVHD was estimated on the basis of cumulative incidence curves.[24] Competing events for chronic GVHD were death or relapse without GVHD. Groups were compared using Gray's test.[25] The Cox proportional hazards model was used to evaluate the effect of confounding variables on chronic GVHD. The following possible confounding variables were considered: recipient age; recipient sex; sex mismatch between recipient and donor (match, male (donor)/female (recipient), or female (donor)/male (recipient)); disease (CML or others); disease risk before transplantation (standard or high risk); donor type (HLA-matched related BM (MR-BM), HLA-matched related PBSCs (MR-PB), HLA-mismatched related BM (MMR-BM), HLA-mismatched related PBSCs (MMR-PB), HLA-matched unrelated BM (MU-BM), HLA-mismatched unrelated BM (MMU-BM) and U-CB); type of conditioning regimen (myeloablative or reduced intensity); type of GVHD prophylaxis (CsA based or tacrolimus based); use of *in vivo* T-cell depletion (yes or no); anti-CMV Ab detection (negative for both recipient and donor, or positive for either recipient or donor), and presence of grade II–IV acute GVHD. Confounding factors were selected in a stepwise manner from the model with a variable retention criterion of $P<0.05$. Reported factors associated with chronic GVHD (recipient age, sex mismatch, donor type, use of *in vivo* T-cell depletion and the presence of grade II–IV acute GVHD) was additionally selected as confounding factors in the analysis of chronic GVHD risk. In the subset analysis, the same variables used in the analysis for the entire cohort were added to the final model. Furthermore, the following variables were also added for the specific group: donor age, presence of an HLA mismatch and the use of PBSCs for the R-BM/PB group; donor age and presence of an HLA mismatch for the U-BM group; and presence of an HLA mismatch for the U-CB group.

We also compared the prevalence of chronic GVHD presentation or organ involvement between MR-BM and other graft types using the χ^2 test. We further evaluated chronic GVHD-specific survival, which is defined as the time from the day of chronic GVHD diagnosis to the day of death in the absence of relapse, among patients who developed chronic GVHD. We also evaluated OS among those who developed chronic GVHD. The probability of developing chronic GVHD-specific survival or OS from the onset of chronic GVHD was estimated using the Kaplan–Meier method, and univariate comparison between groups was performed using the log-rank test. In the analysis of chronic GVHD-specific survival, patients who were alive without disease recurrence were censored at the time of their last follow-up visit and those who experienced disease recurrence were censored at the time of diagnosis of recurrence. The Cox proportional hazards model was used to evaluate the effect of presentation or of each organ's manifestation of chronic GVHD on chronic GVHD-specific survival, after adjusting for donor type and other confounding factors that were selected from the model in a stepwise manner using a variable retention criterion of $P<0.05$. We also evaluated the effect of chronic GVHD on relapse, where the occurrence of chronic GVHD was treated as a time-varying covariate.

All tests were two-sided, and *P*-values <0.05 were considered statistically significant, except for the comparison of prevalence of chronic GVHD organ involvement between MR-BM and other graft types, where *P*-values <0.008 was significant in consideration of multiple comparison. All statistical analyses were performed using Stata version 12 (Stata Corp., College Station, TX, USA) and EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan),[26,27] which is a graphical user interface for R (R Foundation for Statistical Computing, version 2.13.0, Vienna, Austria).

RESULTS

Patient characteristics

Table 1 shows patient characteristics according to the stem cell source. The median age of recipients at the time of the transplant was 47 years (range, 16–82 years) for the entire cohort, and it was significantly higher for patients in the U-CB group. High-risk diseases were more prevalent in the U-CB group. The grafts used were MR-BM ($n=687$), MR-PB ($n=799$), MMR-BM ($n=155$), MMR-PB ($n=218$), MU-BM ($n=1507$), MMU-BM ($n=708$) and U-CB ($n=744$). CsA-based GVHD prophylaxis was received by 74% of the patients in the R-BM/PB group and by only 21% of the U-BM recipients. *In vivo* T-cell depletion was used for only 4% of the entire cohort (ATG, $n=197$; alemtuzumab, $n=7$). Grade II–IV and III–IV acute GVHD occurred in 39% and 11% of the cohort, respectively.

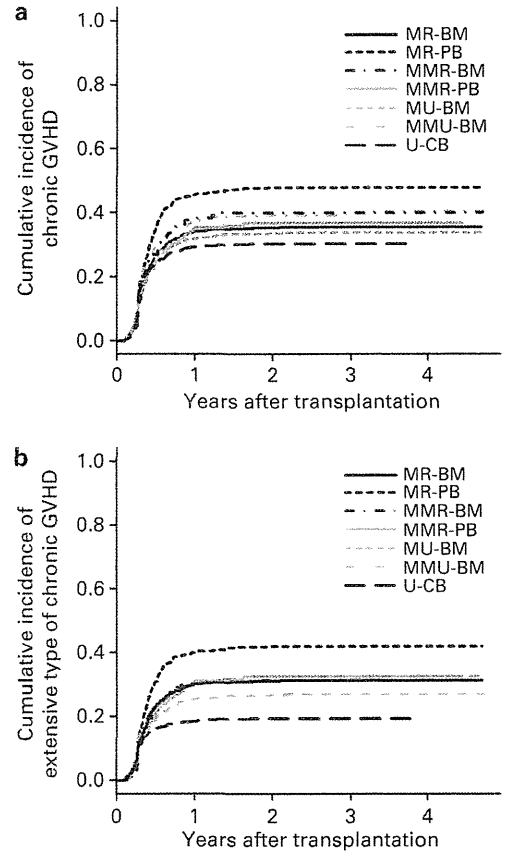

Figure 1. Cumulative incidence of chronic GVHD (**a**) and extensive type of chronic GVHD (**b**).

Chronic GVHD

The incidence of chronic GVHD at 2 years was 37% (95% confidence interval (CI), 35–38%) for the entire cohort, with a median onset of 120 days (range, 30–1203 days), 36% (32–39%) for the MR-BM group, 48% (44–51%) for the MR-PB group, 40% (32–48%) for the MMR-BM group, 37% (30–44%) for the MMR-PB group, 34% (31–36%) for the MU-BM group, 40% (36–44%) for the MMU-BM group and 30% (27–34%) for the U-CB group (Gray's test for the whole group, $P<0.001$; Figure 1a). Female/male mismatch between recipient and donor (hazard ratio (HR), 1.29; $P<0.001$), CMV Ab detection (HR, 1.26; $P=0.015$), the use of MR-PB vs MR-BM graft (HR, 1.49; $P<0.001$), the use of *in vivo* T-cell depletion (HR, 0.48; $P<0.001$) and the occurrence of grade II–IV acute GVHD (HR, 1.62; $P<0.001$) were significantly associated with chronic GVHD development (Table 2). The use of PBSC grafts was significantly associated with chronic GVHD development in the R-BM/PB group (HR, 1.42; $P<0.001$). The impact of CMV Ab positivity on chronic GVHD development was significant only for the U-CB group, but HR was consistently high across donor subtypes. The effect of sex mismatch was significant for the R-BM/PB group, but was not significant for the U-CB group. The effect of grade II–IV acute GVHD occurrence on chronic GVHD development was consistently significant across donor subtypes.

Table 2. Risk factors for chronic GVHD

Variable	*Chronic GVHD (Total)*			*Chronic GVHD (R-BM/PB)*			*Chronic GVHD (U-BM)*			*Chronic GVHD (U-CB)*		
	HR	*95% CI*	*P-value*	*HR*	*95% CI*	*P-value*	*HR*	*95% CI*	*P-value*	*HR*	*95% CI*	*P-value*
Recipient age, per 10 years	1.03	(0.99–1.06)	0.136	1.09	(1.01–1.17)	0.021	1.01	(0.96–1.07)	0.741	0.91	(0.83–1.00)	0.056
Donor age, per 10 years				1.01	(0.94–1.09)	0.730	1.04	(0.95–1.14)	0.429			
Sex match between recipient and donor												
Match	1.00			1.00						1.00		
Male to female	0.97	(0.86–1.10)	0.619	1.01	(0.83–1.23)	0.905	1.00	(0.84–1.19)	0.992	0.78	(0.51–1.19)	0.253
Female to male	1.29	(1.14–1.44)	<0.001	1.45	(1.23–1.71)	<0.001	1.16	(0.96–1.41)	0.127	1.12	(0.78–1.62)	0.535
CMV Ab (donor and recipient)												
Both negative	1.00			1.00			1.00			1.00		
Either positive	1.26	(1.05–1.52)	0.015	1.12	(0.82–1.54)	0.469	1.22	(0.90–1.66)	0.196	1.53	(1.07–2.21)	0.021
Type of donor and stem cell source												
MR-BM	1.00											
MR-PB	1.49	(1.26–1.75)	<0.001									
MMR-BM	1.21	(0.91–1.60)	0.187									
MMR-PB	1.31	(1.00–1.72)	0.054									
MU-BM	0.91	(0.78–1.07)	0.247									
MMU-BM	1.10	(0.92–1.31)	0.306									
U-CB	1.00	(0.81–1.23)	0.991									
Type of stem cell source												
BM				1.00								
PB				1.42	(1.23–1.65)	<0.001						
HLA disparity												
Match				1.00			1.00			1.00		
Mismatch				1.12	(0.92–1.36)	0.274	1.17	(1.00–1.36)	0.043	0.96	(0.55–1.69)	0.887
Use of in vivo *T-cell depletion*												
No		1.00		1.00			1.00			1.00		
Yes	0.48	(0.34–0.66)	<0.001	0.29	(0.18–0.45)	<0.001	0.85	(0.55–1.34)	0.490	0.35	(0.05–2.50)	0.293
Acute GVHD												
Grade 0–I		1.00		1.00			1.00			1.00		
Grade II–IV	1.62	(1.47–1.78)	<0.001	1.44	(1.24–1.66)	<0.001	1.73	(1.50–2.00)	<0.001	1.76	(1.34–2.31)	<0.001

Abbreviations: CI = confidence interval; HR = hazard ratio; MMR-BM = HLA-mismatched related BM; MMR-PB = HLA-mismatched related PBSCs; MMU-BM = HLA-mismatched unrelated BM; MR-BM = HLA-matched related BM; MR-PB = HLA-matched related PBSCs; MU-BM = HLA-matched unrelated BM; R-BM/PB = related BM or PBSC; U-BM; unrelated BM; U-CB = unrelated cord blood.

Extensive chronic GVHD

The incidence of extensive chronic GVHD at 2 years was 30% (29–31%) for the entire cohort, 32% (28–35%) for the MR-BM group, 42% (39–46%) for the MR-PB group, 31% (24–39%) for the MMR-BM group, 33% (26–39%) for the MMR-PB group, 27% (25–29%) for the MU-BM group, 32% (28–36%) for the MMU-BM group and 19% (17–22%) for the U-CB group (Gray's test for the whole group, $P<0.001$; Figure 1b). In addition to being a significant variable in the analysis of chronic GVHD, the use of reduced-intensity conditioning (vs myeloablative conditioning) was inversely associated with the development of extensive chronic GVHD (HR, 0.86; $P=0.019$; Table 3). Compared with MR-BM, MR-PB and MMR-PB were associated with the development of extensive chronic GVHD, whereas MU-BM and U-CB grafts were inversely associated with its development. Grade II–IV acute GVHD occurrence was the only significant variable consistently observed across all donor types.

Organ-specific chronic GVHD

Figure 2 shows the type of presentation and organ involvement associated with chronic GVHD. Among the 1716 patients who developed chronic GVHD, *de novo*, progressive and quiescent chronic GVHD presentations were observed in 467 (27%), 348 (20%) and 901 (53%) patients, respectively. Compared with the MR-BM group, progressive chronic GVHD was more frequently observed in the MMU-BM group (33% vs 15%), and quiescent chronic GVHD was more frequently observed in the U-CB group (62% vs 53%).

Limited type of skin involvement was more frequently observed in the U-CB group than in the MR-BM group (53% vs 29%). We examined the types of chronic GVHD (limited vs extensive) in patients with limited type of skin GVHD to evaluate the effect of limited type of skin GVHD on chronic GVHD type in the U-CB group. Accordingly, extensive chronic GVHD was observed in 73% of patients with limited type of skin GVHD in the MR-BM group, compared with 49% of patients in the U-CB group. Oral cavity (28% vs 55%), eye (12% vs 26%), liver (20% vs 44%), lung (11% vs 25%) and joint (0% vs 6%) involvement was less prevalent in the U-CB group than in the MR-BM group. There was no organ that was more frequently involved in the U-CB group than in the MR-BM group.

Progressive onset of chronic GVHD, extensive skin GVHD, intestinal or genital involvement and extensive type of chronic GVHD were significantly associated with lower chronic GVHD-specific survival rates in multivariate analysis, after adjusting for other confounders (Table 4). Lung involvement in GVHD was marginally significant. On the other hand, limited type of skin GVHD was associated with higher chronic GVHD-specific survival rates. Chronic GVHD-specific survival and OS curves showing a significant difference between the groups are shown in Figure 3 and Supplementary Figure 1. The impact of chronic GVHD on relapse is also an important issue. The occurrence of chronic GVHD

Table 3. Risk factors for extensive type of chronic GVHD

Variable	*Extensive chronic GVHD (Total)*			*Extensive chronic GVHD (R-BM/PB)*			*Extensive chronic GVHD (U-BM)*			*Extensive chronic GVHD (U-CB)*		
	HR	*95% CI*	P-*value*	*HR*	*95% CI*	P-*value*	*HR*	*95% CI*	P-*value*	*HR*	*95% CI*	P-*value*
Recipient age, per 10 years	1.10	(1.05–1.15)	<0.001	1.12	(1.03–1.21)	0.010	1.07	(1.00–1.15)	0.049	1.10	(0.96–1.26)	0.180
Donor age, per 10 years				1.02	(0.94–1.10)	0.662	1.08	(0.98–1.20)	0.136			
Sex match between recipient and donor												
Match	1.00			1.00			1.00			1.00		
Male to female	1.02	(0.89–1.16)	0.822	1.00	(0.81–1.24)	0.977	1.08	(0.90–1.31)	0.409	0.82	(0.49–1.37)	0.442
Female to male	1.32	(1.16–1.50)	<0.001	1.49	(1.25–1.77)	<0.001	1.25	(1.01–1.55)	0.042	0.88	(0.55–1.41)	0.608
CMV Ab (donor and recipient)												
Both negative	1.00			1.00			1.00			1.00		
Either positive	1.32	(1.06–1.64)	0.014	1.17	(0.83–1.64)	0.383	1.37	(0.95–1.97)	0.089	1.54	(0.97–2.44)	0.068
Type of donor and stem cell source												
MR-BM	1.00											
MR-PB	1.41	(1.19–1.58)	<0.001									
MMR-BM	1.08	(0.79–1.49)	0.614									
MMR-PB	1.35	(1.01–1.81)	0.042									
MU-BM	0.78	(0.66–0.93)	0.005									
MMU-BM	0.93	(0.77–1.13)	0.452									
U-CB	0.65	(0.51–0.83)	0.001									
Type of stem cell source												
BM				1.00								
PB				1.42	(1.21–1.66)	<0.001						
HLA disparity												
Match				1.00			1.00			1.00		
Mismatch				1.10	(0.88–1.36)	0.397	1.14	(0.96–1.35)	0.142	0.89	(0.45–1.76)	0.743
Conditioning												
Myeloablative	1.00			1.00			1.00			1.00		
Reduced intensity	0.86	(0.75–0.97)	0.019	0.90	(0.74–1.08)	0.255	0.88	(0.72–1.07)	0.206	0.64	(0.42–0.96)	0.031
Use of in vivo *T-cell depletion*												
No	1.00			1.00			1.00					
Yes	0.39	(0.26–0.58)	<0.001	0.23	(0.13–0.41)	<0.001	0.80	(0.46–1.37)	0.407			
Acute GVHD												
Grade 0–I	1.00			1.00			1.00			1.00		
Grade II–IV	1.74	(1.56–1.93)	<0.001	1.52	(1.30–1.78)	<0.001	1.91	(1.62–2.26)	<0.001	2.02	(1.43–2.86)	<0.001

Abbreviations: CI = confidence interval; HR = hazard ratio; MMR-BM = HLA-mismatched related BM; MMR-PB = HLA-mismatched related PBSCs; MMU-BM = HLA-mismatched unrelated BM; MR-BM = HLA-matched related BM; MR-PB = HLA-matched related PBSCs; MU-BM = HLA-matched unrelated BM; R-BM/PB = related BM or PBSC; U-BM; unrelated BM; U-CB = unrelated cord blood.

was significantly associated with lower incidence of relapse than the absence of chronic GVHD for the total cohort (HR 0.88, $P = 0.018$). However, we did not find any significant different impact of type, onset and organ involvement of chronic GVHD on relapse among those with chronic GVHD.

DISCUSSION

In the present study, we extensively analyzed the risk factors for chronic GVHD, particularly focusing on donor graft sources and organ involvement, using recently obtained national registry data that included a large number of U-CB transplantations. In addition to confirming previously reported chronic GVHD risk factors, we observed a lower incidence of extensive chronic GVHD in recipients of U-CB than in recipients of MR-BM. Moreover, in patients with chronic GVHD, oral cavity, eye, liver, lung and joint involvement was substantially lower in the U-CB group than in the MR-BM group.

Grade II–IV acute GVHD occurrence was a strong risk factor for chronic and extensive chronic GVHD, regardless of the donor type, which is consistent with previous findings.[4–7] The mechanism through which chronic GVHD develops is considered to be different from that of acute GVHD,[28] and the underlying mechanism by which acute GVHD strongly influences chronic GVHD development remains unknown. Acute GVHD causes thymic epithelial damage and functional deterioration, leading to a decrease in thymic output, represented by low T-cell receptor excision circle levels.[29] The association between low T-cell receptor excision circle levels and occurrence of chronic GVHD was reported in HLA-identical sibling transplantation,[30] which may partly explain the association between the history of acute GVHD and the development of chronic GVHD. The combination of female donor/male recipient was significantly associated with the development of chronic GVHD, which is also consistent with previous studies.[4,6] In the subset analysis, the combination of female donor/male recipient was significant for the R-BM/PB group, but not significant for the U-CB group. T cells transplanted from adult female donors can be activated by exposure to Y-chromosome-associated proteins and may cause chronic GVHD, but those from female U-CB units may be less activated against them.[31] Studies on the effect of the CMV Ab on chronic GVHD development have previously yielded controversial results.[2,32] In this study, we observed a significant impact of CMV seropositivity on the incidences of chronic GVHD and extensive chronic GVHD. However, the presence of antigenemia itself was not a significant factor in univariate analysis (data not shown); therefore, the mechanism through which CMV Ab affects chronic GVHD development remains unknown. We also confirmed that the use of a PBSC graft vs a BM graft constituted a strong risk factor for chronic and extensive chronic GVHD development in the R-BM/PB group. On the other

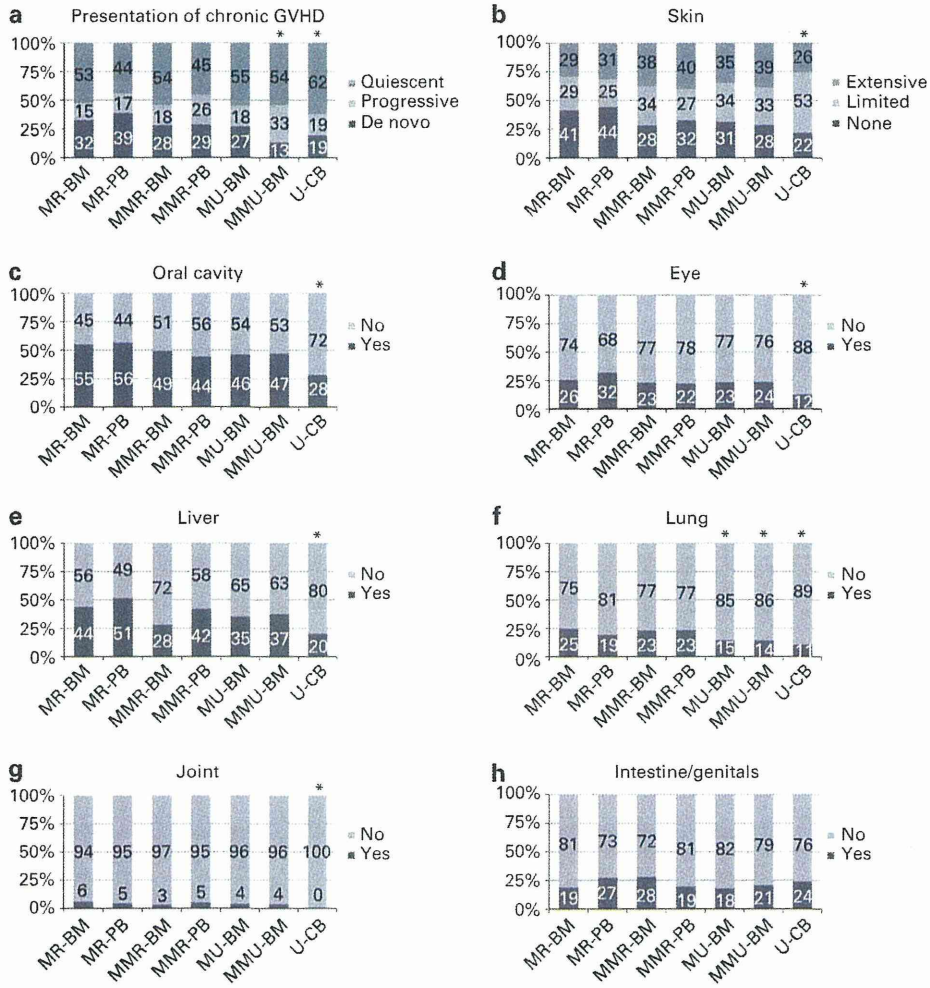

Figure 2. Presentation (**a**) and organ involvement (**b**–**h**) of chronic GVHD according to type of donor and stem cell source. Prevalence was compared between MR-BM and MR-PB, MMR-BM, MMR-PB, MU-BM, MMU-BM or U-CB. *$P<0.008$.

hand, the use of ATG was associated with a lower incidence of chronic GVHD, particularly in the R-BM/PB group. Contrary to previous reports, HLA disparity did not have a strong effect on chronic GVHD development in the R-BM/PB group. In addition, the use of MU-BM grafts was significantly associated with a lower incidence of extensive chronic GVHD. These findings may indicate that GVHD prophylaxis was intensified according to the acknowledged risk of GVHD. Therefore, we performed the same analysis after excluding the use of ATG or in the subgroup of patients who used tacrolimus or CsA as GVHD prophylaxis. However, we obtained the same result, which suggests that some other factor, such as the timing of immunosuppressive agent tapering, may be affecting the results.

In the analysis of chronic GVHD-specific survival, extensive type (vs limited type), progressive onset (vs *de novo* onset), extensive skin involvement (vs none), no skin involvement (vs limited involvement), and intestinal or genital involvement were associated with lower chronic GVHD-specific survival rate. The impact of quiescent onset chronic GVHD has been controversial,[2,33] but chronic GVHD-specific survival in the patients showing quiescent onset chronic GVHD was almost comparable to those showing *de novo* onset in line with several recent reports.[5,34] Although oral involvement was not associated with lower chronic GVHD-specific survival, which is compatible with a previous report,[35] intestinal or genital involvement was associated with lower survival rate. The use of U-CB was not associated with chronic GVHD-specific survival, even when only patients with extensive chronic GVHD were considered (data not shown). This finding suggests that chronic GVHD, if it occurs, does not behave differently regardless of the stem cell source. On the other hand, oral cavity, eye, liver, lung and joint involvement were substantially lower in the U-CB group, which contributed to the significantly lower incidence of extensive GVHD in the U-CB than in the MR-BM group. The high incidence of early TRM, such as that involving graft failure and infection, is considered a disadvantage of U-CB transplantations. However, if a patient survives the first few months following U-CB transplantation without treatment-related complications, the risk of extensive GVHD and GVHD-associated treatment-related complications would then be lower than in other transplantations. The low incidence of chronic GVHD would also contribute to the early discontinuation of immunosuppressive agents, which would allow or even promote immune reconstitution in long-term survivors of U-CB transplantation. Therefore, the choice of using U-CB as an alternative graft source might be prioritized if early treatment-related complications can be avoided through new approaches to ensure engraftment and enhance early immune reconstitution.

Table 4. Impact of type, presentation and organ involvement of chronic GVHD on chronic GVHD-specific survival

Characteristics	*Chronic GVHD-specific survival*		
	HR	*95% CI*	*P-value*
Type of chronic GVHD			
Limited	1.00		
Extensive	2.60	(1.67–4.05)	<0.001
Presentation of chronic GVHD			
de novo	1.00		
Progressive	1.73	(1.10–2.72)	0.017
Quiescent	0.76	(0.51–1.13)	0.173
Skin			
None	1.00		
Limited	0.58	(0.41–0.83)	0.002
Extensive	1.34	(1.01–1.78)	0.043
Oral cavity			
No	1.00		
Yes	0.97	(0.76–1.25)	0.840
Eye			
No	1.00		
Yes	1.03	(0.78–1.35)	0.859
Liver			
No	1.00		
Yes	1.17	(0.91–1.51)	0.225
Lung			
No	1.00		
Yes	1.29	(0.96–1.74)	0.091
Joint			
No	1.00		
Yes	0.93	(0.52–1.66)	0.795
Intestine/genitals			
No	1.00		
Yes	2.15	(1.66–2.78)	<0.001
Others			
No	1.00		
Yes	1.34	(0.85–2.11)	0.206

Abbreviations: CI = confidence interval; HR = hazard ratio. Hazard ratios were adjusted by type of stem cell source, recipient age, disease risk and grade II–IV acute GVHD.

Several limitations of this study should be noted. First, in this study, acute and chronic GVHD were diagnosed on the basis of traditional criteria, whereas chronic GVHD was diagnosed and classified on the basis of NIH criteria in recent studies.[36–39] Therefore, our results cannot be compared with those reported in other studies. In addition, it is possible that late onset acute GVHD was classified as chronic GVHD or early onset of chronic GVHD was defined as acute GVHD. This may bias the association between acute and chronic GVHD. Second, there is a possibility that chronic GVHD that developed a few years after SCT was not reported or was missed. Furthermore, detailed information on the clinical course of GVHD and on the onset of each chronic GVHD organ manifestation was not available; therefore, chronic GVHD-specific survival should be cautiously interpreted. Fourth, because organ involvement of chronic GVHD was not defined in detail in this large retrospective studies, there is a possibility of misclassification regarding organ involvement. Further, the information on intestinal or genital involvement was not separately collected in the questionnaire. Lastly, incidence of chronic GVHD in the present study was relatively low as compared with that in Caucasian cohorts, suggesting that the genetic differences between races may affect occurrence of chronic GVHD. Therefore, the results should be cautiously interpreted when the result is applied for non-Asian populations.

In conclusion, extensive chronic GVHD was less frequently observed in the U-CB group. In addition, among patients who developed chronic GVHD, oral cavity, eye, liver, lung and joint involvement were less frequently observed in the U-CB group. Although limited type of skin GVHD was frequently observed, it remains within the range of limited chronic GVHD. Therefore, the quality of life may be better for long-term survivors of the U-CB group than those of the MR-BM group or the other groups. Progressive onset, extensive chronic GVHD or intestinal or genital involvement was associated with lower chronic GVHD-specific survival, which suggests the need to intensify treatment for patients with these chronic GVHD characteristics. Finally, a prospective study using NIH criteria is needed to compare the

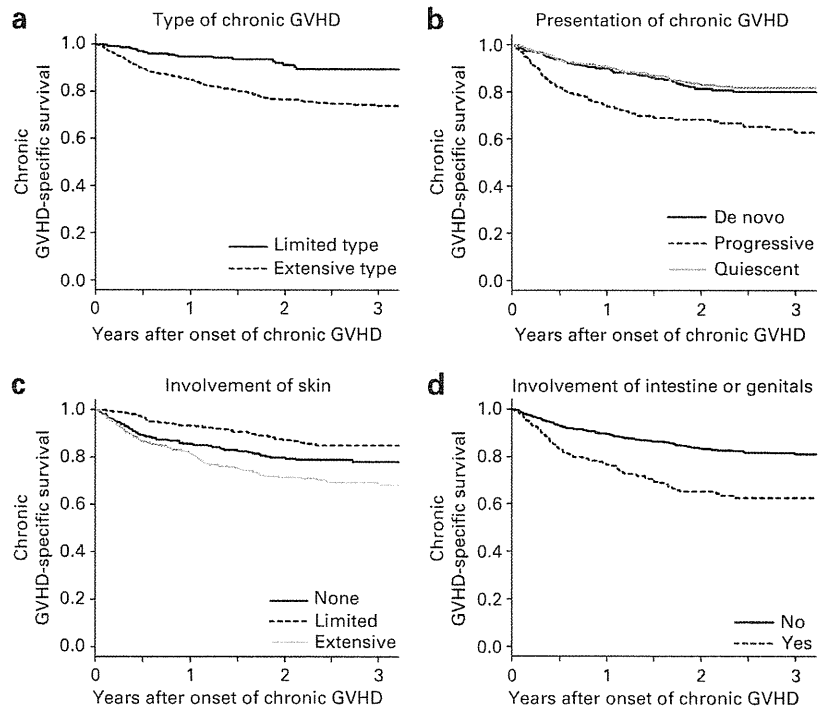

Figure 3. Chronic GVHD-specific survival stratified by type (**a**), presentation (**b**), involvement of skin (**c**) and involvement of intestine or genitals (**d**).

incidences of patients with chronic GVHD between Japan and other countries.

CONFLICT OF INTEREST

The authors declare no conflict interest.

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Continuing increased risk of oral/esophageal cancer after allogeneic hematopoietic stem cell transplantation in adults in association with chronic graft-versus-host disease

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Background: The number of long-term survivors after hematopoietic stem cell transplantation (HSCT) showed steady increase in the past two decades. Second malignancies after HSCT are a devastating late complication. We analyzed the incidence of, risk compared with that in the general population, and risk factors for secondary solid cancers.

Patients and methods: Patients were 17 545 adult recipients of a first allogeneic stem cell transplantation between 1990 and 2007 in Japan. Risks of developing secondary solid tumors were compared with general population by using standard incidence ratios (SIRs).

Results: Two-hundred sixty-nine secondary solid cancers were identified. The cumulative incidence was 0.7% [95% confidence interval (CI), 0.6%–0.9%] at 5 years and 1.7% (95% CI, 1.4%–1.9%) at 10 years after transplant. The risk was significantly higher than that in the general population (SIR = 1.8, 95% CI, 1.5–2.0). Risk was higher for oral cancer (SIR = 15.7, 95% CI, 12.1–20.1), esophageal cancer (SIR = 8.5, 95% CI, 6.1–11.5), colon cancer (SIR = 1.9, 95% CI, 1.2–2.7), skin cancer (SIR = 7.2, 95% CI, 3.9–12.4), and brain/nervous system cancer (SIR = 4.1, 95% CI, 1.6–8.4). The risk of developing oral, esophageal, or skin cancer was higher at all times after 1-year post-transplant. Extensive-type chronic graft-versus-host disease (GVHD) was a significant risk factor for the development of all solid tumors (RR = 1.8, $P < 0.001$), as well as for oral (RR = 2.9, $P < 0.001$) and esophageal (RR = 5.3, $P < 0.001$) cancers. Limited-type chronic GVHD was an independent risk factor for skin cancers (RR = 5.8, $P = 0.016$).

Conclusion: Recipients of allogeneic HSCT had a significantly higher ~2-fold risk of developing secondary solid cancers than the general population. Lifelong screening for high-risk organ sites, especially oral or esophageal cancers, is important for recipients with active, or a history of, chronic GVHD.

Key words: secondary solid cancers, late effect, hematopoietic stem cell transplantation

introduction

Hematopoietic stem cell transplantation (HSCT) is a curative treatment of choice for malignant and non-malignant hematological disorders [1]. The annual number of allogeneic HSCT has increased steadily over the past three decades worldwide [2–6]. Progress in transplant procedures in addition to this steady increase in the number of HSCT procedures worldwide has contributed to an increase in the number of long-term survivors.

Secondary malignancies, including new solid cancers, are an important cause of late mortality. Several studies have reported that survivors of HSCT have a 2–3-fold increased risk of

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developing new solid cancers compared with an age-, sex-, region-, and calendar-year-adjusted population and the risk among long-term survivors ranges from 1% to 6% at 10 years after transplantation [7–14]. Identified risk factors include exposure to radiation as a part of the conditioning regimen and chronic graft-versus-host disease (GVHD), and the latter has been shown to be strongly correlated with the development of squamous cell carcinoma [8, 10, 12, 15–17]. However, a recent long-term follow-up analysis of patients who were transplanted after myeloablative doses of busulfan and cyclophosphamide without total body irradiation (TBI) found a similar increased incidence of 0.6% at 5 years and 1.2% at 10 years after transplantation [13]. We conducted a nationwide, retrospective cohort study with a large and different cohort from those used in previous reports from North America and Europe, to determine the incidence and risks of developing secondary solid cancers.

methods

data source and collection of data

The recipient clinical data were collected by the Japan Society for Hematopoietic Cell Transplantation (JSHCT) using the Transplant Registry Unified Management Program, as described previously [18]. The JSHCT collect recipients' baseline, disease, transplant, and transplant outcome information who received HSCT in the previous year. Patient information regarding survival, disease status, and long-term complications including chronic GVHD and second malignancies are renewed annually. This study was approved by the data management committee of the JSHCT, as well as the institutional review board of Nagoya University Graduate School of Medicine.

patients

Adult patients (at least 16 years of age) who received a first HSCT between 1990 and 2007 were considered as subjects for the present study. Those who were inherently susceptible to developing cancer [Fanconi anemia ($N = 3$) and congenital immunodeficiency ($N = 12$)] were excluded. Three-hundred five recipients (1.7%) were excluded because of insufficient follow-up data. The study included 17 545 recipients; 5358 recipients of related bone marrow, 3587 recipients of related peripheral blood stem cells (including 134 bone marrow and peripheral blood stem cells combined), 6508 recipients of unrelated bone marrow, and 2092 recipients of unrelated cord blood.

statistical analysis

Standard incidence ratios (SIRs) were calculated to determine whether the number of recipients in the present cohort who developed secondary solid tumor after receiving a HSCT was different than that in the general population (supplementary method, available at *Annals of Oncology* online). Cumulative incidences of solid cancer or GVHD were estimated by taking into account the competing risk of death among patients who did not develop a second malignancy or GVHD [19]. The influence of potential risk factors was estimated by using the Cox proportional hazard model [20]. A stepwise multivariate approach was used to identify the most important predictor with respect to the development of secondary solid cancers. The variables considered were age at transplant, patient sex, donor-type (related versus unrelated), graft source, TBI as part of the conditioning regimen, reduced-intensity conditioning, grade 2–4 acute GVHD, and chronic GVHD. The model was stratified into four categories according to the primary disease; acute myeloid leukemia, acute lymphoblastic leukemia, chronic myeloid leukemia, and others. Acute and chronic GVHD were considered as time-dependent covariates. TBI and chronic GVHD were frequent risk factors and were always kept in the model. Risk factors for high-risk cancer sites with adequate numbers of events for analyses were also analyzed: oral cavity/pharynx, esophagus, colon, and skin. The models for high-risk cancer sites were stratified according to the primary disease as described, and patient age at transplantation (<19, 20–29, 30–39, 40–49, 50–59, and >60), and also adjusted by patient age as a continuous variable. All P-values were two-sided.

results

patient and transplant characteristics

Table 1 shows the patient characteristics, their disease, and transplant regimens for 17 545 recipients of a first HSCT. The cumulative incidences of grade 2–4 acute GVHD at 150 days and chronic GVHD at 2 years post-transplant were 35% [95% confidence interval (CI), 35%–36%] and 41% (95% CI, 40%–41%), respectively. The observation period reached 69 465 person-years among the subjects for analyses. Of the 17 545 recipients, 5864 had survived for 5 or more years, and 2192 recipients had survived 10 or more years at the time of analysis (Table 2).

incidence and types of secondary solid cancers

The cumulative incidence of solid cancers was 0.7% (95% CI, 0.6–0.9) at 5 years, 1.7% (95% CI, 1.4–1.9) at 10 years, and 2.9% (95% CI, 2.5–3.4) at 15 years after transplantation (Figure 1). Two-hundred sixty-nine solid cancers were identified. Multiple solid cancers were observed in 11 patients. Nineteen recipients were diagnosed within 1-year post-transplantation (Table 2).

risk compared with the general population

HSCT recipients had a 1.8-fold higher risk of invasive solid cancers compared with the general population (95% CI, 1.5–2.0). SIR was significantly higher for cancers of the oral cavity/pharynx (SIR = 15.7), esophagus (SIR = 8.5), colon (SIR = 1.9), skin (SIR = 7.2), and brain/nervous system (SIR = 4.1; Table 2). The risks of developing secondary cancers of the oral cavity/pharynx, esophagus, and skin were significantly higher than those in the general population throughout all periods after 1 year (Figure 2). The risk for developing colon cancer was elevated during the period of 1–4 years (SIR = 2.7), whereas the risks for developing cancer of the pancreas (SIR = 4.5) were elevated during the period of 5–9 years. Recipients were at higher risk of developing cancers of the rectum (SIR = 3.6) and the brain/nervous system (SIR = 19.1) after 10 years post-transplantation. The risk of developing secondary solid cancers of all types compared with the general population increased with the time since transplantation. This trend was observed for oral/pharynx and esophageal cancer (Table 2; Figure 2).

recipients' age at transplantation and risks for developing secondary solid cancers

SIRs were also analyzed according to the recipient's age at transplantation (Table 3). Compared with the general population in Japan, the SIRs were significantly increased for all solid cancers, oral/pharynx, esophagus, liver, bronchus/lung, and brain/nervous system for recipients who were 16–19 years of age at transplant, all solid cancers, oral/pharynx, and esophagus for recipients who

Table 1. Patient, disease, and transplant characteristics

Characteristics	Number	Percent
Total number	17 545	
Year of transplant		
1990–1994	1630	9
1995–1999	3750	21
2000–2004	7078	40
2005–2007	5087	29
Patient sex		
Male	10 386	59
Female	7149	41
Missing	10	<1
Patient age		
Median (range)	40 (16–85)	
16–19	1399	8
20–29	3506	20
30–39	3787	22
40–49	4167	24
50–59	3549	20
≥60	1137	6
Diagnosis		
Acute myeloid leukemia	6096	35
Acute lymphoblastic leukemia	3334	19
Chronic myeloid leukemia	2514	14
Myelodysplastic syndromes	1716	10
Adult T-cell leukemia	591	3
Other leukemia	130	1
Myeloproliferative disorders	224	1
Non-Hodikin's lymphoma	1652	9
Hodikin's lymphoma	46	<1
Other lymphoma/type missing	54	<1
Multiple myeloma	210	1
Aplastic anemia	745	4
Pure red cell aplasia	4	<1
Paroxysmal nocturnal hemoglobinuria	20	<1
Solid tumor	109	1
Others	86	<1
Data missing	14	<1
Donor		
Related, siblings	7825	45
Related, other relatives	941	5
Related, data missing	179	1
Unrelated	8600	49
Stem cell source		
Bone marrow	11 866	68
Peripheral blood	3453	20
Bone marrow and peripheral blood	134	1
Cord blood	2092	12
Conditioning regimen		
Myeloablative		
Cyclophosphamide + TBI ± other	8298	47
Other TBI regimen	1321	8
Busulfan + cyclophosphamide ± other	2798	16
Other non-TBI regimen	778	4
Reduced intensity		
Fludarabine + busulfan ± other	1527	9
Fludarabine + cyclophosphamide ± other	503	3
Fludarabine + melphalan ± other	1480	8
Other RIST	631	4
Data missing	209	1
GVHD prophylaxis		
No	85	<1
Cyclosporine A + sMTX	10 091	58
Cyclosporine A ± other	1175	7
Tacrolimus + sMTX	4682	27
Tacrolimus ± other	876	5
Other	323	2
Data missing	312	2

TBI, total body irradiation; sMTX, short-term methotrexate.

were 20–29 years of age at transplant, all solid cancers, oral/pharynx, esophagus, and gallbladder for recipients who were 30–39 years of age at transplant, all solid cancers, oral/pharynx, esophagus, and skin for recipients who were 40–49 years of age at transplant, all solid cancers, oral/pharynx, esophagus, colon, and skin for recipients who were 50–59 years of age at transplant (Table 3).

risk factors for the development of secondary solid cancers

Extensive-type chronic GVHD and age at transplantation were important risk factors for the development of secondary solid cancers (Table 4). The risk was not increased in recipients who received TBI for conditioning. The results were similar when subjects were limited to those who received myeloablative conditioning (RR = 1.5, $P = 0.069$ for limited-type chronic GVHD, RR = 1.9, $P < 0.001$ for extensive-type chronic GVHD, and RR = 0.9, $P = 0.751$ for TBI). Risk factor analyses for high-risk organs with more than 10 cancer cases revealed that extensive-type chronic GVHD was an independent risk factor for cancers in the oral cavity/pharynx and esophagus. Limited-type chronic GVHD was a risk factor for cancers of skin (Table 4). For secondary cancers which developed within 1-year post-transplant, the only risk factor identified was older age at transplant (age 60 years or older; supplementary Table, available at *Annals of Oncology* online).

discussion

Our main objective was to determine the incidence of, the risk compared with the general population, and risk factors for secondary solid tumors after allogeneic stem cell transplantation in a large cohort of adult recipients. Allogeneic HCT recipients were at higher risk of developing cancers of the oral cavity, esophagus, colon, and skin. The incidence and SIR of developing all solid cancers continued to increase with follow-up, which suggested a continuous increase as follow-up progressed. Our data are important since we included a large number of subjects and person-years of follow-up, in a transplant cohort that is different from those in previously reported large studies.

Table 2. Standard incidence ratio, ratio of observed versus expected number of secondary solid cancers according to duration post-transplant

	Time since transplantation (years)								Total		
	<1		1–4		5–9		10 or longer				
Number of recipients	17 545		10 210		5864		2192			17 545	
Person-years at risk	12 803		30 599		18 845		7218			69 465	
Secondary cancer sites	O	SIR	O	SIR	O	SIR	O	SIR	O/E	SIR	95% CI
All solid cancers	19	0.7	97	1.5*	90	2.0*	63	3.1*	269/153.6	1.8*	1.5–2.0
Oral/pharynx	0	0.0	16	9.5*	27	23.4*	21	38.5*	64/4.1	15.7*	12.1–20.1
Esophagus	0	0.0	13	6.5*	17	12.6*	11	16.8*	41/4.8	8.5*	6.1–11.5
Stomach	2	0.4	7	0.6	6	0.8	1	0.3	16/26.0	0.6	0.4–1.0
Colon	2	0.8	16	2.7*	5	1.2	4	2.2	27/14.3	1.9*	1.2–2.7
Rectum	0	0.0	1	0.2	0	0.0	5	3.6*	6/10.7	0.6	0.2–1.2
Liver	1	0.6	5	1.4	0	0.0	2	1.8	8/8.6	0.9	0.4–1.8
Gallbladder	2	5.1	2	2.1	2	3.0	0	0.0	6/2.3	2.6	1.0–5.7
Pancreas	0	0.0	2	1.0	6	4.5*	1	1.6	9/4.7	1.9	0.9–3.7
Broncus/lung	3	1.2	4	0.6	9	2.1	3	1.5	19/15.1	1.3	0.8–2.0
Skin	2	7.0	6	8.1*	3	5.7*	2	8.4*	13/1.8	7.2*	3.9–12.4
Female breast	0	0.0	3	0.3	1	0.1	3	0.9	7/24.5	0.3	0.1–0.6
Cervix uteri	1	1.3	4	2.0	1	0.7	1	1.6	7/4.8	1.5	0.6–3.0
Corpus uteri	2	3.7	1	0.7	2	1.8	0	0.0	5/3.6	1.4	0.4–3.2
Ovary	0	0.0	1	0.7	1	1.0	1	2.2	3/3.6	0.8	0.2–2.4
Prostate	1	1.2	0	0.0	1	0.6	1	1.4	3/5.4	0.6	0.1–1.6
Bladder	1	1.9	3	2.4	0	0.0	0	0.0	4/2.9	1.4	0.4–3.5
Kidney	0	0.0	1	0.6	1	0.9	0	0.0	2/4.1	0.5	0.1–1.8
Brain/nervous system	1	3.4	1	1.4	1	2.1	4	19.1*	7/1.7	4.1*	1.6–8.5
Thyroid	0	0.0	2	1.1	2	1.5	0	0.0	4/4.5	0.9	0.2–2.3
Other[a]	1		9		4		3		17		

[a]Other sites included two testicular cancers, four connective tissue cancers, four bone cancers, one larynx cancer, one malignant salivary gland tumor, one duodenum papilla cancer, one germ cell tumor, one carcinomatous pleurisy of origin unknown, and two squamous cell carcinomas of unknown origin.
*$P < 0.05$.

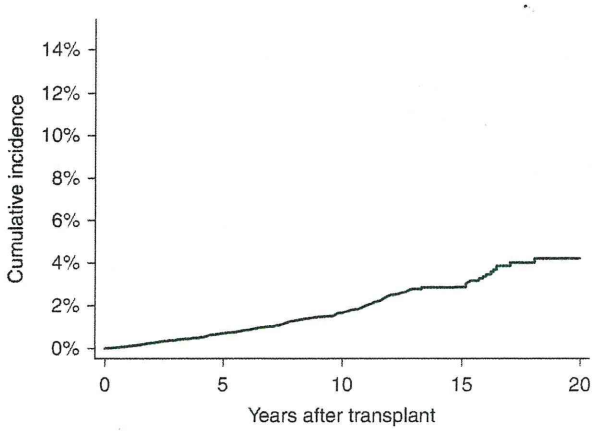

Figure 1. Cumulative incidence of developing a secondary solid cancer. The cumulative incidence of solid cancers was 0.7% [95% confidence interval (CI), 0.6–0.9] at 5 years, 1.7% (95% CI, 1.4–1.9) at 10 years, and 2.9% (95% CI, 2.5–3.4) at 15 years after transplantation.

Extensive-type chronic GVHD has repeatedly been shown to be a significant risk factor for the development of secondary solid tumor and is highly correlated with squamous cell carcinoma [8, 9, 12, 15, 16]. Extensive-type chronic GVHD was also shown to be a significant risk factor for oral cancer in our study. Extensive-type chronic GVHD was shown to be a significant risk factor for esophageal cancer, which was found to be increased in recipients compared with the general population in our study as well as in two other smaller Japanese cohorts in previous studies [11, 14]. Subjects were shown to be at a higher risk for the development of cancers of the oral cavity or esophagus at all time periods after 1 year. Data were not obtained for affected organ sites of chronic GVHD in JSHCT data collection prior to transplants in 2006. Therefore, we could not investigate whether oral or esophageal cancers were related to the chronic GVHD of the same organ. However, results of risk factor analyses for cancer sites of oral, esophagus, colon, and skin which showed high associations of extensive-type chronic GVHD and oral or esophagus cancer, limited-type chronic GVHD, and skin cancer showed that development of secondary solid tumors were likely to be influenced by GVHD-affected sites. Lifelong screening for oral, pharynx, or esophageal cancers for recipients with active or resolved chronic GVHD is important after 1-year post-transplant. The prognosis of solid cancers is highly influenced by the stage of the cancers when they are first detected. Our findings support recently published recommended screening guidelines [21, 22].

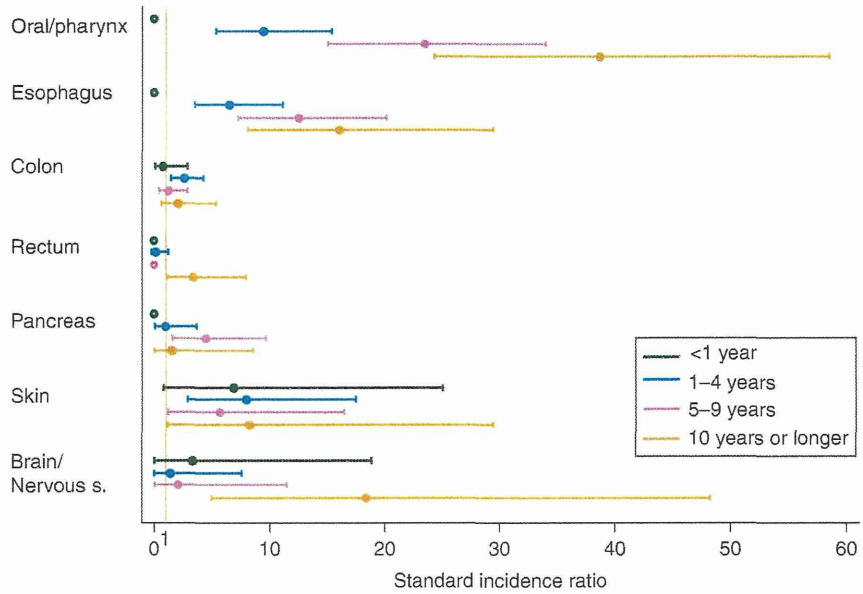

Figure 2. Trends of standard incidence ratios (SIRs) and its 95% confidence intervals (CIs) of high-risk secondary solid cancer sites according to time since transplant. The SIR and 95% CIs for <1, 1–4, 5–9, and 10 years or longer post-transplant were 0.0, 9.5 (5.4–15.4), 23.4 (15.4–34.0), and 38.5 (23.8–58.9) for oral/pharynx cancer, 0.0, 6.5 (3.5–11.2), 12.6 (7.3–20.2), and 16.8 (8.4–30.1) for esophageal cancer, 0.8 (0.1–2.9), 2.7 (1.5–4.3), 1.2 (0.4–2.9), and 2.2 (0.6–5.7) for colon cancer, 0.0, 0.2 (0.0–1.3), 0.0, and 3.6 (1.2–8.4) for rectum cancer, 0.0, 1.0 (0.1–3.7), 4.5 (1.6–9.7), and 1.6 (0.0–8.9) for pancreatic cancer, 7.0 (0.8–25.1), 8.1 (3.0–17.5), 5.7 (1.2–16.7), and 8.4 (1.0–30.3) for skin cancer, and 3.4 (0.1–19.0), 1.4 (0.0–7.7), 2.1 (0.1–11.6), and 19.1 (5.2–49.0) for cancers of brain/nervous system, respectively.

Table 3. Standard incidence ratio according to recipient's age at transplant

	Recipient's age at transplantation											
	16–19		20–29		30–39		40–49		50–59		60 or older	
Number-of-recipients	1399		3506		3787		4167		3549		1137	
Person-years at risk	7083		17 912		17 303		16 198		9126		1843	
Secondary cancer sites	O	SIR	O	SIR	O	SIR	O	SIR	O	SIR	O	SIR
All solid cancers	18	17.0*	28	4.1*	51	2.4*	71	1.4*	79	1.5*	22	1.0
Oral/pharynx	7	140.0*	11	50.7*	19	36.5*	13	10.1*	12	8.1*	2	3.9
Esophagus	1	350.0*	3	131.0*	13	48.5*	10	7.0*	13	5.9*	1	1.1
Stomach	1	13.3	0	0.0	1	0.3	7	0.8	5	0.5	2	0.5
Colon	0	0.0	0	0.0	3	2.0	6	1.3	12	2.1*	6	2.6
Rectum	1	33.1	0	0.0	0	0.0	1	0.3	4	0.9	0	0.0
Liver	1	66.4*	1	8.1	0	0.0	2	0.8	3	0.8	1	0.6
Gallbladder	0	0.0	0	0.0	2	12.0*	1	1.5	2	2.1	1	2.0
Pancreas	0	0.0	0	0.0	2	5.5	1	0.7	4	2.0	2	2.3
Broncus/lung	1	44.3*	0	0.0	2	1.6	7	1.6	7	1.1	2	0.7
Skin	1	28.6	1	6.3	0	0.0	6	11.6*	4	7.4*	1	4.0
Female breast	0	0.0	1	0.7	1	0.2	1	0.1	3	0.5	1	0.9
Cervix uteri	0	0.0	1	1.2	3	1.9	2	1.4	1	1.4	0	0.0
Corpus uteri	0	0.0	1	5.2	0	0.0	2	1.4	2	1.6	0	0.0
Ovary	0	0.0	1	3.2	0	0.0	1	0.7	0	0.0	1	6.4
Prostate	0	0.0	0	0.0	0	0.0	2	2.4	0	0.0	1	0.5
Bladder	0	0.0	0	0.0	0	0.0	2	2.3	2	1.7	0	0.0
Kidney	0	0.0	0	0.0	0	0.0	2	1.4	0	0.0	0	0.0
Brain/nervous system	2	23.9*	1	3.8	1	2.7	1	2.0	1	2.6	1	9.1
Thyroid	0	0.0	2	3.9	0	0.0	1	0.7	1	0.9	0	0.0

*$P < 0.05$.

Table 4. Risk factors for second solid cancers among >1 year survivors after hematopoietic stem cell transplantation

Solid cancer	Risk factor	Number of patients with second cancer	RR	95% CI	*P*-value
All second solid cancers[a]		249			
	Total body irradiation	151	0.9	0.7–1.1	0.294
	Chronic GVHD				
	Limited type	45	1.4	1.0–1.9	0.087
	Extensive type	93	1.8	1.4–2.4	<0.001
	Age at transplant (years)				
	16–29	45	1.0		
	30–39	46	1.6	1.0–2.4	0.042
	40–49	68	2.5	1.7–3.7	<0.001
	50–59	71	5.5	3.7–8.2	<0.001
	60 or older	19	7.9	4.4–14.1	<0.001
Oral cancer[b]		64			
	Total body irradiation	38	1.0	0.8–1.3	0.957
	Chronic GVHD				
	Limited type	10	1.4	0.6–2.9	0.440
	Extensive type	29	2.9	1.6–5.1	<0.001
Esophageal cancer[b]		41			
	Total body irradiation	22	0.6	0.3–1.1	0.108
	Chronic GVHD				
	Limited type	7	2.1	0.8–5.9	0.151
	Extensive type	25	5.3	2.4–11.8	<0.001
Colon cancer[b]		26			
	Total body irradiation	12	0.5	0.2–1.2	0.144
	Chronic GVHD				
	Limited type	6	1.7	0.6–4.9	0.353
	Extensive type	10	1.6	0.6–4.2	0.329
	Grade 2–4 acute GVHD	12	2.0	0.9–4.4	0.101
Skin cancer[b]		13			
	Total body irradiation	12	1.2	0.8–1.6	0.377
	Chronic GVHD				
	Limited type	6	5.8	1.4–23.9	0.016
	Extensive type	2	1.8	0.3–8.9	0.500

RR, relative risk; CI, confidence interval; TBI, total body irradiation; GVHD, graft-versus-host disease.
[a]Stratified for primary disease (acute myeloid leukemia, acute lymphoblastic leukemia, chronic myeloid leukemia, and other).
[b]Stratified for primary disease (acute myeloid leukemia, acute lymphoblastic leukemia, chronic myeloid leukemia, and other) and patient age groups (<19, 20–29, 30–39, 40–49, 50–59, and >60). Adjusted for patient age as a continuous variable.

The incidence of secondary solid tumors in our study was similar to those in previously reported large studies [8, 9, 12, 13]. Rizzo et al. [12] reported that the incidence of secondary solid cancers among 28 874 transplant recipients and 85 583 person-years at risk was 1% at 10 years and 2.2% at 15 years, which were very similar to our results using the same statistical method for cumulative incidence, while treating death before secondary solid tumor as a competing risk. Majhail et al. [13] reported that the incidence of secondary solid cancers after HSCT using non-TBI, busulfan-cyclophosphamide conditioning was also ~1.2% at 10 years. The oral cavity was the most prominent high-risk cancer site compared with the general population, as in previous reports [8, 9, 12, 13]. Despite regional and racial differences in cancer incidence and cancer sites in the general population, the impact of HSCT on secondary cancer was similar.

In previous studies, TBI was reported to be a significant risk factor for the development of secondary cancer, but significant differences were not found in our study [7, 8, 10, 12, 23]. The subjects in this study were adult recipients, which may explain the different findings. Conditioning with radiation was reported to be associated with the development of secondary solid cancer in recipients at a younger age at transplant [12]. Moreover, a recent long-term follow-up analysis of patients who were transplanted after myeloablative doses of busulfan and cyclophosphamide without TBI found a similar increased incidence of secondary solid cancers as previous reports [13].

An older recipient age at transplant was a significant risk factor for the development of secondary solid tumor, as in previous studies [9, 13]. This result was not surprising since it is also the case in the general population. However, it is important to note that older patients are at higher risk of developing

secondary cancer and to promote patient education and preventive practices, since there has been a dramatic increase in the number of transplant recipients who are more than 50 years of age at transplant over the past decade. In comparison with the general population, younger patients were at a higher risk of developing a solid tumor. Several high-risk cancer sites (esophagus, liver, and bronchus/lung) in younger group did have only one observed cases, therefore, these results should not be emphasized and need to be confirmed in other studies. These sites were found to be significant because the expected numbers in general population for these sites were extremely small.

Although this study included a large number of recipients and a large number of person-years of follow-up, there are limitations. The follow-up years for older recipients were still limited, and therefore we may find a higher incidence of and risk of secondary solid cancers among recipients who are 50 years of age or older at transplant in the future. Second limitation involves possible under-reporting by recipients to transplant centers or by transplant centers to the registry. Until recently, transplant recipients have received only limited information regarding screening or the prevention of secondary solid cancers. Another limitation of this analysis was lack of central pathology review for secondary solid tumors. JSHCT data collection does not include the submission of specimen or pathology report. Since this study included transplants from 1990, central pathology review was difficult to perform at the time of analyses. In addition, limiting secondary tumors to centrally diagnosed tumors would decrease the number of identified secondary tumors; therefore, secondary solid tumors were identified as reported from transplant centers.

In conclusion, recipients of allogeneic hematopoietic stem cell transplant had a significantly higher risk of developing secondary solid cancers than the general population. Older recipients are at higher risk of developing secondary solid tumors, as in the general population. Lifelong screening is important for high-risk organ sites, especially for oral, pharynx, and esophageal cancers in recipients with active, or a history of, chronic GVHD.

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disclosure

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Histology and Histopathology

Cellular and Molecular Biology

Review

Ischemic culture of dental pulp-derived cells is a useful model in which to investigate mechanisms of post-ischemic tissue recovery

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Summary. Dental pulp is a soft tissue characterized by unique regenerative properties. It is located in the center of each tooth, and is surrounded by hard tissue (dentin). Vascular access is limited to a small foramen at the root apex. Because of this anatomical limitation, dental pulp can easily lose its blood supply, causing the tissue to become ischemic. This occurs, for example, when a tooth is dislocated by traumatic injury or is subjected to inflammation. Since ischemia is caused by a critical shortage of oxygen and nutrients, ischemic damage is usually irreversible, even when the ischemic event is transient. However, unlike ischemia-sensitive organs such as the brain and heart, dental pulp is relatively ischemia-resistant, and recovers from ischemic injury by regenerating damaged tissue. The mechanisms by which this regeneration occurs are poorly understood, but are being investigated in cell culture models that mimic *in vivo* ischemic conditions using a combination of hypoxia and nutrient deprivation. Here, we review the use of ischemic cell culture to investigate the mechanisms of post-ischemic dental pulp tissue recovery.

Key words: Ischemia, Dental pulp, Stem cells, Odontoblasts, Regeneration

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Introduction

Ischemia occurs when arterial blood flow to a tissue is restricted. Because arterial blood supplies oxygen and nutrients, ischemia is characterized by a lack of these essential elements (Shinzawa and Tsujimoto, 2003). The intensity of tissue damage depends on the length and severity of the ischemic event. Although direct comparison is difficult, tolerance to ischemia appears to differ among various tissues and organs. For example, brain tissue is highly vulnerable to ischemia, and often becomes necrotic even when only transiently exposed to ischemic conditions (Sugiyama et al., 2011). In contrast, dental pulp is relatively tolerant to ischemia and is able to survive transient ischemic events such as tooth extraction and replantation (Tsukamoto-Tanaka et al., 2006). Dental pulp recovers its functions by regenerating damaged tissue after ischemia exposure. This unique response suggests that dental pulp contains ischemia-tolerant cells that play important roles in post-ischemic tissue regeneration.

Dental pulp contains a postnatal stem cell population named dental pulp stem cells (DPSCs) (Gronthos et al., 2000). DPSCs possess multi-lineage differentiation abilities (odontogenic, osteogenic, chondrogenic, adipogenic and neurogenic lineage), and are considered potent stem cells for use in tissue engineering and regenerative medicine (Gronthos et al., 2002; Iohara et al., 2006). Although the characteristics and functions of DPSCs within the pulp remain largely unknown, these cells appear to play an important role in tissue development, homeostasis and regeneration and are a

particularly interesting target for investigations into the mechanisms of post-ischemic tissue regeneration.

Evaluating dental pulp tissue reactions during and after ischemia would ideally be undertaken *in vivo*, but this is experimentally difficult due to the anatomic location of the tissue and its complex cellular composition (Liu et al., 2006). Thus, *in vitro* ischemic culture of dental pulp-derived cells has been developed as an alternative experimental model, though studies differ in the culture conditions utilized (Agata et al., 2008; Wang et al., 2010). In this review, we discuss *in vitro* cell culture conditions that best approximate *in vivo* ischemia. Next, we evaluate the relevance of these conditions in dental pulp-derived cell culture. Finally, we examine the characteristics of dental pulp-derived cells that survive ischemic culture conditions and explore possible mechanisms of post-ischemic pulp tissue regeneration.

Approximating *in vivo* ischemic conditions in experimental cell culture systems

Under ischemic conditions, cells experience both low oxygen tension and nutrient deprivation. Hence, ischemia can be mimicked *in vitro* by exposing cells to both hypoxia and a low-glucose environment (Jones et al., 2011). These appear to be the two most influential factors for tissue survival (Acosta et al., 1978). When PC12 cells (derived from a pheochromocytoma and able to differentiate into neurons) are cultured under hypoxic conditions and in a low-glucose environment, they are severely damaged, often to the point of necrosis (Shinzawa and Tsujimoto, 2003). Unfortunately, most of the current literature on ischemic culture of dental pulp-derived cell or DPSCs is limited to investigation of the effect of low oxygen tension alone; the number of studies using both low oxygen tension and nutrient deprivation is limited (Agata et al., 2008; Wang et al., 2010) (Table 1). In fact, low nutrient supply may enhance the effect of low oxygen tension. It has been shown that caspase-independent cell death, which is commonly seen under ischemic conditions, is significantly upregulated when cells are deprived of both oxygen and glucose (Agata et al., 2008).

Another important consideration when developing *in vitro* models of ischemia is the level of hypoxia used in the experiments. Conventional cell culture experiments use approximately 20% oxygen, with a partial pressure of oxygen (pO_2) of 140 mmHg (Rodrigues et al., 2010). However, pO_2 in the arterial blood of normal human subjects ranges from 60-90 mmHg and pO_2 in bone marrow is even lower (47-49 mmHg). The discrepancy between *in vitro* and *in vivo* conditions suggests that conventional cell culture may occur in a relatively hyperoxic environment, while traditional "hypoxic" culture environments actually reproduce normal physiologic conditions. This may explain why mesechymal stem cells grow and survive better in low oxygen cell culture environments. Mesenchymal stem cells cultured under low oxygen tension (5%) have a greater number of colonies as primary isolates, proliferate more rapidly and produce more bone (Lennon et al., 2001). In fact, "hypoxic" cell culture conditions can increase proliferation rates and enhance differentiation along multiple mesenchymal lineages (Das et al., 2010), providing further evidence that the

Table 1. Effect of hypoxia on DPSC culture.

Species	Oxygen tension	culture conditions	proliferation (compared to 20% O2)	cell properties (compared to 20% O2)	References
Human	1%	Monolayer culture for 24 hours	Cell proliferaton ↑	HIF-1α↑, CXCR4↑, SDF1↓	Gong et al. 2010
Human	1%	Monolayer culture for 24 hours /Endothelial cells culture in conditioned medium from hypoxic pulp cells for 72 hours	Endothelial cell proliferation ↑	HIF-1α↑, VEGF↑, bFGF→	Aranha et al. 2010
Human	2%	Monolayer culture for 24 or 48 hours	Cell proliferation ↓	SP cells↑, ABCG2↑, Oct4↑	Wang et al. 2010
Human	2%	Monolayer culture for 24 hours after80% confluent in normoxic conditions	none	Erythropoietin↑, Erythropoinetinreceptor↑	Gong et al. 2010
Human	3%	Monolayer culture for 14 days	Cell proliferation ↑	CD133↓, STRO-1↑	Sakdee et al. 2009
Human	3%	Monolayer culture with or without osteogenic supplements for 14 days	Cell proliferation ↑	STRO-1↑, osteogenic differetiation ↓	Iida et al. 2010
Human	5%	Monolayer culture with or without osteogenic supplements for 21 days	viability ↑	OCN↑, DMP1↑, BSP↑, DSPP↑ von kossa, alizarin↑(at 21 days of culture)	Li et al. 2011
Porcine	0.1% or 5%	Monolayer culture with or without osteogenic supplements under various glucose concentrations for 24 hours /Re-oxygenation after 24 hours ano/hypoxic culture + 3 day normoxic culture	Cell proliferation; Hypoxia ↑, Anoxia ↓	Oct4 ↑, Sox2↑ (at 6 hours in non-induced cells), ALP activity↑ (at 7 days after re-oxygen)	Agata et al. 2008

HIF-1α, hypoxia-inducible factor-1α; CXCR4, CXC chemokine receptor 4; SDF1, stromal cell-derived factor 1; VEGF, vascular endothelial growth factor; bFGF, basicfibroblast growth factor; SP cells, side population cells; ABCG2, ATP-binding cassette sub-family G member 2; Oct4, octamer binding transcription factor 4; OCN,osteocalcin; DMP1, dentin matrix acidic phosphoprotein 1; BSP, bone sialoprotein; DSPP, dentin sialoprotein; Sox2, SRY-box 2; ↑, increas; ↓, decrease; →, unchanged