overexpression of the *PI3-kinase*, which then leads to the characteristic clinicopathologic features of the acute type. The most frequent gain characteristic of the lymphoma type is gain of 7q11-36.3 (57%), which was absent from the acute type. The most frequent loss characteristic of the lymphoma type is that of 13q21.1-q32.1 (41%), which was found in only 1 (6%) of 17 patients with the acute type. The candidate genes for these regions, as well as several other regions of lymphoma type which showed significant alterations, may well be responsible for the characteristic clinicopathologic features of the lymphoma type.

The comparative analysis of the genome profiles of acute and lymphoma types identified distinct patterns of genomic alteration, suggesting that these entities might use distinct genomic pathways to develop tumors. The acute type of ATLL may develop tumors via a different mechanism such as translocation or/and mutation with fewer genomic alterations, whereas the lymphoma type may do so through a stepwise process with accumulation of genetic alterations.

We were able to demonstrate that *CARMA1* is a possible target of 7p22 amplification in the lymphoma type, making this the first report of aberrant *CARMA1* expression in human cancer. *CARMA1* is a lymphocyte-specific member of the membrane-associated guanylate kinase (MAGUK) family of scaffolding proteins. It interacts with BCL10 via its caspase-recruitmant domain (CARD), functions as an intermediate in the T-cell receptor (TCR) signal transduction pathway[23-25] and is a critical regulator of TCR inducing nuclear factor-κB (NF-κB) activation. Because NF-κB is known to be constitutively activated in primary adult T-cell leukemia cells, its activation may be essential for ATLL development.[26]

Overexpression of *CARMA1* leading to activation of the NF-κB pathway might play a crucial role in the tumorigenesis of acute and lymphoma types. In the lymphoma type, overexpression of *CARMA1* was found in patients with 7p22 amplification, while in the acute type, *CARMA1* was overexpressed without any 7p22 genomic gain. This makes it likely that a still unknown mechanism other than genomic amplification is responsible for *CARMA1* activation in the acute type.

It is known that the viral protein Tax induces the activation and nuclear translocalization of NF-κB,[27-30] but primary ATLL cells express only a low level of Tax mRNA and protein.[31,32] These facts indicate that there must be a mechanism other than Tax for NF-κB activation. *CARMA1* overexpression detected in our study is one of the mechanisms leading to constitutive activation of NF-κB in ATLL cells regardless of the level of Tax. However, the presence of patients with lymphoma-type ATLL lacking *CARMA1* overexpression indicates that other pathways for NF-κB activation may exist in ATLL.

RQ-PCR examination of *BCL11B* gene[33] expression for acute and lymphoma types showed that genomic amplifications of 14q32 did not correlate well with the expression levels of this gene. This means that *BCL11B* gene is not a target for 14q32 genomic amplification. Interestingly, however, considerably higher levels of *BCL11B* expression irrespective of 14q32 amplification were found in the acute type, suggesting that a mechanism other than genomic amplification induces *BCL11B* overexpression. Since several studies have shown that inv(14)(q11;q32) and t(14;14)(q11;q32) are recurrent chromosomal aberrations in ATLL,[34,35] aberrant overexpression of *BCL11B* may be due to translocation of 14q.[18] A candidate gene other than *BCL11B* must therefore exist in this 14q32 region of amplification for the lymphoma type and warrants further investigation. The difference in the expression profile of *BCL11B* between acute and lymphoma types also supports the concept that acute and lymphoma types are different disease entities.

In conclusion, genome profiling by array CGH demonstrated that the lymphoma type is characterize by more frequent gains and losses than is the acute type, and it was found that the patterns of genome profiles are also different for these 2 types. *CARMA1* was identified as a possible target of 7p22 amplification in the lymphoma type but no such amplification was found in the acute type although the expression of 7p22 was high. *BCL11B* was not overexpressed in the lymphoma type with 14q32 amplification but a relatively high expression of *BCL11B* was found in the acute type regardless of 14q32 amplification. These results suggest that acute and lymphoma types are distinct subtypes, and may develop via distinct genomic/genetic pathways.

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ORIGINAL ARTICLE

Increased risk for treatment-related mortality after bone marrow transplantation in GSTM1-positive recipients

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Treatment-related mortality (TRM) is a major obstacle to successful allogeneic hematopoietic stem cell transplantation (HSCT). A variety of drugs are used in allogeneic HSCT, and a genetic polymorphism in metabolic enzymes could affect the metabolism of drugs and potentially influence TRM. Here, we focused attention on GSTM1 and GSTT1 enzymes, which metabolize chemotherapeutic agents, chemical carcinogens and by-products of oxidative stress and are absent from more than 50% of some populations. To assess the significance of homozygous *GSTM1* and *GSTT1* gene deletion in HSCT, we analyzed DNA from 373 patients with hematological disease and their HLA-identical unrelated bone marrow donors using PCR. Homozygous *GSTM1* and *GSTT1* gene deletions were observed in 56 and 45% of patients, respectively, and 57 and 46% of donors, respectively. There was no significant association between GSTT1 polymorphism and any outcome. However, a GSTM1-positive genotype in recipients was significantly associated with higher TRM and lower survival. These results suggest that a *GSTM1*-null genotype in recipients protects against TRM after allogeneic HSCT. Further studies are needed to elucidate a mechanism of increased risk for TRM in GSTM1-positive recipients.

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Keywords: hematopoietic stem cell transplantation; GSTM1; GSTT1; polymorphism; treatment-related mortality; survival

Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) is a curative therapy for hematological diseases but also carries a risk of treatment-related mortality (TRM) resulting from chemoradiotherapy, graft-versus-host disease (GVHD) or its treatment, and infections.[1] A variety of drugs are used in allogeneic HSCT, such as chemotherapeutic agents, immunosuppressants, antibiotics, antifungal and antiviral drugs; thus, a genetic polymorphism in metabolic enzymes could affect the metabolism of drugs and influence subsequent TRM after HSCT.

We recently reported a metabolic enzyme, UDP glycosyltransferase 2 family, polypeptide B17 (UGT2B17), has a null phenotype at frequencies of 11 and 85% among Caucasians and Japanese populations, respectively,[2] and identified the use of a UGT2B17-positive donor as an independent risk factor for higher TRM and lower survival after HSCT.[3] Here, we focused attention on glutathione *S*-transferase (GST) M1 and GSTT1 enzymes. These enzymes mainly metabolize chemotherapeutic agents, chemical carcinogens and by-products of oxidative stress,[4] and are absent from more than 50% of some populations.[5–7] We analyzed the association between homozygous *GSTM1* and *GSTT1* gene deletions in the donor or recipient with various outcomes of transplantation and found GSTM1-positive recipients were significantly associated with higher TRM and lower survival. These results suggest that some reactive intermediates or toxic metabolites generated by GSTM1 may initiate or promote the development of TRM.

Patients and methods

Patients

The study population was selected from the patients who received bone marrow transplantation from an unrelated donor through the Japanese Marrow Donor Program (JMDP) between January 1993 and March 2000. The selection criteria for the patients and donors in the study population were (1) donor/recipient pairs matched for all genotypes of HLA-A, -B, -C and DRB1, (2) an intensive myeloablative pretransplant conditioning regimen, (3) an unmanipulated marrow graft, (4) the use of cyclosporin A (CsA) or tacrolimus as GVHD prophylaxis, (5) DNA samples were stored and available for genotyping, and (6) clinical outcome data were available. The genotypes of

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each allele at the HLA-A, -B, -C and DRB1 loci were determined by high-resolution DNA typing as described previously.[8,9]

The characteristics of the 373 patients are summarized in Table 1. Standard-risk disease was defined as acute myeloid leukemia or acute lymphoblastic leukemia in first remission, chronic myeloid leukemia in first chronic phase or myelodysplastic syndrome classified as refractory anemia. All other hematological malignancies including Hodgkin's lymphoma and non-Hodgkin's lymphoma were considered advanced disease. The pretransplant conditioning regimen varied according to disease or stage of disease at transplantation. GVHD prophylaxis consisted of either CsA plus short-term methotrexate (sMTX) ± anti-thymocyte globulin, or tacrolimus plus sMTX.

TRM was defined as any death that occurred while the patient was in remission. The assessment and grading of acute and chronic GVHD were performed as described previously.[10,11] A final clinical survey of these patients was carried out on July 1, 2001, and the median follow-up period was 45 months (range, 0–111 months). Stored DNA was available from all 373 recipients and from 313 donors for analysis of *GSTM1* and *GSTT1* genotype. Informed consent was obtained from all patients and donors, and approval was obtained from the ethics committee at Nagoya University School of Medicine.

Table 1 Patient characteristics

Number (male/female)	373 (211/162)
Median age (years (range))	24 (1–51)
Disease	
Acute myeloid leukemia	101
Acute lymphoblastic leukemia	92
Chronic myeloid leukemia	98
Myelodysplastic syndrome	28
Hodgkin's lymphoma	11
Non-Hodgkin's lymphoma	18
Severe aplastic anemia	25
Status of malignant disease	
Standard	164
Advanced	177
Unknown	7
Pretransplant conditioning regimen	
TBI containing	303
TBI + CY	102
TBI + CY + BU	33
TBI + CY + other(s)	130
TBI + BU	1
TBI + BU + other(s)	13
TBI + other(s)	24
Non-TBI containing	70
BU + CY	33
BU + CY + other(s)	23
BU + other(s)	2
CY + other(s)	12
GVHD prophylaxis	
CsA + sMTX (+ anti-thymocyte globulin)	299 (43)
Tacrolimus + sMTX	31

BU = busulfan; CsA = cyclosporin A; CY = cyclophosphamide; GVHD = graft-versus-host disease; other(s) = chemotherapy drug(s) other than cyclophosphamide and busulfan; sMTX = short-term methotrexate; TBI = total body irradiation.

Determination of homozygous deletion of the GSTM1 *and* GSTT1 *genes*

The homozygous deletion of the *GSTM1* and *GSTT1* genes was determined by PCR on genomic DNA from donor and recipient cells. The sense and antisense primers used for PCR to detect the *GSTM1* gene were 5′-GAACTC CCTGAAAAGCTAAAGC-3′ and 5′-GTTGGGCTC AAATATACGGTGG-3′, respectively. The sense and antisense primers used for PCR to detect the *GSTT1* gene were 5′-TTCCTTACTGGTCCTCACATCTC-3′ and 5′-TCACCGGATCATGGCCAGCA-3′, respectively. The sense and antisense primers for PCR to detect the *β-globin* gene as an internal control in each assay were 5′-ACACAACTGTGTTCACTAGC-3′ and 5′-CAACTT CATCCACGTTCACC-3′, respectively. Thirty cycles of amplification were performed using thermalcycler (Model 9600; Perkin-Elmer, Boston, MA, USA) on 0.5 μl genomic DNA extracted from peripheral blood before transplantation or Epstein–Barr virus-transformed lymphoblastoid cells, which were established from pretransplant cryopreserved peripheral blood mononuclear cells. Each reaction contained 0.4 μl of Advantage 2 Polymerase Mix (Clontech Laboratories Inc., Palo Alto, CA, USA), 0.2 mmol/l of each of the four deoxyribonucleotides, 4 pmol of each primer for *GSTT1*, *GSTM1* and *β-globin*, and PCR buffer in a volume of 20 μl. Each cycle consisted of denaturation (95°C; 30 s), annealing (65°C; 15 s) and elongation (72°C; 30 s). A 10 μl volume of the PCR product was analyzed by electrophoresis on a 1.5% agarose gel.

Statistical analysis

A χ^2 test with 2 × 2 contingency tables was used to evaluate differences of the frequencies of homozygous *GSTM1* and *GSTT1* genes deletion between patients and donors. The Cox proportional-hazard model was applied to multivariate analysis for TRM, acute and chronic GVHD, relapse, disease-free survival (DFS) and overall survival (OS).[12] The following variables were evaluated in a univariate analysis: patient age (continuous variable); disease status of hematological malignancy at the time of transplantation (advanced disease versus standard disease); GVHD prophylaxis (CsA containing regimen versus tacrolimus containing regimen); pretransplant conditioning regimen (TBI containing versus non-TBI containing); incidence of acute GVHD (only for analysis about chronic GVHD) (grade II–IV versus grade 0–I and grade III–IV versus grade 0–II); patient *GSTM1* and *GSTT1* genotype (deleted versus positive); and donor *GSTM1* or *GSTT1* genotype (deleted versus positive). All variables with $P<0.10$ were entered into the multivariate logistic regression using a backward, stepwise method. $P<0.05$ was regarded as statistically significant, and those between 0.05 and 0.1 as suggestive of a trend. The TRM, DFS and OS were estimated by using the Kaplan–Meier method, and log-rank test was used to analyze differences.[13]

Results

Frequencies of homozygous deletion of the GSTM1 *and* GSTT1 *genes*

A homozygous deletion of the *GSTM1* gene was found in 207 (55.5%) of 373 patients and in 177 (56.5%) of 313 healthy unrelated donors. A homozygous deletion of the *GSTT1* gene was found in 168 (45.0%) of 373 patients and in 145 (46.3%) of 313 healthy unrelated donors. There were no statistically significant differences in the frequencies of homozygous *GSTM1* and *GSTT1* deletion between each disease group and donor group (data not shown).

TRM

Of 367 evaluable patients for TRM, 115 (31.3%) were dead without relapse at the time of survey. In a univariate analysis, higher patient age, advanced disease and GSTM1-positive patient were associated with higher TRM (Table 2). There was no significant association between *GSTT1* genotype in either patient or donor and TRM. In a multivariate analysis, GSTM1-positive patient as well as higher patient age and advanced disease were significantly associated with higher TRM. TRM was analyzed in relation to *GSTM1* genotype in the patient by the Kaplan–Meier method (Figure 1a). TRM in the GSTM1-positive patients was significantly higher than that in the GSTM1-negative patients (42.9 versus 29.3%; $P=0.04$). Because death after day 200 may include treatment-'unrelated' death, we analyzed TRM within 200 days after transplant in relation to *GSTM1* genotype in the patient (Figure 1b). TRM within 200 days in the GSTM1-positive patients was also significantly higher than that in the GSTM1-negative patients (28.8 versus 18.7%; $P=0.04$).

Forty-four of GSTM1-positive patients died within 200 days after transplant while they were in remission. Causes of death in the patients were rejection/graft failure ($n=3$; 6.8%), GVHD ($n=11$; 25.0%), interstitial pneumonia ($n=8$; 18.2%), sepsis ($n=7$; 15.9%), bleeding ($n=3$; 6.8%), veno-occlusive disease (VOD) ($n=2$; 4.5%), adenovirus infection ($n=1$; 2.3%), myocardiopathy ($n=1$; 2.3%), cerebral infarction ($n=1$; 2.3%), hemolytic uremic syndrome ($n=1$; 2.3%), leukoencephalitis ($n=1$; 2.3%), pneumonia ($n=1$; 2.3%) and unknown ($n=4$; 9.6%). Thirty-seven of GSTM1-negative patients died within 200 days after transplant while they were in remission. Causes of death in the patients were rejection/graft failure ($n=1$; 2.7%), GVHD ($n=7$; 18.9%), interstitial pneumonia ($n=13$; 35.1%), sepsis ($n=6$; 16.2%), bleeding ($n=3$; 8.1%), VOD ($n=1$; 2.7%), heart failure ($n=2$; 5.4%), renal failure ($n=1$; 2.7%), hemosiderosis ($n=1$; 2.7%), thrombotic microangiopathy ($n=1$; 2.7%) and unknown ($n=1$; 2.7%). There was no significant difference in the frequencies of each cause of death between two patient groups ($P=0.52$).

Table 2 Univariate and multivariate analyses of risk factors for transplant outcome

Outcome and significant factor	*Univariate analysis*	*Multivariate analysis*	
	P-*value*	*Odds ratio (95% CI)*	P-*value*
TRM			
Higher patient age	0.001	1.03 (1.02–1.04)	<0.0001
Advanced disease	0.015	1.80 (1.23–2.63)	0.002
GSTM1-positive patient	0.043	1.49 (1.03–2.16)	0.036
Acute GVHD (II–IV)			
Advanced disease	0.025	1.46 (1.04–2.07)	0.031
CsA as GVHD prophylaxis	0.008	6.23 (1.54–25.2)	0.010
Chronic GVHD			
Acute GVHD (II–IV)	0.002	1.81 (1.25–2.61)	0.002
Relapse rate			
Advanced disease	<0.0001	3.64 (2.21–5.99)	<0.0001
DFS			
Higher patient age	0.02	1.02 (1.01–1.03)	0.0002
Advanced disease	<0.0001	2.54 (1.85–3.50)	<0.0001
GSTM1-positive patient	0.025	1.50 (1.08–1.97)	0.013
OS			
Higher patient age	0.0013	1.03 (1.02–1.04)	<0.0001
Advanced disease	<0.001	2.54 (1.83–3.53)	<0.0001
GSTM1-positive patient	0.070	1.41 (1.04–1.92)	0.030

CI = confidence interval.

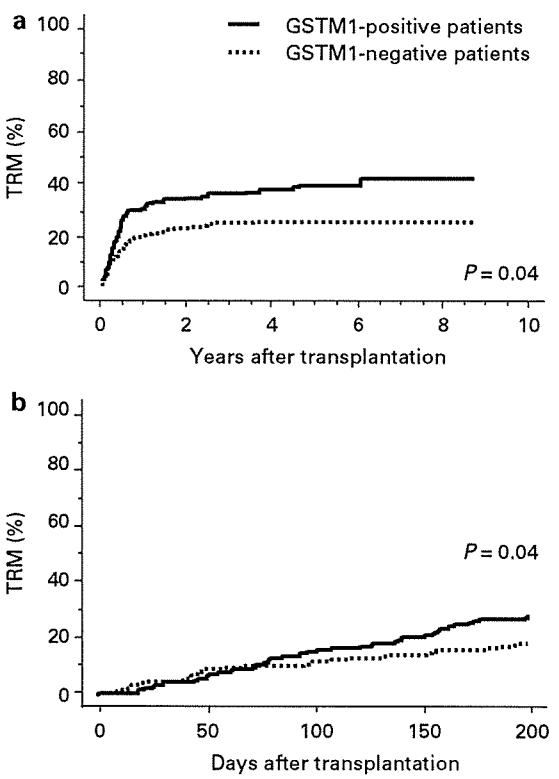

Figure 1 Impact of homozygous deletion of *GSTM1* on TRM after unrelated donor bone marrow transplantation. (**a**) TRM in the GSTM1-positive patients with a hematological disease was 42.9% ($n=166$) and that in GSTM1-negative patients was 29.3% ($n=201$). (**b**) TRM within 200 days after transplantation in the GSTM1-positive patients with a hematological disease was 28.8% ($n=166$) and that in GSTM1-negative patients was 18.7% ($n=201$).

It is suggested that a polymorphism of GSTM1 may be associated with risk of VOD development, especially if the patients received busulfan (BU) and/or cyclophosphamide (CY) as a pretransplant conditioning regimen.[14,15] We therefore analyzed the association between GSTM1 polymorphism and death from VOD in the subgroups of patients. In the patients who received BU (+others) ($n=105$), none of the GSTM1-positive patients ($n=47$) died of VOD, whereas one of the GSTM1-negative patients ($n=58$) died of VOD (not significant). In the patients who received CY (+others) ($n=333$), two of the GSTM1-positive patients ($n=145$) died of VOD, whereas none of the GSTM1-negative patients ($n=188$) died of VOD (not significant).

GVHD

Of 370 evaluable patients, 132 (35.7%) developed grade II–IV acute GVHD. No significant association was detected between the *GSTM1* or *GSTT1* deletion in the patient or donor with the incidence of grade II–IV acute GVHD (Table 2). Forty-one (11.1%) patients developed grade III–IV acute GVHD, but no factor was detected as a risk factor for a higher incidence of grade III–IV acute GVHD.

Of 304 evaluable patients, 124 (40.8%) developed chronic GVHD, including 48 (15.8%) with a limited type and 76 (25.0%) with an extensive type. No significant association was detected between the *GSTM1* or *GSTT1* deletion in the patient or donor with the incidence of chronic GVHD (Table 2).

Relapse

Of 348 evaluable patients with a malignant disease, 74 (21.3%) relapsed after transplantation. No significant association was detected between the *GSTM1* or *GSTT1* deletion in the patient or donor with a relapse rate (Table 2).

DFS and OS

Of 348 evaluable patients with a malignant disease, 177 (50.9%) were alive at the time of survey, including 165 (47.4%) patients who were alive without relapse. In a univariate analysis, higher patient age, advanced disease and GSTM1-positive patient were significantly associated with lower DFS and OS (Table 2). *GSTT1* genotype in either patient or donor was not associated with DFS and OS. In a multivariate analysis, GSTM1-positive patient as well as higher patient age and advanced disease were significantly associated with lower DFS and OS. DFS and OS were analyzed in relation to *GSTM1* genotype in the patient by the Kaplan–Meier method (Figure 2). Both DFS and OS in the GSTM1-positive patients were lower than those in the GSTM1-negative patients (DFS, 38.3 versus 54.0%, $P=0.02$; OS, 41.1 versus 55.1%, $P=0.07$).

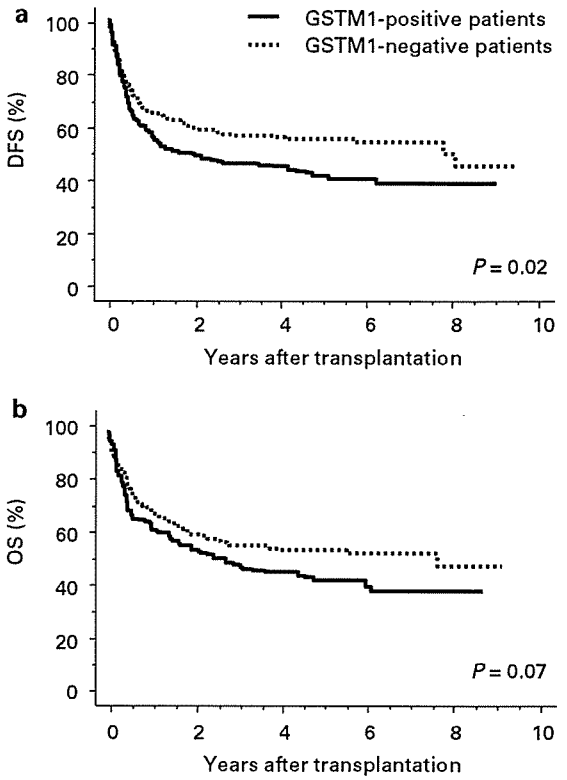

Figure 2 Impact of homozygous deletion of *GSTM1* on DFS and OS after unrelated donor bone marrow transplantation. (a) DFS in the GSTM1-positive patients with a malignant disease was 38.3% ($n=152$) and that in GSTM1-negative patients was 54.0% ($n=192$). (b) OS in those two groups were 41.1% ($n=152$) and 55.1% ($n=191$), respectively.

Discussion

To evaluate a potential contribution of *GSTM1* and *GSTT1* genotype to allogeneic HSCT outcome, we determined the frequencies of homozygous deletion of *GSTM1* and *GSTT1* genes in the patients with a hematological disease and healthy marrow donors. A homozygous deletion of the *GSTM1* and *GSTT1* genes was found in ~57 and ~46% of healthy donors, respectively, which was comparable to the previous Japanese studies.[7,16] Of patients with a hematological malignancy, homozygous deletion of the *GSTM1* and *GSTT1* genes was found in ~56 and ~45%, respectively, and there was no difference in the frequencies between each disease group and donor group. The findings, of no association between both *GSTM1*- and *GSTT1*-null genotypes and risk of each hematological malignancy, were consistent with our previous study in a different Japanese population;[7] however, many studies have emphasized the importance of these genetic polymorphisms in susceptibility to hematological diseases.[17,18] The analysis in a larger study size may reveal the role of *GSTM1*- and *GSTT1*-null genotypes in the development of hematological malignancies in Japanese people.

The present study demonstrated that a GSTM1-positive patient was significantly associated with higher TRM. GSTs are classified into phase II-metabolizing enzyme group. After phase I, enzymes, in which the cytochrome *P*450 has a central role, convert xenobiotics into a variety of reactive hydrophobic and electrophilic intermediates, phase II enzymes generally detoxify them by neutralizing

reactive electrophiles.[4] GSTs catalyze the nucleophilic addition of thiol of glutathione (GSH) to electrophilic acceptors of various substrates. However, in a minority of the reactions, GSTM1 can activate certain molecules instead of detoxification. GSH conjugates originated from such substrates are relatively unstable and the reaction product requires further detoxification, or is reversely converted into the original compound. Thus, it is possible some reactive intermediates or toxic metabolites can be generated by GSTM1 and initiate or promote the development of TRM.

Although the majority of GST substrates are either xenobiotics or by-products of oxidative stress, GSTs also contribute to the metabolism of endogenous compounds including leukotriene (LT) A4, prostaglandin (PG) D2, PGH2 and PGJ2, as part of their normal biosynthetic pathways.[4] LTB4 and PGI2 were described as markers for the inflammatory processes during GVHD development,[19] suggesting that the absence of GSTM1 may protect the recipient against the production of some inflammatory mediators.

Recently, GSTM1 deficiency in the patients with β-thalassemia major was found to be a risk factor for VOD after bone marrow transplantation using BU plus CY as a pretransplant conditioning regimen.[14] BU is metabolized by GSTs including GSTA1, GSTM1 and GSTP1 to form a positively charged sulfonium ion that is toxic to sinusoidal endothelial cells and hepatocytes. CY is metabolized to 4-hydroxycyclophosphamide (HCY) by cytochrome *P*450, and subsequently to iminocyclophosphamide. Iminocyclophosphamide is conjugated with GSTs and its metabolite is excreted into the bile. As a competing pathway for this detoxification pathway for HCY, the ring-opened form of HCY, aldophosphamide, enters cells and decomposes to phosphoramide mustard, the final cytotoxic metabolite, and acrolein through β-elimination.[15] Thus, BU metabolites and/or CY metabolites may increase risk of VOD. We analyzed the association between GSTM1 polymorphism and death from VOD in the subgroup of patients who received BU (+others) or CY (+others); however, we could not find the effect of GSTM1 polymorphism on fatal VOD. Further studies are clearly needed to determine whether GSTM1 polymorphism is associated with VOD development.

In summary, we assessed the significance of homozygous *GSTM1* and *GSTT1* gene deletions in the donor or recipient with the outcome of unrelated donor bone marrow transplantation. GSTT1 deficiency was not significantly associated with any outcome. However, the GSTM1-positive recipient was an independent risk factor for higher TRM and lower DFS and OS. It is possible that the presence of the *GSTM1*-null genotype in recipient may provide better protection against TRM after allogeneic HSCT. Further studies are needed to elucidate a mechanism of increased risk for TRM in GSTM1-positive recipients.

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ORIGINAL ARTICLE

Risk and prognostic factors for Japanese patients with chronic graft-versus-host disease after bone marrow transplantation

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The incidence and prognostic factors for chronic graft-versus-host disease (cGVHD) were evaluated for 255 Japanese patients who survived more than 100 days after bone marrow transplantation, and of whom 119 (47%) developed cGVHD. Prior acute GVHD (grade 2–4) and use of an unrelated donor were significantly associated with the onset of cGVHD. Presence of cGVHD did not have an impact on mortality (hazard ratio (HR) = 0.89; 95% confidence interval (CI), 0.59–1.3). Three factors at diagnosis were associated with cGVHD-specific survival: presence of infection (HR = 4.1; 95% CI, 1.6–10.3), continuing use of corticosteroids at the onset of cGVHD (HR = 3.9; 95% CI, 1.7–9.1), and a Karnofsky performance score <80 (HR = 4.7; 95% CI, 2.0–11.3). The probability of cGVHD-specific survival at 4 years was 79% (95% CI, 70–86%). The severity and death rate of Japanese patients with cGVHD was lower than those for populations in Western countries, which might be the result of greater genetic homogeneity of Japanese ethnics. Our patients could not be accurately classified when the proposed prognostic models from Western countries were used, thus indicating the need for a different model to identify high-risk patients.

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Introduction

Chronic graft-versus-host disease (cGVHD) is an important complication of allogeneic hematopoietic stem cell transplantation (HSCT) with a reported incidence from 30 to 70%.[1,2] To date, a variety of factors have been found to be associated with the development of cGVHD (reviewed by Higman and Vogelsang[3]). The most important factor is the presence of prior aGVHD, which has been repeatedly identified in several studies.[2 7] Other factors include older age, diagnosis of chronic myelogenous leukemia, female donor to male recipient, and the use of an unrelated donor.[2,4,6,7] A recent meta-analysis confirmed that use of peripheral blood stem cells significantly increases the risk of cGVHD.[8]

Chronic GVHD can be classified according to the extent of disease with the most commonly used grading system dividing cGVHD into limited or extensive disease. This system was proposed in 1980 based on the clinicopathologic findings of 20 patients.[9] However, the majority of patients come under the extensive cGVHD category, which is characterized by highly heterogeneous manifestations. For this reason, this grading system, although highly reproducible and useful for clinical decision making as to whether to initiate treatment, provides only limited information on the prognosis of patients.[10,11]

Several clinical and biologic features have been identified as prognostically significant in previous studies of cGVHD, including categorization as extensive cGVHD,[9,12] Karnofsky performance status (KPS),[12] thrombocytopenia ($<100\,000\,mm^3$),[12,13] progressive-type onset of cGVHD,[14] lichenoid histology,[14] and elevated bilirubin.[14] According to recently introduced new clinical grading systems for cGVHD,[10,11] thrombocytopenia, progressive-type onset, and extensive skin GVHD, involving more than 50% of body surface area at the diagnosis of cGVHD, are independent risk factors affecting relapse-free survival.[10] One of these systems uses a novel scoring method based on the results of 151 patients[10] and the validity and significance of this system were confirmed in 1105 patients from

multiple institutions.[15] Lee *et al.*[11] identified high KPS (≥80%) and oral involvement as favorable prognostic signs, and cutaneous involvement, diarrhea, and weight loss as unfavorable prognostic factors.

Because of the historically determined genetic homogeneity of the Japanese population[16] compared to those in Western countries, Japanese patients who receive allogeneic HSCT show a lower incidence of acute GVHD.[17 19] This finding prompted us to analyze a series of Japanese patients in an attempt to identify the incidence and risk factors of cGVHD as well as the prognostic factors for patients who developed cGVHD in the Japanese context.

Patients and methods

Patients

In the 5-year period from January 1995 to December 1999, a total of 400 patients with allogeneic HSCT were treated consecutively at eight centers belonging to the Nagoya Blood and Marrow Transplantation Group (NBMTG). This is a collaborative group consisting of 22 adult and three child HSCT centers. Eight centers of NBMTG which perform allogeneic transplants participated in this study. Of the 400 patients, 301 received bone marrow transplantation (BMT) from either an HLA genotypically matched relative or from HLA A, B, DR genotypically matched unrelated donors. Only patients who underwent BMT were included in this study, and those who received a peripheral blood stem cell transplant or who underwent two or more HSCT procedures were not. The study population comprised 255 patients who survived longer than 100 days post transplant, while patients who developed GVHD after donor leukocyte infusion ($n=2$) or who decided themselves to abandon immunosuppressive agents ($n=1$) were not included.

Diagnosis and staging of cGVHD

Diagnosis of cGVHD was based on clinical criteria of skin, oral and other affected sites as previously described.[9,20] The diagnosis of cGVHD was confirmed by histologic studies of skin, oral, or other affected sites in 60 of 110 patients (54.6%) who were clinically diagnosed. Established criteria were used for the histologic diagnosis.[9,21] Staging of cGVHD was originally performed by institutional physicians, and was reviewed on the basis of these physicians' data sheets by three independent hematologists (YA, RS and KY) using the most commonly used criteria.[9] The stage in agreement was used for the analyses.

Analysis of demographic variables for risk factors of extensive cGVHD

Patient age, sex, diagnosis of primary disease, disease status at transplant, donor type, preparative regimen, GVHD prophylaxis, and prior aGVHD grade were examined for risk factors of cGVHD.

Demographics, clinical, and laboratory variables analyzed for prognostic factors for those who developed cGVHD

The following variables documented at the time of diagnosis of cGVHD were used for our study: age, sex, donor type, donor/recipient sex matching, pretransplant disease status, preparative regimen, GVHD prophylaxis, prior occurrence and severity of aGVHD, positive cytomegalovirus status (donor and/or recipient), mode of presentation of cGVHD, serum alkaline phosphatase and total bilirubin, peripheral blood platelet count, absolute serum immunoglobulin level, blood eosinophil percentage, and use of corticosteroids before initiation of treatment of cGVHD. The extent and severity of cGVHD in the affected organs were also assessed. Other surrogate parameters indicating disease severity, such as weight loss after BMT, presence of chronic diarrhea, performance status measured by KPS, and the presence of infectious complications, were also included for analysis.

Coding system of the clinical variables analyzed for prognostic factors for patients who developed cGVHD

The most common sites of involvement were coded at the time of diagnosis of cGVHD into two or three grades by using a coding system based on the one by Akpek *et al.*[10] with several modifications. These codes were then entered into the database for analyses. The coding system or cutoff points of other variables are listed in Table 1.

Data collection instrument and methods

Subject-, disease-, and transplant-related variables were collected on standardized forms provided by the NBMTG. Survival, relapse, and complication data in the database, including those for cGVHD, are revised annually. Informed consent is obtained from all patients when they are registered with the NBMTG. This study was approved by the board of directors of NBMTG.

Patients diagnosed with cGVHD were selected from the NBMTG database. Data collection forms were sent out for the collection of clinical and laboratory data at the time of diagnosis of cGVHD, at the initiation of primary therapy, and, if used, at the initiation of secondary therapy. Immunosuppressive therapy regimens and responses to primary and secondary therapy were reviewed. Data on survival and relapse were collected as of March 2003.

Statistical analyses

Cox regression model was used for both univariate and multivariate analyses of risk factors for extensive cGVHD. Factors found to be significant ($P<0.05$) in the univariate analysis were included in the multivariate analysis.

To identify the prognostic factors for patients who developed cGVHD, the major statistical end point was 'cGVHD-specific survival' from the time of diagnosis of cGVHD. Although the term 'cGVHD-specific survival' does not completely exclude all deaths without recurrent malignancy, we used this term in its traditional meaning of recurrence censored survival.[10,15,22] Death because of relapse of underlying hematological disorders was censored at the time of relapse. Cox regression model was used to analyze data for the identification of prognostic factors. All clinical factors and laboratory data were initially categorized as several numeric variables (see above), and subsequently binarized during univariate analyses. The cutoff points were chosen to make optimal use of information,

Table 1 Prognostic significance of cGVHD parameters for cGVHD-specific survival

	N	*HR (95% CI)*	P-*value*
Mode of presentation of cGVHD			
De novo	33	1.0	
Quiescent	73	1.1 (0.44–2.6)	0.89
Progressive	4	1.8 (0.83–4.0)	0.14
Grade of cGVHD			
Limited	30	1.0	
Extensive	80	1.6 (0.60–4.3)	0.34
Cutaneous extent			
Code 1 (no skin involvement)	45	1.0	
Code 2 (50% or less of all skin surface area)	54	0.61 (0.26–1.5)	0.27
Code 3 (more than 50% of all skin surface area)	9	1.3 (0.71–2.2)	0.42
Oral involvement			
Code 1 (no clinical evidence of cGVHD)	25	1.0	
Code 2 (clinically evident oral symptoms)	78	0.62 (0.25–1.5)	0.31
Code 3 (severe oral cGVHD causing functional impairment)	4	1.1 (0.37–3.1)	0.91
Thrombocytopenia			
Code 1 ($\geqslant 100\,000/mm^3$)	56	1.0	
Code 2 ($<100\,000/mm^3$ and $\geqslant 50\,000/mm^3$)	40	1.2 (0.48–2.8)	0.73
Code 3 ($<50\,000/mm^3$)	14	2.1 (0.74–6.2)	0.16
Eosinophilia (>4%)	45	3.5 (1.3–9.5)	0.01
Hyperbilirubinemia (>1.2 mg/dl)	22	1.7 (0.71–4.2)	0.23
Elevated alkaline phosphatase (>2.5 × ULN IU/l)	26	1.8 (0.79–4.2)	0.16
Weight loss			
None	61	1.0	
<10% from baseline; body weight before BMT	22	0.65 (0.22–2.0)	0.45
⩾10% from baseline; body weight before BMT	9	1.4 (0.75–2.6)	0.29
Chronic diarrhea	7	2.1 (0.62–6.9)	0.24
Presence of infection	20	5.7 (2.5–13.2)	<0.0001
Reduced serum IgG (<500 mg/dl)	33	2.0 (0.84–4.7)	0.12
KPS<80%	30	5.8 (2.5–13.3)	<0.0001
Use of corticosteroids	23	3.9 (1.8–8.7)	0.001

GVHD = graft-versus-host disease; HR = hazard ratio; ULN = upper limit of normal; BMT = bone marrow transplantation; KPS = Karnofsky performance score.

with the proviso that smaller groups contain at least 20% of the patients. A forward stepwise method was applied for detecting sets of independent significant factors. In the previously reported studies of prognostic factor analyses of cGVHD, the statistical end point was either cGVHD-specific survival,[10,15,22] or overall survival.[11] Our analyses were therefore also performed with overall survival as the statistical end point.

Survival distributions were estimated with the method of Kaplan and Meier, and compared by means of the long-rank test. Cumulative incidence of cGVHD was calculated by treating death without cGVHD or relapse as a competing risk for cGVHD. The proportional hazards regression model with cGVHD entered as a time-dependent covariate was used to determine the effect of cGVHD on relapse, non-relapse mortality, and overall survival. All statistical analyses were performed with STATA software version 8.0 (College Station, TX, USA).

Results

Patient characteristics and incidence of cGVHD

Patient characteristics and BMT information are summarized in Table 2.

Of the 255 BMT recipients, 119 (47%) developed cGVHD. The cumulative incidence of cGVHD 2 years after transplant was 42% (95% confidence interval (CI), 35–48%) (Figure 1a). Cumulative incidence of clinical extensive cGVHD 2 years after transplant was 31% (95% CI, 25–37%).

Risk factors for developing cGVHD

Among the factors evaluated as risks for the development of clinical cGVHD, only the presence of prior grades 2–4 aGVHD was identified as significant by univariate analysis (Table 3). When the same clinical characteristics were analyzed for clinical extensive cGVHD, univariate analysis showed two factors to be significant, unrelated donor type (hazard ratio (HR) = 1.8; 95% CI, 1.2–2.7; $P = 0.008$), and prior grades 2–4 aGVHD (HR = 1.9; 95% CI, 1.2–3.0; $P = 0.005$). Multivariate analysis demonstrated that the factors constituting independent risk factors for clinical extensive cGVHD were unrelated donor type (HR = 1.6; 95% CI, 1.1–2.5; $P = 0.02$), and prior grades 2–4 aGVHD (HR = 1.8; 95% CI, 1.1–2.7; $P = 0.02$).

Effect of cGVHD on relapse and mortality

The median follow-up of survivors was 4.1 years. The probability of overall survival 4 years after the transplant

Table 2 Patient characteristics

	N
Total number of patients	255
Median age, years (range)	36 (16–54)
Male/female	174/81
Diagnosis	
Acute leukemia/myelodysplastic syndrome	143
Chronic leukemia	69
Lymphoma/multiple myeloma	27
Aplastic anemia	14
Disease status (malignant disease, only)	
Standard risk (CR1 and chronic phase)	120
Advanced risk (all others)	130
Donor type	
HLA-identical, related	155
HLA-identical, unrelated	100
Preparative regimen	
Chemotherapy alone	21
Total body irradiation based	202
GVHD prophylaxis	
Cyclosporine + short-term methotrexate	189
FK506 + short-term methotrexate	54
Others	12
Acute GVHD grade	
0	96
1	97
2–4	62

CR1 = first complete remission; HLA = human leukocyte antigen; GVHD = graft-versus-host disease.

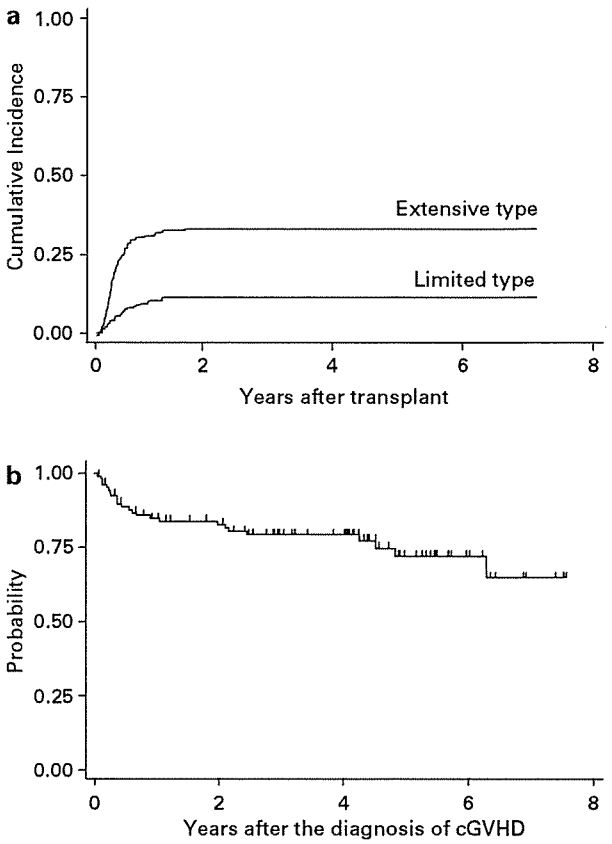

Figure 1 Cumulative incidence of limited and extensive cGVHD after allogeneic hematopoietic stem cell transplantation was 10% (95% CI, 7–14%) and 31% (95% CI, 25–37%) at 2 years, respectively. (**a**) cGVHD-specific survival from the time of diagnosis of cGVHD for 110 patients who developed cGVHD was 79% (95% CI, 70–86%) at 4 years (**b**).

was 60% (95% CI, 53–66%), and cumulative incidence of relapse after 2 years was 21% (95% CI, 16–27%).

The analysis of the effects of cGVHD as a time-dependent covariate with adjustments for patient age, sex, disease status, and donor type showed no statistical significance for relapse (HR = 0.70; 95% CI, 0.37–1.3), non-relapse mortality (HR = 1.4; 95% CI, 0.72–2.6) or survival (HR = 0.97; 95% CI, 0.64–1.5). The results were similar to the results obtained for the effects of clinical extensive cGVHD on relapse (HR = 0.67; 95% CI, 0.33–1.3), non-relapse mortality (HR = 1.6; 95% CI, 0.92–2.7), and survival (HR = 1.05; 95% CI, 0.69–1.6).

Characteristics and survival of patients who developed clinical cGVHD

Detailed information at the time of diagnosis of cGVHD was available for 110 of the 119 patients who developed cGVHD. Their characteristics are shown in Table 4, and their clinical manifestations and laboratory data at the time of diagnosis of cGVHD are given in Table 1.

Follow-up of survivors after the diagnosis of cGVHD (median length: 4.2 years) showed that 69 (63%) of the 110 patients were alive at the time of data collection. The probability of cGVHD-specific survival and overall survival 4 years after the diagnosis of cGVHD was 79% (95% CI = 70–86%) (Figure 1b) and 65% (95% CI = 55–74%), respectively. Of the 41 deaths, 16 (39%) were the result of relapse and 25 (61%) of other complications. The latter consisted of various infections (seven patients) and organ failures associated with cGVHD (12 patients). Detailed information on their cause of death is summarized in Table 5. The patient who died of gastric cancer was censored from the analysis of cGVHD-specific survival when the disease was diagnosed. For the 80 patients with clinical extensive cGVHD, cGVHD-specific survival and overall survival 4 years after the diagnosis of cGVHD were 76% (95% CI = 65–84%) and 62% (95% CI = 50–71%), respectively.

Prognosis of patients with cGVHD

Univariate analysis showed that age of 36 or older (HR = 2.6; 95% CI, 1.1–6.3), <4% eosinophils at the time of diagnosis of cGVHD (HR = 3.5; 95% CI, 1.3–9.5), Karnofsky score of <80% (HR = 5.8; 95% CI, 2.5–13.3), presence of infectious complication (HR = 5.7; 95% CI, 2.5–13.1), and use of corticosteroids before initiating treatment for cGVHD (HR = 3.9; 95% CI, 1.8–8.7) were significant prognostic factors (Table 4, Table 1). Corticosteroids were being administered to 23 patients (22%) at

Table 3 Univariate analysis of risk factors for development of cGVHD

Factor	*No. of patients*	*HR (95% CI)*	P-*value*
Patient age			
Younger than 36 years	117	1.0	
36 years or older	138	0.93 (0.65–1.3)	0.73
Patient sex			
Female	81	1.0	
Male	174	1.1 (0.76–1.6)	0.56
Diagnosis			
Acute leukemia/myelodysplastic syndrome	143	1.0	
Chronic leukemia	69	1.2 (0.81–1.8)	0.38
Lymphoma/multiple myeloma	27	0.85 (0.51–1.4)	0.54
Aplastic anemia	14	—	—
Disease status (malignant disease, only)			
Standard risk (CR1 and chronic phase)	120	1.0	
Advanced risk (all others)	130	0.76 (0.52–1.1)	0.13
Donor type			
HLA-identical, related	155	1.0	
HLA-identical, unrelated	100	1.4 (0.94–1.9)	0.10
Preparative regimen			
Chemotherapy alone	21	1.0	
Total body irradiation based	202	1.00 (0.54–1.9)	1.00
GVHD prophylaxis			
Cyclosporine + short-term methotrexate	189	1.0	
FK506 + short-term methotrexate	54	1.1 (0.68–1.6)	0.84
Acute GVHD grade			
0–1	193	1.0	
2–4	62	1.8 (1.2–2.6)	0.005

GVHD = graft-versus-host disease; HR = hazard ratio; CR1 = first complete remission; HLA = human leukocyte antigen.

the onset of cGVHD. All patients received steroids for treatment of acute GVHD and were in the process of tapering off when the cGVHD developed. Multivariate analysis identified three factors at the time of diagnosis of cGVHD as independent predictors for non-relapse mortality: presence of infection (HR = 4.1; 95% CI, 1.6–10.4), use of corticosteroids (HR = 3.9; 95% CI, 1.7–9.1), and KPS <80% (HR = 4.7; CI, 2.0–11.3) (Table 6). These results enabled us to establish a prognostic model for cGVHD-specific survival or relapse-censored survival in terms of how many of these three risk factors are present. Figure 2 shows the probability of cGVHD-specific survival for patients divided into three risk groups according to this model. The estimated 4-year cGVHD-specific survival was 91.8% for patients without any prognostic factors (PFs) ($n = 51$), 72.6% for those with one or two factors ($n = 42$), and 14.3% for those with all three factors ($n = 7$) ($P < 0.0001$; log rank test). Six of the seven patients who showed all three prognostic factors died within 8 months after the diagnosis of cGVHD. The same three risk groups were used successfully

Table 4 Patient characteristics and their prognostic significance for cGVHD-specific survival

	N	*HR (95% CI)*	P-*value*
Total number of patients	110		
Median age, years (range): 36 years (17–62)			
Younger than 36 years	52	1.0	
36 years or older	58	2.6 (1.1–6.3)	0.03
Sex			
Female	34	1.0	
Male	76	1.0 (0.44–2.4)	0.96
Donor and recipient, sex mismatch			
None	56	1.0	
Female to male	34	0.96 (0.62–1.5)	0.83
Male to female	15	1.0 (0.29–3.5)	0.99
Disease status			
Standard risk (CR1 and chronic phase)	61	1.0	
Advanced risk (all others)	49	0.74 (0.33–1.7)	0.48
Donor type			
HLA-identical, related	65	1.0	
HLA-identical, unrelated	45	0.84 (0.37–1.9)	0.67
GVHD prophylaxis			
Cyclosporine + short-term methotrexate	84	1.0	
FK506 + short-term methotrexate	24	1.2 (0.47–3.0)	0.73
Others	2		
Acute GVHD grade			
0	33	1.0	
1 (cutaneous)	43	1.3 (0.43–3.8)	0.67
2–4 (systemic)	34	1.6 (0.94–2.7)	0.08

GVHD = graft-versus-host disease; HR = hazard ratio; CR1 = first complete remission; HLA = human leukocyte antigen.

Table 5 Cause of death of patients who developed cGVHD

Cause of death	N
Relapse	16
Other than relapse	25
Infection	7
Organ failures associated with cGVHD	12
Lung failure	10
Liver failure	2
Other causes	6

GVHD = graft-versus-host disease.

for overall survival. The estimated 4-year overall survival was 80% for patients without any prognostic factors (PFs) ($n = 51$), 60% for those with one or two factors ($n = 42$), and 0% for those with all three factors ($n = 7$) ($P < 0.0001$; log rank test).

Univariate and multivariate analyses confirmed the same three factors as independent prognostic factors when the subjects were limited to 80 patients with clinical extensive cGVHD. These three risk groups thus resulted in successful categorization of patients with clinical extensive cGVHD for prognosis of cGVHD-specific survival ($P < 0.0001$;

Table 6 Multivariate analysis for non-relapse mortality of patients newly diagnosed with cGVHD

Prognostic factor	N	*HR*	*95% CI*	P-*value*
KPS≥80%	76	1.0	—	
KPS<80%	30	4.7	2.0–11.3	<0.001
No use of corticosteroid	82	1.0	—	
Use of corticosteroid	23	3.9	1.7–9.1	0.002
Absence of infection	88	1.0	—	
Presence of infection	20	4.1	1.6–10.4	0.001

GVHD = graft-versus-host disease; HR = hazard ratio; CI = confidence interval; KPS = Karnofsky performance score.

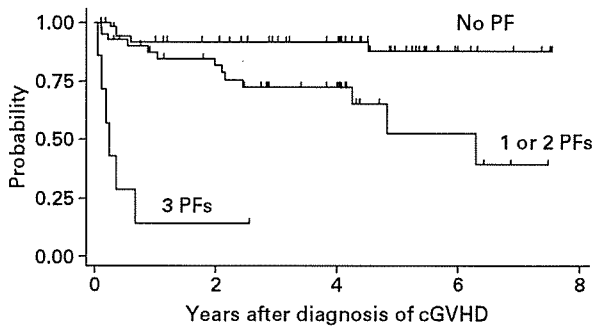

Figure 2 cGVHD-specific survival after the diagnosis of cGVHD for patients grouped by the total number of prognostic factors (PFs) was 92% (95% CI, 80–97%) ($n=51$) for no PF, 73% (95% CI, 55–84%) ($n=42$) for 1 or 2 PFs at 4 years, and 14% (95% CI, 1–46%) ($n=7$) for 3PFs at 2 years.

log rank test) as well as of overall survival ($P<0.0001$; log rank test).

Comparison with other grading systems

The classical grading system of limited/extensive-type cGVHD produced the survival curves in Figure 3a for our patients after the diagnosis of cGVHD. The result shows that the probability of cGVHD-specific survival 4 years after the diagnosis of cGVHD was 76% (95% CI, 65–84%) for the extensive type and 89% (95% CI, 70–96%) for the limited type, without any statistically significant difference (log rank test; $P=0.34$). There was no significant difference either when the statistical end point was overall survival (log rank test; $P=0.25$). In this case, the probability of overall survival 4 years after the diagnosis of cGVHD was 62% (95% CI, 50–72%) for the extensive type and 76% (95% CI, 56–88%) for the limited type.

Another grading system by Akpek *et al.*[15] was also used. None of the factors used in this grading system, for example, progressive-type onset, extensive involvement of more than 50% of total skin surface area, and thrombocytopenia proved to be prognostic for our patients (Table 1). Categorization according to this system for cGVHD-specific survival when matched to the system's statistical end point could not produce a useful stratification of our patients (Figure 3b). Finally, the grading system by Lee *et al.*[11] was used for our patients for overall survival matched to that system's statistical end point. The results in

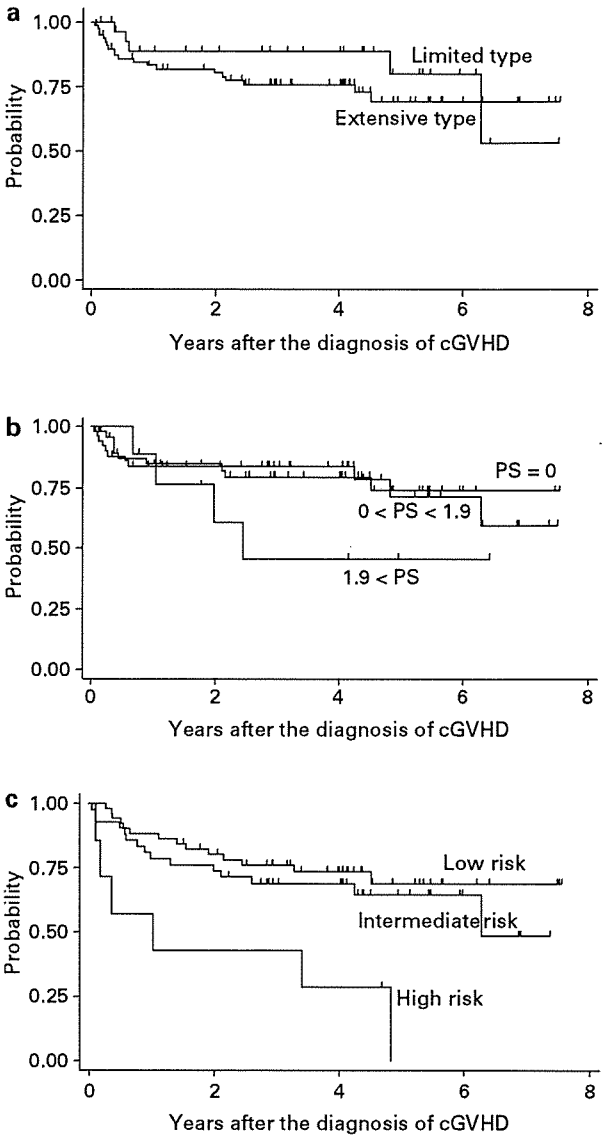

Figure 3 Survival curves after the diagnosis of cGVHD according to the classical limited/extensive grading system. cGVHD-specific survival at 4 years was 89% (95% CI, 70–96%) ($n=30$) and 76% (95% CI, 65–84%) ($n=80$), respectively (**a**). With Akpek's prognostic scoring (PS) system, the cGVHD-specific survival at 4 years was 79% (95% CI, 70–92%) ($n=48$) for PS = 0, 84% (95% CI, 70–92%) ($n=51$) for 0<PS<1.9, and 46% (95% CI, 11–76%) ($n=9$) for 1.9<PS of cGVHD-specific survival at 4 years (**b**). With Lee's grading system, overall survival at 4 years was 73% (95% CI, 59–84%) ($n=51$) for low risk, 69% (95% CI, 53–81%) ($n=42$) for intermediate risk, and 29% (95% CI, 4–61%) ($n=7$) for high risk (**c**).

Figure 3c show that the majority of patients were classified into the low or intermediate risk group, and only seven patients as high risk, with no statistically significant difference between the low- and intermediate-risk groups (log rank test; $P=0.33$). The results were quite similar when Akpek *et al.*'s and Lee *et al.*'s grading systems were applied to the 80 subjects with clinical extensive cGVHD. More than 90% of our patients were classified in the lower

risk groups with no significant difference between the two groups (log rank test; $P=0.47$ and 0.59, respectively).

Discussion

The aim of this study was to identify the incidence and risk factors of cGVHD, as well as the survival and prognostic factors for patients who developed cGVHD among Japanese BMT patients, who show a lower incidence and severity of acute GVHD than patients in Western countries.[17,19,23] Our analyses concerning cGVHD showed that the incidence and risk factors of our study group were comparable to those of Western patients. The most important risk factor among various factors found to be associated with the development of cGVHD is the existence of prior acute GVHD.[2,7] A recent report comparing GVHD and survival after HLA-identical sibling BMT in ethnic populations showed no differences in the risk for cGVHD.[23] The results of our analysis were consistent with those reported in the literature.

We also used recently reported prognostic scoring systems, which were developed on the basis of clinical findings for Western patients, on our Japanese patients, and unexpectedly found that these scoring systems did not produce an effective categorization. None of the three prognostic factors at the diagnosis of cGVHD in Akpek *et al.*'s scoring system were found to be prognostic in our analyses. When we compared the percentage of patients with Akpek *et al.*'s prognostic factors recorded in published data from Western countries with the corresponding percentages for our patients, we found some major differences. The proportion of progressive type onset of cGVHD is reportedly 20–70% in Western countries,[3,11,15] but was only 4% for our patients. The extent of cutaneous cGVHD was also different, with the incidence of those presenting with cutaneous lesions covering more than 50% of the total skin area was 9.5% for our patients, but 25–72% for those reported by Akpek *et al.*[15] These differences are likely to result in different results for prognostication and point to the need for the establishment of a different model for Japanese patients.

Our analyses identified the ongoing use of corticosteroids at the onset of cGVHD as a significant prognostic factor, which has not been included in previous studies.[10,11,14] Whether the use of a certain agent can be a prognostic factor is associated with how it is used. Approximately 20% of our patients were using corticosteroids at the time of diagnosis of cGVHD, and their steroid use was associated with prior acute GVHD. Wagner *et al.*[4] reported that 59% of patients were still under corticosteroid treatment on day 100, which was higher than for our series. They further showed that steroid use on day 100 was an adverse prognostic factor for patients after HSCT. Przepiorka *et al.* found that 40% of patients were on steroid treatment on day 100 and showed poorer prognosis,[5] but neither of these studies examined steroid use at the time of cGVHD onset. Because the occurrence of cGVHD despite the use of corticosteroids indicates resistance to immunosuppressive therapies and an immunodeficient status, it is reasonable to assume that steroid use at the diagnosis of cGVHD acts as an adverse prognostic factor. We believe therefore that the use of corticosteroids should be more widely examined for its prognostic significance.

The probability of cGVHD-specific survival of our patients was fairly high, 79% at 4 years, while the probability was $<60\%$ at 4 years in the study by Akpek *et al.*[10] A recent Korean study reported a cGVHD-specific survival of 74.2% at 5 years, which is similar to our result.[22] The presence of cGVHD did not show prognostic significance for our patients, which might be the result of the neutralization of the positive and negative effects of cGVHD on survival. The result of our analyses limited to those with clinical extensive cGVHD indicate that it is not due to the higher proportion of patients with limited cGVHD in Japanese patients which contribute to the different outcomes from the Westerns.

In conclusion, we identified three prognostic factors for patients who developed cGVHD. Since the manifestations of cGVHD in Japan are different from those in Western countries, the morbidity and mortality of cGVHD were found to be lower for Japanese patients. This indicated the need for a different prognostic model to identify high-risk patients.

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bjh research paper

A UGT2B17-positive donor is a risk factor for higher transplant-related mortality and lower survival after bone marrow transplantation

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Summary

We recently identified a human minor histocompatibility (H) antigen, encoded by UDP glycosyltransferase 2 family, polypeptide B17 (UGT2B17), whose immunogenicity results from differential expression in donor and recipient cells as a consequence of a homozygous deletion of the *UGT2B17* gene. UGT2B17 is highly expressed in the liver and colon, which are major targets for graft-*versus*-host disease (GVHD). To assess the significance of homozygous *UGT2B17* gene deletion in allogeneic haematopoietic stem cell transplantation (HSCT), we analysed DNA from 435 stem cell transplant recipients with a haematological malignancy and their human leucocyte antigen-identical unrelated bone marrow donors using sequence-specific primer polymerase chain reaction. Homozygous deletion of the *UGT2B17* gene was observed in 85% of normal donors and in 82% of patients. The analysis showed no significant association between UGT2B17 mismatch in the GVHD direction and the incidence of acute GVHD, chronic GVHD, relapse, or survival. However, the use of a UGT2B17-positive donor was an independent risk factor for higher transplant-related mortality and lower survival after transplantation. UGT2B17 is a metabolic enzyme for hormones, drugs, and potentially toxic exogenous compounds and is expressed in subsets of haematopoietic cells. Thus, the enzyme function of UGT2B17 in donor cells may affect the outcome of allogeneic HSCT.

Keywords: haematopoietic stem cell transplantation, UDP glycosyltransferase, minor histocompatibility antigen, transplant-related mortality, graft-*versus*-host disease.

Allogeneic haematopoietic stem cell transplantation (HSCT) from a human leucocyte antigen (HLA)-identical donor is curative for various haematological malignancies but also carries a risk of transplant-related mortality (TRM) caused by graft-*versus*-host disease (GVHD), toxicity from chemo-radiotherapy, and infections (Storb *et al*, 1986). Development of GVHD results from donor T-cell responses to recipient minor histocompatibility (H) antigens, which consist of HLA-bound peptides derived from cellular proteins encoded by polymorphic genes that differ between transplant donor and recipient (Goulmy, 1997). The identification and characterization of genes that encode minor H antigens may lead to strategies to improve the outcome of allogeneic HSCT. However, only a small number of human minor H antigens have been molecularly characterized, and their influence on HSCT outcome remains speculative (Roopenian *et al*, 2002).

A minor H antigen encoded by the *UDP glycosyltransferase 2 family, polypeptide B17* (*UGT2B17*) gene was recently identified (Murata *et al*, 2003). This minor H antigen is presented by HLA-A29 and was recognized by a T-cell clone isolated from a patient with GVHD involving the gastrointestinal tract, liver and skin. UGT2B17 is highly expressed in the liver and colon, and antigen-presenting cells, prepared from the pretransplant patient blood, stimulated the T-cell clone to release interferon-γ consistent with a role for recipient antigen-presenting cells in the induction of acute GVHD (Shlomchik *et al*, 1999). UGT2B17 is immunogenic because of differential expression in donor and recipient cells as a consequence of a homozygous deletion of the *UGT2B17* gene in the donor involving a large

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portion of the gene, including the ATG initiation codon and the last exon (Murata *et al*, 2003).

UGT2B17 is a member of UGT2B multigene family, which serve a major role in the conjugation and subsequent elimination of potentially toxic exogenous compounds, as well as endogenous compounds including steroid hormones and bilirubin (Tukey & Strassburg, 2000). There is a high degree of homology in the amino acid sequences of UGT2B17 and other UGT2B family members, and overlapping substrate specificity presumably explains the lack of an observed clinical phenotype in individuals with UGT2B17 deletion. However, there is some disparity in the amino acid sequence of UGT2B17 and other family members and it is possible that UGT2B17 provides minor H antigens in addition to the epitope presented by HLA-A29. It is also possible that differences in UGT2B17 enzymatic function could affect the metabolism of drugs used for HSCT. A recent study showed that UGT2B7, another UGT2B family member, glucuronidates ciclosporin A (CsA) and tacrolimus, and additional UGT members have been suggested to participate in the metabolism of these drugs (Strassburg *et al*, 2001). Thus, the *UGT2B17* genotype of the donor or recipient could influence the outcome of allogeneic HSCT, including GVHD and TRM, by serving as a source of minor H antigens or by modifying metabolism of drugs used in transplantation.

In this study, we determined the frequency of the homozygous deletion of *UGT2B17* gene in patients with haematological diseases and their unrelated bone marrow donors, and analysed the association between homozygous *UGT2B17* deletion either in the donor or recipient with outcome after allogeneic HSCT. Homozygous *UGT2B17* deletion was observed in 85% of normal Japanese donors, which is dramatically higher than that (11%) observed in normal White donors (Murata *et al*, 2003). There was no significant association between transplant of UGT2B17-positive recipient from UGT2B17-deleted donor and incidence of GVHD. However, the use of an UGT2B17-positive donor was an independent risk factor for higher TRM and lower survival after transplantation. These results suggest the enzymatic function of UGT2B17 in donor haematopoietic cells may affect the outcome of allogeneic HSCT.

Patients and methods

Patients

The study population was selected from the patients who received a bone marrow transplantation from an unrelated donor through the Japan Marrow Donor Program (JMDP) between January 1993 and March 2000. The selection criteria for the patients and donors in the study population were (i) recipient/donor pairs matched for all genotypes of HLA-A, B, C and DRB1, (ii) an unmanipulated marrow graft, (iii) the use of CsA or tacrolimus as GVHD prophylaxis, (iv) DNA samples were stored and available for genotyping, and (v) clinical outcome data were available. The genotypes of each allele at the HLA-A, B, C and DRB1 loci were determined by high-resolution DNA typing as described previously (Sasazuki *et al*, 1998; Morishima *et al*, 2002).

The characteristics of the 435 patients are summarized in Table I. Standard-risk disease was defined as acute myeloid leukaemia or acute lymphoblastic leukaemia in first remission, chronic myeloid leukaemia (CML) in first chronic phase, or myelodysplastic syndrome classified as refractory anaemia. There was no patient with refractory anaemia with ringed sideroblasts in the study population. All other haematological malignancies including Hodgkin's disease and non-Hodgkin's lymphoma were considered advanced disease. Severe aplastic anaemia was also included in this study population.

All patients received an intensive myeloablative pretransplant conditioning regimen, which varied according to disease or stage of disease at transplantation. A total of 349 patients were conditioned with total body irradiation (TBI) and chemotherapeutic agent(s), while 86 patients received non-TBI-containing regimen. GVHD prophylaxis consisted of either CsA and short-term methotrexate (sMTX) ± anti-thymocyte globulin, or tacrolimus + sMTX.

The assessment and grading of acute and chronic GVHD were performed as previously described (Sullivan *et al*, 1981; Przepiorka *et al*, 1995). A final clinical survey of these patients was carried out on 1 July 2001, and the median follow-up period was 45 months (range, 0–111). Stored DNA was available from all 435 transplant recipients and from 377

Table I. Patient characteristics.

Number (male/female)	435 (250/185)
Median age [years (range)]	24 (0–51)
Disease	
AML	118
ALL	113
CML	112
MDS	31
HD	13
NHL	21
SAA	27
Status of malignant disease	
Standard	188
Advanced	213
Unknown	7
Preconditioning regimen	
TBI containing	349
Non-TBI containing	86
GVHD prophylaxis	
CsA + sMTX (+anti-thymocyte globulin)	351 (48)
Tacrolimus + sMTX	36

AML, acute myeloid leukaemia; ALL, acute lymphoblastic leukaemia; CML, chronic myeloid leukaemia; MDS, myelodysplastic syndrome; HD, Hodgkin's disease; NHL, non-Hodgkin's lymphoma; SAA, severe aplastic anaemia; TBI, total body irradiation; GVHD, graft-*versus*-host disease; sMTX, short-term methotrexate; ciclosporin A (CsA).

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donors for analysis of *UGT2B17* genotype. Informed consent was obtained from all patients and donors, and approval was obtained from the ethics committee at Nagoya University.

Determination of homozygous deletion of the UGT2B17 gene

The homozygous deletion of the *UGT2B17* gene was determined by sequence-specific primer-PCR on genomic DNA from donor and recipient cells. The sense and antisense primers used for polymerase chain reaction (PCR) were 5′-TGTTGGGAATATTCTGACTATAA-3′ and 5′-CCCACTTC TTCAGATCATATGC-3′ respectively. The sense and antisense primers for PCR to detect the β-*globin* gene as an internal control in each assay were 5′-ACACAACTGTGTTCACTAGC-3′ and 5′-CAACTTCATCCACGTTCACC-3′ respectively. Thirty cycles of amplification were performed using a thermalcycler (Model 9600; Perkin-Elmer, Boston, MA, USA) on 0·5 μl genomic DNA extracted from peripheral blood before transplantation or Epstein–Barr virus-transformed lymphoblastoid cells, which were established from pretransplant cryopreserved peripheral blood mononuclear cells. Each reaction contained 0·4 μl of Advantage 2 Polymerase Mix (Clontech Laboratories, Inc., Palo Alto, CA, USA), 0·2 mmol/l of each of the four deoxyribonucleotides, 8 pmol of each primer for UGT2B17, 8 pmol of each primer for β-globin, and PCR buffer in a volume of 20 μl. Each cycle consisted of denaturation (95°C; 30 s), annealing (63°C; 15 s) and elongation (72°C; 25 s). A 10 μl of the PCR product was analysed by electrophoresis on a 1·5% agarose gel.

Statistical analysis

A chi-squared test with 2 × 2 contingency tables was used to evaluate differences of the frequencies of homozygous *UGT2B17* gene deletion between patients and donors. The Cox proportional hazard model was applied to multivariate analysis for acute and chronic GVHD, relapse, TRM, survival and disease-free survival (DFS) (Cox, 1972). TRM was defined as any death that occurred while the patient was in remission. The following variables were evaluated: patient age (continuous); patient sex; donor sex; sex mismatch (female donor to male patient *versus* other pairs); disease status of haematological malignancy at the time of transplantation (advanced disease *versus* standard disease); GVHD prophylaxis (CsA *versus* tacrolimus); pretransplant conditioning regimen (TBI-containing *vs* non-TBI containing); incidence of acute GVHD (grade II–IV *versus* grade 0–I and grade III–IV *versus* grade 0–II); patient UGT2B17 genotype (deleted *versus* positive); donor UGT2B17 genotype (deleted *versus* positive); UGT2B17 mismatch in GVHD direction (UGT2B17-deleted donor to UGT2B17-positive patient *versus* other pairs). $P < 0·05$ were regarded as statistically significant, and those between 0·05 and 0·1 as suggestive of a trend. The TRM, survival and DFS were estimated by using the Kaplan–Meier method, and log rank test was used to analyse differences (Kaplan & Meier, 1958).

Results

UGT2B17 encodes peptides predicted to bind to class I HLA molecules

The amino acid sequence of UGT2B17 was scanned using the SYFPEITHI algorithm (http://www.syfpeithi.de) to identify peptides that are predicted to bind to common class I HLA molecules. UGT2B17 peptides that are identical to corresponding sequences encoded by other UGT2B family members are not likely to be immunogenic since donor T cells should be tolerant to these epitopes, even if they were displayed on recipient cells. However, we found many UGT2B17 peptides that were predicted to bind to class I HLA molecules including HLA-A*01, A*0201, A*03, A*2402, A*26, B*0702, B*08, B*1510, B*4402, and B*5101 and were distinct from all other UGT2B family members (data not shown). Thus, transplantation of UGT2B17 positive recipients who expressed one or more of these HLA alleles with bone marrow from UGT2B17 negative donors might be associated with GVHD if these peptides were presented as minor H antigens.

Frequencies of homozygous deletion of the UGT2B17 gene

To evaluate a potential contribution of UGT2B17 genotype to transplant outcome, we analysed 435 recipients who received an HLA-matched bone marrow transplant from an unrelated donor. A homozygous deletion of the *UGT2B17* gene was found in 358 (82·3%) of 435 patients and in 320 (84·9%) of 377 healthy unrelated donors (not significant, $P = 0·32$). There were no statistical significant differences in the frequencies of homozygous *UGT2B17* deletion between each disease group and donor group. However, of the 112 patients with CML, 88 (78·6%) lacked the *UGT2B17* gene, which was relatively lower than that (84·9%) in unrelated healthy donors ($P = 0·12$).

Graft-versus-host disease

Acute GVHD developed in 324 (75%) of 432 evaluable patients. The severity of GVHD was grade I in 170 (39%), grade II in 105 (24%), grade III in 31 (7%), and grade IV in 18 (4%). In a univariate analysis, a significant association with a higher incidence of grades II–IV acute GVHD was observed with the use of CsA as GVHD prophylaxis ($P = 0·03$) (Table II). A trend for a higher incidence of grades II–IV acute GVHD was found in the patients with advanced disease ($P = 0·099$). No significant association was detected between the incidence of grades II–IV acute GVHD and UGT2B17 deletion in the patient or UGT2B17 deletion in the donor. Recipients who were mismatched for the UGT2B17 genotype with their donors, in which UGT2B17 could serve as a target of

Table II. Univariate and multivariate analysis of risk factors for outcome.

Outcome and significant factor	Univariate analysis (*P*-value)	Relative risk (95% CI)	Multivariate analysis (*P*-value)
aGVHD (II–IV)			
Advanced disease	0·099		NS
CsA as GVHD prophylaxis	0·03	2·76 (1·13–6·76)	0·026
cGVHD (Lt + Ex)			
Male patient	0·08		NS
aGVHD (II–IV)	0·001	1·78 (1·26–2·52)	0·001
aGVHD (III–IV)	0·03		NS
UGT2B17 deletion in donor*	0·08		NS
Relapse			
Advanced disease	<0·001	4·18 (2·42–7·19)	<0·0001
TRM			
Higher patient age	<0·001	1·04 (1·03–1·06)	<0·0001
Advanced disease	0·01	1·85 (1·22–2·79)	0·004
aGVHD (II–IV)	0·008		NS
aGVHD (III–IV)	<0·001	3·16 (1·97–5·05)	<0·0001
UGT2B17 deletion in donor*	0·007	0·53 (0·33–0·84)	0·007
Survival			
Higher patient age	0·0002	1·03 (1·02–1·05)	<0·0001
Advanced disease	<0·001	2·42 (1·71–3·41)	<0·0001
aGVHD (II–IV)	0·05		NS
aGVHD (III–IV)	<0·001	2·44 (1·62–3·68)	<0·0001
UGT2B17 deletion in donor*	0·008	0·57 (0·39–0·83)	0·004
DFS			
Higher patient age	0·001	1·03 (1·02–1·04)	<0·0001
Advanced disease	<0·0001	2·48 (1·78–3·48)	<0·0001
aGVHD (II–IV)	0·07		NS
aGVHD (III–IV)	<0·0001	2·04 (1·36–3·04)	<0·0005
UGT2B17 deletion in donor*	0·02	0·65 (0·44–0·95)	0·024

*Favourable factor.

CI, confidence interval; aGVHD, acute GVHD; cGVHD, chronic GVHD; NS, not significant.

GVHD (combination of UGT2B17-positive patient and UGT2B17-deleted donor) did not exhibit a higher incidence of GVHD. In a multivariate analysis, the use of CsA as GVHD prophylaxis was significantly associated with higher incidence of grades II–IV acute GVHD. No factor was significantly associated with higher incidence of grades III–IV acute GVHD.

Of 305 evaluable patients, 129 (42%) developed chronic GVHD including 48 (16%) with limited cGVHD, and 81 (27%) with extensive cGVHD. In a univariate analysis, significant associations with a higher incidence of chronic GVHD were observed for grades II–IV acute GVHD ($P = 0·001$) and grades III–IV acute GVHD ($P = 0·03$). A trend for a higher incidence of chronic GVHD was found in the male patients ($P = 0·08$) and UGT2B17 deletion in the donor ($P = 0·08$). However, in a multivariate analysis, only grades II–IV acute GVHD was significantly associated with a higher incidence of chronic GVHD.

Relapse

Of 365 evaluable patients with malignant disease, 73 (21%) relapsed after transplantation. In a univariate analysis, a significant association with higher rate of relapse was observed in the patients with advanced disease ($P < 0·001$) (Table II). No significant association was detected between the relapse rate and UGT2B17 deletion in the patient, UGT2B17 deletion in the donor, or UGT2B17 mismatch between patient and donor in the GVHD direction. In a multivariate analysis, advanced disease was significantly associated with higher rate of relapse.

Transplant-related mortality

In a univariate analysis of 360 patients evaluable for TRM, significant associations with increased TRM were observed for higher patient age ($P < 0·001$), advanced disease ($P = 0·01$), grades II–IV acute GVHD ($P = 0·008$) and grades III–IV acute GVHD ($P < 0·001$) (Table II). In a multivariate analysis, higher patient age, advanced disease and grades III–IV acute GVHD were significantly associated with higher TRM.

Surprisingly, UGT2B17 deletion in the donor was a favourable factor for TRM in the univariate analysis ($P = 0·007$), and remained a significant factor in the multivariate analysis [relative risk, 0·53; 95% confidence interval (CI), 0·33–0·84;

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