**Table 4**
Response to Corticosteroid Therapy in Each Stem Cell Source

| Stem Cell Source | No. of Cases | Patients with Improved Response, n (%) |
|---|---|---|
| MRD-BM | 445 | 328 (73.7) |
| MRD-PB | 481 | 312 (64.9) |
| MUD-BM | 783 | 468 (59.8) |
| UCB | 839 | 614 (73.2) |
| MMRD-BM | 155 | 66 (42.9) |
| MMRD-PB | 161 | 78 (48.4) |
| MMUD-BM | 572 | 324 (56.6) |
| Total | 3436 | 2190 (63.7) |

MRD-BM indicates HLA-matched related donor bone marrow; MRD-PB, HLA-matched related donor peripheral blood stem cells; MUD-BM, HLA-matched unrelated donor bone marrow; UCB, umbilical cord blood; MMRD-BM, HLA-mismatched related donor bone marrow; MMRD-PB, HLA-mismatched related donor peripheral blood stem cells; MMUD-BM, HLA-mismatched unrelated donor bone marrow.

was still significantly lower in patients with a stable or progressive response to corticosteroid therapy than in patients with an improved response (hazard ratio, 1.66; 95% confidence interval, 1.49 to 1.85).

## DISCUSSION

The present nationwide study revealed that the response rate of grade II to IV acute GVHD to systemic corticosteroid therapy in Japanese patients was approximately 64%, which is comparable to that in Caucasian patients. In a retrospective analysis of 456 patients who were treated with methylprednisolone 2 mg/kg/day for grade II to IV acute GVHD after allogeneic BM transplantation at the Fred Hutchinson Cancer Research Center, 59% of the patients experienced a complete, partial, or mixed response [10]. In another retrospective analysis of 864 patients who were treated with prednisone 60 mg/m$^2$/day for grade II to IV acute GVHD after BM, PBSC, or UCB transplantation at the University of Minnesota, 65% of the patients experienced a complete, very good partial, or partial response [16].

The factors associated with poor response to corticosteroid therapy were MUD-BM, HLA-mismatched stem cell sources other than UCB (MMRD-BM, MMRD-PB, and MMUD-BM), more severe acute GVHD, and multiple organ involvement including gut of acute GVHD (Table 3). The previous studies also found these features as risk factors for an increased treatment failure rate [9,10], suggesting that these subgroups may be targets for alternate first-line immunosuppressive therapies.

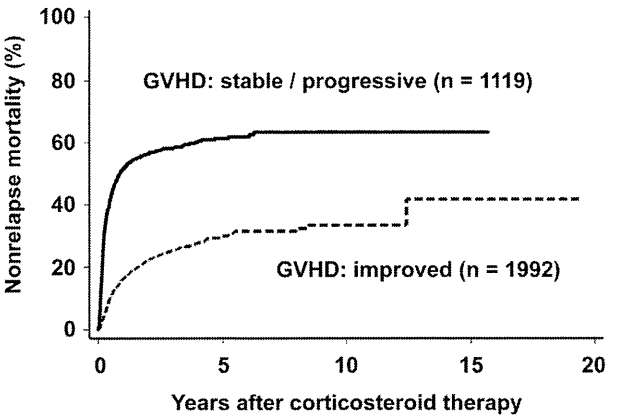


**Figure 1.** Nonrelapse mortality (NRM) after systemic corticosteroid therapy for patients with grade II to IV acute GVHD. Cumulative incidence rates of NRM after systemic corticosteroid therapy in patients (n = 1992) with an improved response to corticosteroid therapy (dashed line, 22.2% [95% confidence interval, 20.1% to 24.4%] at 2 years, 30.1% [27.1% to 33.0%] at 5 years, 33.5% [29.4% to 37.6%] at 10 years, and 41.8% [26.2% to 56.7%] at 15 years) and patients (n = 1119) with a stable or progressive response to corticosteroid therapy (solid line, 56.3% [53.1% to 59.5%] at 2 years, 61.4% [57.7% to 64.9%] at 5 years, 63.4% [59.2% to 67.3%] at 10 years, and 63.4% [59.2% to 67.3%] at 15 years) are shown ($P < .0001$).

On the other hand, UCB was identified as a factor associated with a higher response to first-line corticosteroid therapy in the present study (Table 3). Although several studies have demonstrated a significantly lower incidence of acute GVHD in UCB transplantation than in unrelated BM transplantation [23-29], no study has compared the response to treatment of acute GVHD between them. The present study demonstrated, for the first time, a higher response of grade II to IV acute GVHD to systemic corticosteroid therapy in patients after UCB transplantation than in those after BM or PBSC transplantation.

Nevertheless, UCB transplantation had no impact on NRM after corticosteroid therapy in the multivariate analysis and, in fact, had higher NRM than MRD-BM transplantation in the univariate analysis (Table 5). Thus, even though there was a higher response of acute GVHD to systemic corticosteroid therapy in patients after UCB transplantation, careful management is required for patients who suffer from grade II to IV acute GVHD after UCB transplantation, as well as those after transplantation with other stem cell sources.

Unexpectedly, adult patient (ages 18 to 49 years) was predictive of a good response to systemic corticosteroid therapy compared with child patient (age <18 years). Additional analysis was performed, and it was found that patients with grade II acute GVHD accounted for 61.4% of adult patient group, whereas 56.1% of child patient group (Fisher exact test, $P = .019$). This difference might affect the above result because severity of acute GVHD was the most significant factor associated with response to corticosteroid therapy (Table 3). Nonetheless, adult patients were likely to have higher NRM than child patients (Table 5). Our data indicate that although adult patients may be more responsive to corticosteroid therapy for acute GVHD, they have a higher risk of transplant-related toxicity than children with acute GVHD.

Despite the fact that multivariate analysis showed a significantly higher response rate to corticosteroid therapy in UCB transplantation than MRD-BM transplantation, the actual percentage was similar between UCB (73.2%) and MRD-BM (73.7%) transplantations (Table 4). Additional analysis found that patients in the age group 18 to 49 years (predictive factor of good response) accounted for only 32.2% of UCB transplantation, but constituted 58.4% of the MRD-BM population (Fisher exact test, $P < .001$) and that patients with grade II acute GVHD (predictive factor of good response) accounted for only 58.6% of UCB transplantation, but constituted 70.1% of the MRD-BM population (Fisher exact test, $P < .001$). These data suggested that the UCB population included fewer patients having predictive factors of good response to corticosteroid therapy compared with the MRD-BM population. This could explain why the actual percentage of patients with an improved response in UCB transplantation was almost the same as the percentage of patients with an improved response in MRD-BM transplantation.

Interestingly, multiorgan involvement that includes the gut was less likely to respond to first-line therapy with corticosteroids (Table 3); however, patients with liver involvement are more likely to have higher NRM (Table 5). Further study is required to elucidate the mechanisms of the difference in the effect of gut and liver GVHD on

**Table 5**
Factors Associated with Nonrelapse Mortality after Corticosteroid Therapy

| Factor (n) | Univariate Analysis Hazard Ratio* (95% CI) | *P* Value | Multivariate Analysis Hazard Ratio* (95% CI) | *P* Value |
|---|---|---|---|---|
| Patient age | | | | |
| <18 yr (554) | 1 | | 1 | |
| 18 to 49 yr (1503) | 1.50 (1.21 to 1.85) | <.001 | 1.72 (1.38 to 2.14) | <.001 |
| ≥50 yr (1054) | 2.74 (2.22 to 3.38) | <.001 | 3.34 (2.67 to 4.17) | <.001 |
| Stem cell source | | | | |
| MRD-BM (402) | 1 | | 1 | |
| MRD-PB (447) | 1.43 (1.11 to 1.83) | .005 | .88 (.68 to 1.15) | .344 |
| MUD-BM (726) | 1.40 (1.11 to 1.77) | .004 | 1.02 (.80 to 1.30) | .866 |
| UCB (720) | 1.35 (1.06 to 1.71) | .014 | 1.15 (.90 to 1.48) | .265 |
| MMRD-BM (141) | 1.63 (1.16 to 2.28) | .005 | 1.15 (.82 to 1.62) | .415 |
| MMRD-PB (153) | 1.74 (1.26 to 2.39) | .001 | .97 (.69 to 1.37) | .882 |
| MMUD-BM (522) | 1.79 (1.41 to 2.27) | <.001 | 1.25 (.97 to 1.60) | .082 |
| GVHD prophylaxis | | | | |
| Cyclosporine A-based (1528) | 1 | | | |
| Tacrolimus-based (1520) | 1.06 (.94 to 1.21) | .332 | | |
| Other (50) | 1.28 (.81 to 2.04) | .296 | | |
| In vivo T cell depletion | | | | |
| No (3004) | 1 | | | |
| Yes (91) | .98 (.66 to 1.44) | .919 | | |
| Onset of acute GVHD | | | | |
| Day ≤28 (2212) | 1 | | | |
| Day ≥29 (899) | 1.05 (.92 to 1.20) | .476 | | |
| Grade of acute GVHD | | | | |
| II (1864) | 1 | | 1 | |
| III (917) | 2.21 (1.92 to 2.56) | <.001 | 1.56 (1.31 to 1.86) | <.001 |
| IV (330) | 7.93 (6.67 to 9.43) | <.001 | 3.53 (2.84 to 4.38) | <.001 |
| Organ involvement | | | | |
| Skin only (1010) | 1 | | 1 | |
| Gut only (266) | 1.11 (.84 to 1.47) | .448 | .80 (.59 to 1.08) | .139 |
| Liver only (28) | 4.11 (2.20 to 7.69) | <.001 | 2.22 (1.19 to 4.16) | .013 |
| Skin and gut, no liver (1083) | 1.27 (1.06 to 1.51) | .008 | .97 (.79 to 1.18) | .753 |
| Skin and liver, no gut (160) | 2.42 (1.83 to 3.21) | <.001 | 1.54 (1.13 to 2.08) | .006 |
| Gut and liver, no skin (75) | 3.64 (2.57 to 5.16) | <.001 | 1.88 (1.29 to 2.73) | .001 |
| Skin, gut, and liver (448) | 4.82 (4.03 to 5.77) | <.001 | 2.07 (1.64 to 2.62) | <.001 |
| Response to systemic corticosteroid therapy | | | | |
| Improved (1992) | 1 | | 1 | |
| Stable/progressive (1119) | 3.63 (3.20 to 4.12) | <.001 | 2.45 (2.14 to 2.82) | <.001 |

MRD-BM indicates HLA-matched related donor bone marrow; MRD-PB, HLA-matched related donor peripheral blood stem cells; MUD-BM, HLA-matched unrelated donor bone marrow; UCB, umbilical cord blood; MMRD-BM, HLA-mismatched related donor bone marrow; MMRD-PB, HLA-mismatched related donor peripheral blood stem cells; MMUD-BM, HLA-mismatched unrelated donor bone marrow; GVHD, graft-versus-host disease; CI, confidence interval.
* Values >1.0 indicate higher probability of non relapse mortality; values <1.0 indicate lower probability.

transplantation outcome. Nevertheless, lack of response to initial therapy is an important risk factor in predicting high NRM in patients with grade II to IV acute GVHD (Table 5).

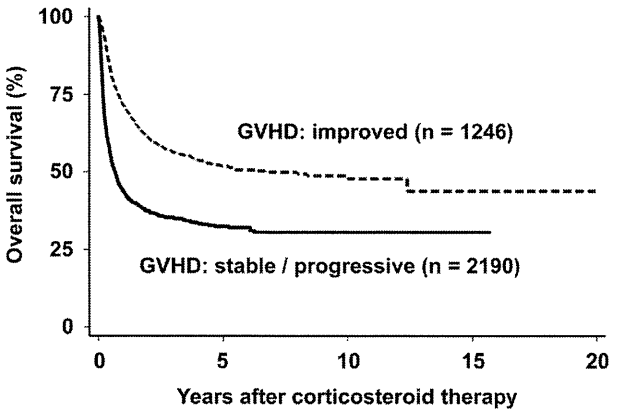


**Figure 2.** Overall survival (OS) for patients with grade II to IV acute GVHD. OS for patients (n = 2190) with an improved response (dashed line; 61.3% [95% confidence interval, 59.0% to 63.5%] at 2 years, 51.9% [49.2% to 54.5%] at 5 years, 47.8% [44.0% to 51.5%] at 10 years, and 43.8% [35.5% to 51.8%] at 15 years) and OS for patients (n = 1246) with a stable or progressive response (solid line; 37.4% [34.6% to 40.3%] at 2 years, 32.5% [29.5% to 35.6%] at 5 years, 30.6% [27.3% to 34.1%] at 10 years, and 30.6% [27.3% to 34.1%] at 15 years) are shown ($P < .0001$).

The patients who did not achieve improvement of acute GVHD by corticosteroid therapy had approximately 2.5-times higher NRM and approximately .6-times lower OS rates. It is well known that the incidence of acute GVHD in Japanese patients is lower than that in Caucasian patients [30,31]. However, the present data clearly demonstrate that, if the systemic corticosteroid therapy is ineffective, even Japanese patients cannot achieve a satisfactory survival rate. Another important message of this study is that the establishment of second-line treatment for corticosteroid-refractory acute GVHD is required for not only Caucasian, but also for Japanese patients.

This study had several limitations. First, the sort and dose of corticosteroids are not collected in the Japan Society for Hematopoietic Cell Transplantation database. In patients with grade II to IV acute GVHD, initial treatment with prednisone-equivalent steroid doses higher than 2.5 mg/kg has not been shown to provide better outcomes [32], although in patients with grade II acute GVHD, lower-dose initial treatment at 1.0 mg/kg has not been shown to provide worse outcomes [33]. The intensity of corticosteroid therapy may differ by each transplantation team or each patient, as shown by a survey in Europe [34], and this information may give us additional findings. Second, criteria for improvement, or for stable or progressive acute GVHD, had been previously defined in the

database, which did not allow for analysis by outcomes such as complete, partial, or mixed response, as has been performed in previous studies [10,16]. Third, the time of the evaluation of GVHD is not defined in the database. Thus, the response was evaluated using a nonfixed time point, although GVHD sometimes shows a waxing and waning course. This also prevented us from analyzing the speed of the response to therapy. A recent study has reported that the day-28 response to corticosteroid therapy can predict the outcomes for patients with acute GVHD [16]. Fourth, this study was a retrospective analysis, which is challenging given the heterogeneous background. Multivariate analysis was used to attempt to reduce statistical bias, but a prospective study is required to validate the present findings.

The results of this large retrospective study showed a higher response of acute GVHD to systemic corticosteroid therapy in patients after UCB transplantation than for patients after BM and PBSC transplantation, and confirmed the factors previously reported. These results should be considered in the design of future clinical trials of acute GVHD treatment.

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# Different effects of HLA disparity on transplant outcomes after single-unit cord blood transplantation between pediatric and adult patients with leukemia

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## ABSTRACT

Recent advances in unrelated cord blood transplantation have increased chances and options available in allogeneic stem cell transplantation. The effect of HLA disparity on outcomes after cord blood transplantation was studied recently in mainly pediatric populations. Results showed that HLA matching in combination with total nucleated cell dose positively affects survival. The effect of HLA disparity after single-unit cord blood transplantation may be different in adults because their total nucleated cell dose is much lower compared to pediatric patients. We investigated the effect of HLA disparity on the outcome of single-unit unrelated cord blood transplantation separately in 498 children aged 15 years or under (HLA-A, HLA-B low-resolution, and HLA-DRB1 high-resolution matched [6/6], n=82, and one locus- [5/6], n=222, two loci- [4/6], n=158, three loci- [3/6] mismatched, n=36) and 1,880 adults (6/6, n=71; 5/6, n=309; 4/6, n=1,025; 3/6, n=475) with leukemia. With adjusted analyses, in children, 4/6 showed significantly increased risks of overall mortality (relative risk [RR]=1.61, $P$=0.042) and transplant-related mortality (RR=3.55, $P$=0.005) compared to 6/6. The risk of grade 2 to 4 acute GVHD was increased in 5/6 (RR=2.13, $P$=0.004) and 4/6 (RR=2.65, $P$<0.001). In adults, the risk of mortality did not increase with the number of mismatched loci (RR=0.99, $P$=0.944 for 5/6; RR=0.88, $P$=0.436 for 4/6). The risk of relapse was significantly decreased in 4/6 (RR=0.67, $P$=0.034). The risk of transplant-related mortality (TRM) or acute GVHD was not increased in 5/6 or 4/6. The effect of HLA disparity on transplant outcome differed between children and adults. In children, an increased number of mismatched HLA loci correlated with an increased risk of mortality. In adults, there was no increase in mortality with an increase in the number of mismatched HLA loci.

## Introduction

Recent advances in unrelated cord blood transplantation (UCBT) have provided increased opportunities for patients with hematologic malignancies to receive hematopoietic stem cell transplantation (HSCT). This has led to an increased number of UCBT procedures over the past decade.[1,2] Clinical comparison studies of cord blood and bone marrow from unrelated donors have shown comparable results, which indicates that cord blood is a reasonable alternative donor / stem cell source.[3-12] These studies support the use of HLA-A, HLA-B, low-resolution and HLA-DRB1 zero- to two-loci-mismatched UCB for patients with leukemia in the absence of an HLA-A, HLA-B, HLA-C, and HLA-DRB1 allele matched unrelated adult donor, and the use of UCB as a first-line option when a transplant is urgently required.

The effect of HLA mismatches after bone marrow transplantation from unrelated donors (UBMT) has been well studied, and HLA-A, HLA-B, HLA-C, and HLA-DRB1 allele matched bone marrow is currently the first alternative for HLA-identical sibling donors.[13-16] An increase in the number of HLA mismatches, antigen-level, or high-resolution, at HLA-A, HLA-B, HLA-C, or HLA-DRB1 loci from 8/8 to 7/8, or 7/8 to 6/8 was associated with higher mortality with an approximately 10% reduction in survival in UBM recipients.[12,13,15] Since HLA mismatches are better tolerated after UCB with a lower incidence of severe graft-*versus*-host disease (GVHD), up to two HLA antigen mismatches of HLA-A, HLA-B, low resolution and HLA-DRB1 high resolution are considered in the current CB selection algorithm. Several reports have recently described the effect of HLA disparity on the transplant outcomes after UCBT.[9,17,18] Eapen *et al.* reported the pos-

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sibility of a better outcome in HLA 6/6 matched UCB in 35 recipients, and Barker *et al.* confirmed these results with a larger number of UCB recipients.[9,18] However, these studies, which assessed the effect of HLA disparity on the outcome of single-unit CBT, were mainly conducted in pediatric populations in which the infused cell dose is much greater than that in adult recipients.

The aim of this study was to assess the effect of HLA disparity on the transplant outcomes after single-unit UCBT in pediatric and adult recipients. The accumulation of single-unit CBT in adult recipients has enabled us to assess separately the effect of HLA disparity on CBT outcomes in children and adults.

## Design and Methods

### Study design and data source

For this retrospective observational study, recipients' clinical data were provided by the Japan Cord Blood Bank Network (JCBBN). All 11 cord blood banks in Japan are affiliated with the JCBBN. JCBBN collected the recipients' clinical information at 100 days post-transplant through the Transplant Registry Unified Management Program (TRUMP) of the Japan Society of Hematopoietic Cell Transplantation (JSHCT).[19] Information on survival, disease status, and long-term complications including chronic graft-*versus*-host disease and second malignancies is renewed annually. Patient consent is not required for TRUMP registration of the JSHCT for the registry data consists of anonymized clinical information. This study was approved by the data management committees of the JSHCT and the JCBBN, and by the institutional review boards of Saitama Medical Center, Jichi Medical University and Nagoya University Graduate School of Medicine, Japan.

### Patients

The subjects were patients with acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), chronic myeloid leukemia (CML), or myelodysplastic syndrome (MDS), who were recipients of their first UCBT between January 2000 and December 2009. Among 2,461 recipients of single-unit UCB with complete HLA-A, HLA-B, low-resolution and HLA-DRB1 high-resolution data, 51 recipients with 4 HLA mismatches were excluded. Thirty recipients who did not receive GVHD prophylaxis and 2 recipients for whom information regarding the conditioning regimen was missing were excluded. A total of 2378 single-unit UCB recipients (498 children aged 15 years or under at transplant, and 1880 adults aged 16 years or over at transplant) were subjects for analysis.

### HLA typing

Histocompatibility data for low-resolution typing for the HLA-A, HLA-B, and HLA-DR loci and high-resolution typing for HLA-DRB1 were obtained from the TRUMP database which includes HLA information provided by cord blood banks or transplant centers. The level of HLA typing in the present study was HLA-A, HLA-B, low-resolution, and HLA-DRB1 high-resolution, as in other studies in Europe and North America. However, according to current practice in Japan, mismatches in HLA-DR loci were counted at the low-resolution level at UCB unit selection. Therefore, results regarding the effect of HLA mismatches in HLA-A, HLA-B, and HLA-DR low-resolution are also provided (*Online Supplementary Table S1*). Analyses from the Japan Marrow Donor Program (JMDP) showed better survival in HLA class II mismatched recipients compared to HLA class I mismatched recipients. Thus, in Japan, a single-DRB1-mismatched UBM donor is preferred over a single-A-mismatched UBM or single-B-mismatched UBM donor.[15,20] This background affected HLA typing strategy of HLA-DR low-resolution typing instead of high-resolution typing for selection of cord blood units in Japan. This observation may explain the fact that the frequency of 4/6 grafts is higher in this cohort than in cohorts in Europe and the USA.

### Definitions

The primary outcome of the analyses was overall survival, defined as time from transplant to death from any cause. Several secondary end points were also analyzed. Neutrophil recovery was defined as an absolute neutrophil count of at least $0.5x10^9$/L cells per cubic millimeter for three consecutive points; platelet recovery was defined as a count of at least $50x10^9$ platelets per cubic millimeter without transfusion support. The recipients of reduced-intensity conditioning were also defined with the criteria above, according to the previous report that confirmed complete donor chimeras of all engrafted patients after CBT with reduced-intensity conditioning.[21] Diagnosis and clinical grading of acute GVHD were performed according to the established criteria.[22,23] Relapse was defined as the recurrence of underlying hematologic malignant diseases. Transplant-related death was defined as death during a continuous remission.

### Statistical analysis

Descriptive statistical analysis was performed to assess patient baseline characteristics, diagnosis, disease status at conditioning, donor-patient ABO mismatches, preparative regimen, and GVHD prophylaxis. Medians and ranges are provided for continuous variables and percentages are shown for categorical variables. Cumulative incidence curves were used in a competing-risks setting to calculate the probability of acute and chronic GVHD, relapse and transplant-related mortality (TRM).[24] Gray's test was used for group comparisons of cumulative incidences.[25] An adjusted comparison of the groups with regard to overall survival (OS) was performed with the use of the Cox's proportional-hazards regression model.[26] For other outcomes with competing risks, Fine and Gray's proportional-hazards model for the subdistribution of a competing risk was used.[27] For neutrophil and platelet recovery, death before neutrophil or platelet recovery was the competing event. For GVHD, death without GVHD and relapse were competing events. For relapse, death without relapse was the competing event, and for transplant-related mortality (TRM), relapse was the competing event.[28] For acute GVHD, subjects were limited to those who engrafted, and for chronic GVHD, subjects were limited to those who engrafted and survived at least 100 days after transplantation.

The variables considered were the patient's age at transplant (5 years or over *vs.* under 5 years for pediatric recipients, and 50 years or over *vs.* under 50 years for adult recipients; cut-off points were around the median in each group), patient's sex, donor-patient sex mismatch (matched *vs.* male to female *vs.* female to male), donor-patient ABO mismatch (major mismatch *vs.* matched or minor mismatch), diagnosis (AML, ALL, CML or MDS), disease status at conditioning (first or second complete remission (CR) of AML, 1CR of ALL, first chronic phase of CML, and refractory anemia or refractory anemia with ringed sideroblasts as standard-risk diseases *vs.* advanced for all others), the conditioning regimen (reduced-intensity conditioning *vs.* myeloablative conditioning), and the type of prophylaxis against GVHD (tacrolimus-based *vs.* cyclosporine-based). Conditioning regimens were classified as myeloablative if total-body irradiation >8 Gy, oral busulfan ≥9 mg/kg, intravenous busulfan ≥7.2 mg/kg, or melphalan >140 $mg/m^2$ was used based on the report from the Center for International Blood and Marrow Transplant Research.[29,30] We cat-

egorized patients for whom there was insufficient information regarding the doses of agents or radiation used for the conditioning regimen according to information on the conditioning intensity (i.e. whether or not the conditioning regimen was intended to be myeloablative) as reported by the treating clinicians. The cryopreserved total nucleated cell dose was categorized as >10.0x10$^7$/kg, 5.0-9.9 x 10$^7$/kg, 2.5-4.9x10$^7$/kg, or <2.5 x 10$^7$/kg for children, and >3.0x10$^7$/kg, 2.5-2.9x10$^7$/kg, 2.0-2.4x10$^7$/kg, or <2.0x10$^7$/kg for adults. HLA disparity and nucleated cell dose were maintained in the model. Since patient age was highly correlated with the total nucleated cell dose in children, age was excluded from multivariate analyses for pediatric recipients. Other variables were selected in a backward stepwise manner with a variable retention criterion of $P$<0.05. Interaction between HLA disparity and adult (patient age at transplant 16 years or over) or child (patient age at transplant 15 years or under) was tested for overall survival by using a Cox's proportional-hazards regression model adjusted by other significant covariates in the final model for adult and pediatric recipients except for patient age. All $P$ values were two-sided.

## Results

### Patients' characteristics

Table 1 shows patients' characteristics, their disease, and transplant regimens. Median age at transplant was five years (range 0-15) in 498 pediatric and 49 years (range 16-82) in 1880 adult recipients of single-unit CBT. The proportion of females was 45% in both children and adults. Among children, the proportion of patients with ALL was greatest (58%) followed by that of patients with AML (34%). Among adults, the most frequent disease was AML (59%), followed by ALL (22%) and MDS (13%). The median number of cryopreserved total nucleated cells received in children was 5.30 x 10$^7$/kg, which was significantly greater (approximately double) than the number of nucleated cells received in adult patients (2.52 x 10$^7$/kg). In adults, only 33 patients (2%) received CB with a total nucleated cell dose greater than or equal to 5.0 x 10$^7$/kg. In children, 82 patients (16%) received HLA-matched (6/6) UCB, 222 (45%) received one-locus-mismatched (5/6), 158 (32%) received two-loci-mismatched (4/6), and 36 (7%) received three-loci-mismatched (3/6) UCB. For adults, the numbers and proportions of recipients were 71 (4%) for 6/6, 309 (16%) for 5/6, 1025 (55%) for 4/6, and 475 (25%) for 3/6. Among those who received 3/6 UCB, only 2 pediatric and 11 adult patients received three HLA-A, HLA-B, HLA-DR low-resolution mismatched UCB. Eighty-eight percent (TBI regimen 62%, non-TBI regimen 26%) and 62% (TBI regimen 56%, non-TBI regimen 6%) of children and adults, respectively, received myeloablative conditioning. Fludarabine-based reduced-intensity conditioning was given to 34% of adult recipients. T-cell depletion *in vivo* with antithymocyte globulin or antilymphocyte globulin was performed in only 6 (2%) child recipients and 26 (1%) adult recipients. The median follow-up period for survivors was 2.4 years (range 0.1-9.5) for pediatric recipients and 2.1 (range 0.1-9.0) years for adult recipients.

### Outcome

Overall survival, relapse, and transplant-related mortality: among children, overall mortality in 4/6 UCB recipients was significantly higher than that in 6/6 UCB recipients (RR=1.61, 95% confidence interval [CI], 1.02-2.56, $P$=0.042) (Table 2). Overall mortality increased with the number of mismatched loci in children ($P$ for trend 0.043). The increased mortality in 4/6 UCB recipients was mainly affected by increased transplant-related mortality (TRM) (RR=3.55, 95%CI: 1.47-8.58, $P$=0.005) ($P$ for trend 0.002) but not by the risk of relapse (RR=0.77, 95%CI: 0.48-1.24, $P$=0.392) in children. Among children, there were no differences in the risks of mortality and relapse between 5/6 UCB recipients (RR=1.07, $P$=0.765 for overall mortality; RR=1.06, $P$=0.794 for relapse; and RR=1.29, $P$=0.58 for TRM) and 6/6 UCB recipients (Table 2).

In adults, the number of HLA mismatches was not significantly associated with increased mortality (for overall mortality: RR=0.99, $P$=0.944 for 5/6; RR=0.88, $P$=0.436 for 4/6; RR=0.95, $P$=0.751 for 3/6; for TRM, RR=1.41, $P$=0.205 for 5/6; RR=1.24, $P$=0.408 for 4/6; RR=1.29, $P$=0.339 for 3/6). A two-loci mismatch was associated with a decreased risk of relapse in adult recipients (RR=0.70, $P$=0.075 for 5/6; RR=0.67, $P$=0.034 for 4/6; RR=0.70, $P$=0.07 for 3/6) (Table 2). The risks of mortality were similar when subjects were limited to those with standard risk disease status or to those with advanced risk disease status at transplant, to those who received myeloablative conditioning or to those who received reduced-intensity conditioning (*Online Supplementary Table S2*). A decreased risk of relapse was more prominent in patients with acute myeloid leukemia, and those who received reduced-intensity conditioning (*Online Supplementary Table S2*).)

Figure 1 shows unadjusted overall survival curves in children and adults. In children, the unadjusted probabilities of survival at three years post-transplant were 66% for 6/6, 62% for 5/6, 45% for 4/6, and 62% for 3/6 ($P$=0.032) (Figure 1A). In adults, the survival probabilities in all of the HLA disparity groups were similar (38% for 6/6, 37% for 5/6, 39% for 4/6, and 40% for 3/6 at three years post-transplant, $P$=0.567) (Figure 1B). A similar trend was seen when subjects were limited to standard-risk disease status at transplant (81% for 6/6, 76% for 5/6, 57% for 4/6, and 81% for 3/6 at three years post-transplant, $P$=0.035, for children; 51% for 6/6, 57% for 5/6, 58% for 4/6, and 55% for 3/6 at three years post-transplant, $P$=0.375, for adults) (*Online Supplementary Figure S1*).

A test of the interaction between HLA disparity and age (adult *vs.* child) revealed that the effect of HLA disparity on overall survival differed significantly between the pediatric and adult patient groups ($P$=0.009 for HLA disparity of 0-1 mismatches *vs.* 2-3 mismatches).

### Hematologic recovery

The cryopreserved total nucleated cell dose significantly affected neutrophil and platelet recovery in children and neutrophil recovery in adults (Table 3). HLA disparity did not significantly affect neutrophil or platelet recovery in adults or children for neutrophil recovery: RR=1.03, $P$=0.823 for 5/6; RR=0.96, $P$=0.799 for 4/6; RR=0.67, $P$=0.068 for 3/6 in children; RR=0.89, $P$=0.436 for 5/6; RR=0.92, $P$=0.576 for 4/6; RR=0.84, $P$=0.243 for 3/6 in adults; for platelet recovery: RR=0.89, $P$=0.438 for 5/6; RR=0.75, $P$=0.09 for 4/6; RR=0.71, $P$=0.164 for 3/6 in children; RR=1.05, $P$=0.775 for 5/6; RR=1.05, $P$=0.791 for 4/6; RR=0.99, $P$=0.951 in 3/6 in adults (Table 3).

Table 1. Patients', disease, and transplant characteristics of pediatric and adult recipients of single-unit cord blood.

| Characteristics | Children (age<16) N. | (%) | Adult (age>16) N. | (%) |
|---|---|---|---|---|
| N. of transplants | 498 | | 1880 | |
| Patient age at transplant | | | | |
| Median (range) | 5 (0-15) | | 49 (16-82) | |
| 0-9 years | 378 | (76) | | |
| 10-19 years | 120 | (24) | 88 | (5) |
| 20-29 years | | | 236 | (13) |
| 30-39 years | | | 317 | (17) |
| 40-49 years | | | 351 | (19) |
| 50-59 years | | | 492 | (26) |
| ≥60 years or older | | | 396 | (21) |
| Patient sex | | | | |
| Male | 275 | (55) | 1039 | (55) |
| Female | 223 | (45) | 841 | (45) |
| Sex matching | | | | |
| Matched | 207 | (42) | 696 | (37) |
| Male to female | 114 | (23) | 391 | (21) |
| Female to male | 125 | (25) | 485 | (26) |
| Unknown | 52 | (10) | 308 | (16) |
| Diagnosis | | | | |
| AML | 170 | (34) | 1115 | (59) |
| ALL | 290 | (58) | 418 | (22) |
| CML | 7 | (1) | 106 | (6) |
| MDS | 31 | (6) | 241 | (13) |
| Disease status | | | | |
| Standard | 247 | (50) | 673 | (36) |
| Advanced | 236 | (47) | 1127 | (60) |
| Unknown | 15 | (3) | 80 | (4) |
| ABO matching | | | | |
| Matched | 182 | (37) | 602 | (32) |
| Minor mismatch | 127 | (26) | 522 | (28) |
| Major mismatch | 113 | (23) | 451 | (24) |
| Bidirectional | 75 | (15) | 301 | (16) |
| Unknown | 1 | (<1) | 4 | (<1) |
| HLA mismatched number | | | | |
| Matched (6/6) | 82 | (16) | 71 | (4) |
| One locus mismatched (5/6) | 222 | (45) | 309 | (16) |
| Two loci mismatched (4/6) | 158 | (32) | 1025 | (55) |
| Three loci mismatched (3/6) | 36 | (7) | 475 | (25) |
| N. of cryopreserved nucleated cells (x10$^7$/kg) | | | | |
| Median | 5.30 | | 2.52 | |
| Range | 0.81-38.7 | | 0.71-9.98 | |
| N. of cryopreserved CD34-positive cells (x10$^5$/kg) | | | | |
| Median | 1.68 | | 0.83 | |
| Range | 0.072-65.66 | | 0.07-14.02 | |
| Preparative regimen* | | | | |
| MAST | | | | |
| CY+TBI | 216 | (43) | 891 | (47) |
| Other TBI regimen | 93 | (19) | 162 | (9) |
| BU+CY | 86 | (17) | 65 | (3) |
| Other non-TBI regimen | 41 | (8) | 47 | (3) |
| RIST | | | | |
| FL+BU+other | 6 | (1) | 172 | (9) |
| FL+CY+other | 12 | (2) | 119 | (6) |
| FL+Mel+other | 21 | (4) | 357 | (19) |
| Other RIST | 23 | (5) | 67 | (4) |
| T-cell depletion *in vivo*** | | | | |
| ATG or ALG use | 9 | (2) | 26 | (1) |
| GVHD prophylaxis*** | | | | |
| Cyclosporine A + sMTX | 157 | (32) | 748 | (40) |
| Cyclosporine A + MMF/steroid | 37 | (7) | 99 | (5) |
| Cyclosporine A alone | 31 | (6) | 142 | (8) |
| Tacrolimus + sMTX | 216 | (43) | 434 | (23) |
| Tacrolimus + MMF/steroid | 24 | (5) | 132 | (7) |
| Tacrolimus alone | 20 | (4) | 304 | (16) |
| Others | 13 | (3) | 21 | (1) |

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*CY: cyclophosphamide; CA: citarabine; BU: busulfan; TBI: total body irradiation; FL: fludarabine; Mel: melphalan, **ATG: antithymocyte globulin; ALG: antilymphocyte globulin; ***sMTX: short-term methotrexate; MMF: mycophenolate mofetil.

### Acute and chronic graft-versus-host disease

The risk of grade 2 to 4 acute GVHD was significantly higher in HLA-mismatched UCB pediatric recipients (RR=2.13, *P*=0.004 for 5/6; RR=2.65, *P*<0.001 for 4/6; RR=2.39, *P*=0.0015 for 3/6; P for trend 0.001) (Table 4). The risk of chronic GVHD and extensive-type chronic GVHD was also significantly higher in 4/6 UCB recipients (RR=2.99, *P*=0.005 for chronic GVHD, and RR=7.62, *P*=0.047 for extensive-type chronic GVHD), and the risks increased according to the number of mismatches (P for trend, 0.002 for chronic GVHD, 0.005 for extensive-type chronic GVHD). In adults, in contrast to the results in children, there were no differences in the risks of grade 2 to 4 acute GVHD in 5/6 and 4/6 UCB recipients (for grade 2 to 4 acute GVHD, RR=1.03, *P*=0.916 for 5/6, RR=1.27, *P*=0.276 for 4/6). The risk of grade 2 to 4 acute GVHD was higher for 3/6 (RR=1.72, *P*=0.017). In adult recipients, the risk of chronic GVHD was increased in recipients of 4/6 UCB (RR=1.90, *P*=0.04), however, there were no differences in the risk of extensive-type chronic GVHD (RR=1.15, *P*=0.758 for 5/6; RR=1.62, *P*=0.253 for 4/6; RR=1.28, *P*=0.574 for 3/6) (Table 4).

### Effect of total nucleated cell dose on outcome

An increase in the cryopreserved total nucleated cell dose increased the incidence of neutrophil recovery in both children and adults, as well as the incidence of platelet recovery in children (Table 3). The cumulative incidences of neutrophil recovery were 94% for >10 x $10^7$/kg, 88% for 5.0-9.9 x $10^7$/kg, 82% for 2.5-4.9 x $10^7$/kg, and 86% for <2.5 x $10^7$/kg in children (*P*<0.001) (Figure 2A). The cell dose was significantly correlated with the recipient's age at transplant in children (the median ages were one year for >10 x $10^7$/kg, 3 years for 5.0-9.9 x $10^7$/kg, 8 years for 2.5-4.9 x $10^7$/kg, and 12 years for <2.5 x $10^7$/kg). The cumulative incidences of neutrophil recovery were 76% for >2.5 x $10^7$/kg and 74% for <2.5 x $10^7$/kg in adults (*P*=0.007) (Figure 2B). The cumulative incidences of TRM at three years post-transplant were 13% for >10 x $10^7$/kg, 14% for 5.0-9.9 x $10^7$/kg, 14% for 2.5-4.9 x $10^7$/kg, and 14% for <2.5x$10^7$/kg in children (*P*=0.98) and 29% for >2.5x$10^7$/kg and 28% for <2.5 x $10^7$/kg in adults (*P*=0.77) (*Online Supplementary Figure S2*). The probabilities of overall survival at three years post-transplant were 68% for >10x$10^7$/kg, 53% for 5.0-9.9 x $10^7$/kg, 57% for 2.5-4.9 x $10^7$/kg, and 55% for <2.5x$10^7$/kg in children (*P*=0.30) and 36% for >2.5 x 107/kg and 41% for <2.5x$10^7$/kg in adults (*P*=0.13). A lower total nucleated cell dose was neither associated with increased mortality in children or adults in multivariate analyses (Table 2). Thus, there was no combined effect of HLA disparity and total nucleated cell dose on mortality neither in children nor in adults (cumulative

incidence of TRM at three years post-transplant, 8% for 6/6, 11% for 5/6 and >5 x $10^7$/kg, 11% for 5/6 and 2.5-4.9 x $10^7$/kg, 0% for 5/6 and <2.5 x $10^7$/kg, 23% for 4/6 and >5 x $10^7$/kg, 24% for 4/6 and 2.5-4.9 x $10^7$/kg, 25% for 4/6 and <2.5 x $10^7$/kg in children, and 23% for 6/6, 29% for 5/6 and >2.5 x $10^7$/kg, 30% for 5/6 and <2.5 x $10^7$/kg, 27% for 4/6 and >2.5 x $10^7$/kg, 27% for 4/6 and <2.5 x $10^7$/kg in adults (*Online Supplementary Figure S3*).

### Association of outcomes with the type of HLA mismatches for 4/6 adult recipients

The large number of adult recipients of 4/6 CB enabled us to analyze association of outcomes with the type of HLA mismatches in this population. The number of recipients were 7 for HLA-A double mismatch, 170 for HLA-A and HLA-B mismatch, 190 for HLA-A and HLA-DRB1 mismatch, 36 for HLA-B double mismatch, 581 for HLA-B and HLA-DRB1 mismatch, and 41 for HLA-DRB1 double mismatch. With adjusted analyses, adjusted with same variables in the final model of all adult recipients, there was no significant effect of HLA mismatch types on overall mortality with HLA-A and HLA-B mismatch as the reference (*Online Supplementary Table S3*). The risk of relapse was significantly decreased in HLA-A and HLA-DRB1

**Table 2. Multivariate analyses of overall survival, relapse, and transplant-related mortality.**

| Outcome | N. | Overall mortality RR | 95%CI | P | Relapse RR | 95%CI | P | Transplant-related mortality RR | 95%CI | P |
|---|---|---|---|---|---|---|---|---|---|---|
| Children 15 years or younger | | | | | | | | | | |
| HLA disparity | | | | | | | | | | |
| Matched (6/6) | 82 | 1.00 | | | 1.00 | | | 1.00 | | |
| 5/6 | 222 | 1.07 | (0.68-1.69) | 0.765 | 1.06 | (0.68-1.65) | 0.794 | 1.29 | (0.52-3.23) | 0.58 |
| 4/6 | 158 | 1.61 | (1.02-2.56) | 0.042 | 0.77 | (0.48-1.24) | 0.282 | 3.55 | (1.47-8.58) | 0.005 |
| 3/6 | 36 | 1.25 | (0.65-2.42) | 0.498 | 0.91 | (0.45-1.86) | 0.802 | 1.56 | (0.43-5.63) | 0.497 |
| Total nucleated cell dose | | | | | | | | | | |
| ≥10.0x$10^7$/kg | 85 | 1.00 | | | 1.00 | | | 1.00 | | |
| 5.0-9.9x$10^7$/kg | 169 | 1.14 | (0.72-1.79) | 0.579 | 1.10 | (0.69-1.75) | 0.684 | 0.82 | (0.40-1.68) | 0.592 |
| 2.5-4.9x$10^7$/kg | 190 | 0.92 | (0.58-1.45) | 0.707 | 0.90 | (0.56-1.44) | 0.651 | 0.90 | (0.45-1.80) | 0.77 |
| <2.5x$10^7$/kg | 43 | 0.88 | (0.47-1.67) | 0.701 | 0.98 | (0.53-1.83) | 0.961 | 0.67 | (0.24-1.88) | 0.443 |
| Adults 16 years or older | | | | | | | | | | |
| HLA disparity | | | | | | | | | | |
| Matched (6/6) | 71 | 1.00 | | | 1.00 | | | 1.00 | | |
| 5/6 | 309 | 0.99 | (0.71-1.38) | 0.944 | 0.70 | (0.47-1.04) | 0.075 | 1.41 | (0.83-2.41) | 0.205 |
| 4/6 | 1025 | 0.88 | (0.65-1.21) | 0.436 | 0.67 | (0.47-0.97) | 0.034 | 1.24 | (0.75-2.04) | 0.408 |
| 3/6 | 475 | 0.95 | (0.69-1.31) | 0.751 | 0.70 | (0.48-1.03) | 0.07 | 1.29 | (0.77-2.16) | 0.339 |
| Total nucleated cell dose | | | | | | | | | | |
| ≥3.0x$10^7$/kg | 439 | 1.00 | | | 1.00 | | | 1.00 | | |
| 2.5-2.9x$10^7$/kg | 492 | 0.99 | (0.83-1.17) | 0.876 | 0.86 | (0.70-1.06) | 0.167 | 1.10 | (0.86-1.42) | 0.445 |
| 2.0-2.4x$10^7$/kg | 705 | 0.86 | (0.72-1.01) | 0.06 | 0.79 | (0.65-0.97) | 0.021 | 1.05 | (0.83-1.33) | 0.694 |
| <2.0x$10^7$/kg | 183 | 0.93 | (0.73-1.18) | 0.562 | 0.79 | (0.59-1.07) | 0.126 | 1.00 | (0.70-1.45) | 0.983 |

*For overall mortality, other predictive variables were advanced disease status at transplant in children, and age at transplant over 50 years, male sex, advanced disease status at transplant, chronic myeloid leukemia (associated with a lower risk of mortality), and reduced-intensity conditioning in adults. For relapse, other predictive variables were advanced disease status at transplant, and acute lymphoblastic leukemia or myelodysplastic syndrome (associated with a lower risk of relapse) in children, and advanced disease status at transplant and myelodysplastic syndrome (associated with a lower risk of relapse) in adults. For transplant-related mortality, there was no other predictive variable in children. Other predictive variables for adults were age at transplant over 50 years and female to male donor-recipient sex mismatch.*

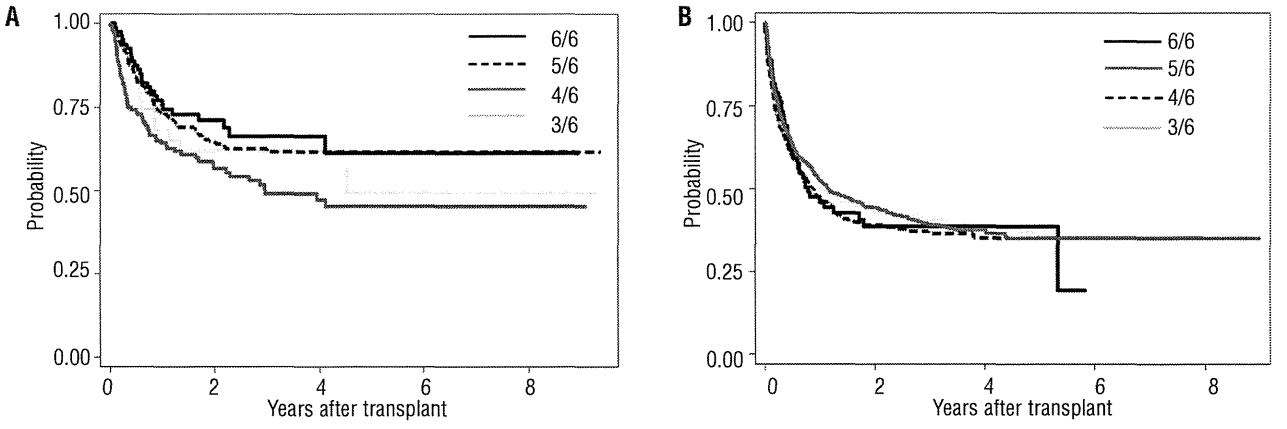


**Figure 1. Unadjusted probabilities of overall survival in HLA disparity groups for pediatric (A) and adult (B) recipients with leukemia. (A) In children, the unadjusted probabilities of survival at three years post-transplant were 66% for recipients of HLA matched (6/6), 62% for one-locus-mismatched (5/6), 45% for two-loci-mismatched (4/6), and 62% for three-loci-mismatched (3/6) single-unit unrelated cord blood (*P*=0.032). (B) In adults, these probabilities were 38% 37%, 39%, and 40% respectively (*P*=0.567) (B).**

mismatch, HLA-B and HLA-DRB1 mismatch, and HLA-DRB1 double mismatch recipients (RR=0.70, *P*=0.045; RR=0.76, *P*=0.047; and RR=0.46, *P*=0.03, respectively). The risk of transplant-related mortality was significantly increased in HLA-DRB1 double mismatch recipients (RR=2.06, *P*=0.025). There was no significant effect of HLA mismatch types for risks of grade 2 to 4 and grade 3 to 4 acute GVHD (*Online Supplementary Table S3*).

## Discussion

Our main objective was to assess the effect of HLA disparity on survival after single-unit UCBT in children and adults, and to obtain data that could be useful for the selection of an appropriate cord blood unit for patients with leukemia. Our study is the first to assess the effect of UCB HLA-matching on the transplant outcome in a large

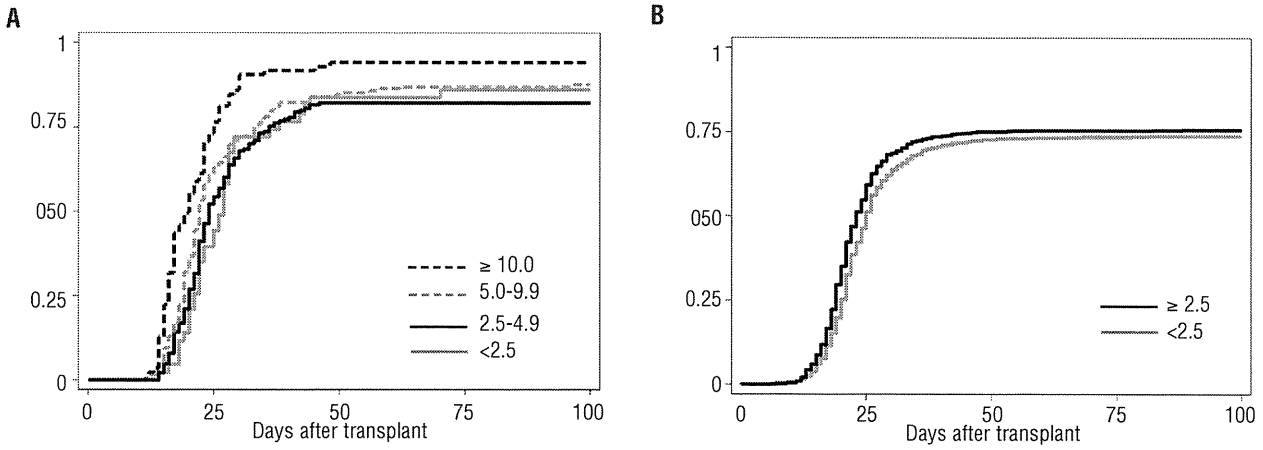


**Figure 2. Unadjusted cumulative incidences of neutrophil recovery in total nucleated cell dose groups for pediatric (A) and adult (B) recipients with leukemia. (A) In children, the unadjusted cumulative incidences of neutrophil recovery were 94% for >10x10$^7$/kg, 88% for 5.0-9.9 x10$^7$/kg, 82% for 2.5-4.9x10$^7$/kg, and 86% for <2.5x10$^7$/kg (*P*<0.001). (B) In adults, these incidences were 76% for >2.5x10$^7$/kg and 74% for <2.5 x10$^7$/kg (*P*=0.007).**

**Table 3. Multivariate analyses of neutrophil and platelet recovery.**

| Outcome | Children ≤15 years or younger N | RR | 95%CI | *P* value | | Adults ≥16 years or older N | RR | 95%CI | *P* |
|---|---|---|---|---|---|---|---|---|---|
| Neutrophil recovery | | | | | | | | | |
| HLA disparity | | | | | | | | | |
| Matched (6/6) | 82 | 1.00 | | | | 71 | 1.00 | | |
| 5/6 | 222 | 1.03 | (0.77-1.39) | 0.823 | | 309 | 0.89 | (0.66-1.19) | 0.436 |
| 4/6 | 158 | 0.96 | (0.71-1.30) | 0.799 | | 1025 | 0.92 | (0.70-1.22) | 0.576 |
| 3/6 | 36 | 0.67 | (0.44-1.03) | 0.068 | | 475 | 0.84 | (0.64-1.12) | 0.243 |
| Total nucleated cell dose | | | | | | | | | |
| ≥>10.0x10$^7$/kg | 85 | 1.00 | | | ≥3.0x10$^7$/kg | 439 | 1.00 | | |
| 5.0-9.9x10$^7$/kg | 169 | 0.66 | (0.49-0.89) | 0.007 | 2.5-2.9x10$^7$/kg | 492 | 0.84 | (0.72-0.97) | 0.021 |
| 2.5-4.9x10$^7$/kg | 190 | 0.50 | (0.37-0.67) | <0.001 | 2.0-2.4x10$^7$/kg | 705 | 0.79 | (0.68-0.90) | 0.001 |
| <2.5x10$^7$/kg | 43 | 0.54 | (0.38-0.77) | 0.001 | <2.0x10$^7$/kg | 183 | 0.78 | (0.64-0.94) | 0.009 |
| Platelet recovery | | | | | | | | | |
| HLA disparity | | | | | | | | | |
| Matched (6/6) | 82 | 1.00 | | | | 71 | 1.00 | | |
| 5/6 | 222 | 0.89 | (0.66-1.20) | 0.438 | | 309 | 1.05 | (0.73-1.52) | 0.775 |
| 4/6 | 158 | 0.75 | (0.54-1.05) | 0.09 | | 1025 | 1.05 | (0.74-1.48) | 0.791 |
| 3/6 | 36 | 0.71 | (0.44-1.15) | 0.164 | | 475 | 0.99 | (0.69-1.41) | 0.951 |
| Total nucleated cell dose | | | | | | | | | |
| ≥10.0x10$^7$/kg | 85 | 1.00 | | | ≥3.0x10$^7$/kg | 439 | 1.00 | | |
| 5.0-9.9x10$^7$/kg | 169 | 0.93 | (0.68-1.29) | 0.681 | 2.5-2.9x10$^7$/kg | 492 | 0.84 | (0.70-1.01) | 0.058 |
| 2.5-4.9x10$^7$/kg | 190 | 0.70 | (0.51-0.97) | 0.03 | 2.0-2.4x10$^7$/kg | 705 | 0.86 | (0.73-1.02) | 0.078 |
| <2.5x10$^7$/kg | 43 | 0.70 | (0.45-1.07) | 0.101 | <2.0x10$^7$/kg | 183 | 0.72 | (0.57-0.91) | 0.007 |

*For neutrophil recovery, other predictive variables were acute lymphoblastic leukemia in children (with a higher neutrophil recovery), and advanced disease status at transplant in adults. For platelet recovery, other predictive variables were advanced disease status at transplant in children, and age at transplant over 50 years, male sex, and advanced disease status at transplant in adults.*

Table 4. Multivariate analyses of grade 2 to 4/grade 3 to 4 acute graft-*versus*-host disease, and chronic/extensive-type chronic graft-*versus*-host disease.

| Outcome | Grade 2 to 4 acute GVHD N. | RR | 95%CI | P | Grade 3 to 4 acute GVHD RR | 95%CI | P | Chronic GVHD N. | RR | 95%CI | P | Extensive-type chronic GVHD RR | 95%CI | P |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Children 15 years or younger | | | | | | | | | | | | | | |
| HLA disparity | | | | | | | | | | | | | | |
| Matched (6/6) | 72 | 1.00 | | | 1.00 | | | 67 | 1.00 | | | 1.00 | | |
| 5/6 | 196 | 2.13 | (1.28-3.58) | 0.004 | 1.75 | (0.73-4.24) | 0.212 | 186 | 1.79 | (0.85-3.75) | 0.123 | 4.15 | (0.54-31.81) | 0.17 |
| 4/6 | 136 | 2.65 | (1.55-4.52) | <0.001 | 2.25 | (0.94-5.41) | 0.07 | 114 | 2.99 | (1.42-6.30) | 0.004 | 7.62 | (1.03-56.63) | 0.047 |
| 3/6 | 28 | 2.39 | (1.18-4.84) | 0.015 | 2.60 | (0.82-8.26) | 0.105 | 23 | 2.61 | (0.96-7.11) | 0.061 | 7.49 | (0.81-69.63) | 0.077 |
| Adults 16 years or older | | | | | | | | | | | | | | |
| HLA disparity | | | | | | | | | | | | | | |
| Matched (6/6) | 56 | 1.00 | | | 1.00 | | | 49 | 1.00 | | | 1.00 | | |
| 5/6 | 227 | 1.03 | (0.64-1.65) | 0.916 | 0.95 | (0.38-2.37) | 0.919 | 193 | 1.58 | (0.83-3.02) | 0.161 | 1.15 | (0.47-2.80) | 0.758 |
| 4/6 | 765 | 1.27 | (0.82-1.97) | 0.276 | 1.27 | (0.55-2.94) | 0.573 | 650 | 1.90 | (1.03-3.51) | 0.04 | 1.62 | (0.71-3.72) | 0.253 |
| 3/6 | 341 | 1.72 | (1.10-2.70) | 0.017 | 1.13 | (0.47-2.68) | 0.788 | 288 | 1.81 | (0.96-3.38) | 0.065 | 1.28 | (0.54-3.02) | 0.574 |

*For grade 2 to 4 acute GVHD, other predictive variables were total nucleated cell dose (>10x10$^7$/kg as the reference, RR=1.94 P=0.009 for 5.0-9.9x107/kg, RR=1.73 P=0.028 for 2.5-4.9x107/kg, and R=1.68 P=0.094 for <2.5x10$^7$/kg) in children, and cyclosporine-based GVHD prophylaxis (vs. tacrolimus-based) in adults. For grade 3 to 4 acute GVHD, male sex and advanced disease status in children, and male sex and male to female donor-recipient sex mismatch and reduced-intensity conditioning in adults. For chronic GVHD, no other predictive variables in children, and other predictive variable for adults was ABO major mismatch, and male to female sex mismatch and advanced risk disease status for decreased risk. For extensive-type chronic GVHD, no other predictive variables in children, and other predictive variable for adults was ABO major mismatch.*

number of adult recipients. Our findings in children were similar to those in previous reports.[9,17,18,31,32] An increase in the number of HLA mismatches resulted in an increased risk of acute and chronic GVHD, which led to an increased risk of overall and transplant-related mortality. In contrast to the results in children, the probability of overall or relapse-free survival did not decrease with the number of mismatched antigens in adults. An increase in the number of HLA mismatches in UCB increased the incidence of cGVHD in 4/6 CB recipients; however, there was no increase in the risk of grade 2 to 4 or severe acute GVHD, or extensive-type chronic GVHD. These differences may have contributed to the decreased incidence of relapse without affecting TRM after HLA-mismatched UCBT in adults.

A major potential contributor to the different findings in children and adults is the difference in the nucleated cell dose. There was a dramatic difference in the nucleated cell dose between children and adults. TNC dose in adults is highly concentrated in a very small, low-dose area that is quite different from the doses used in children in our study and from the doses in previous reports, mainly in pediatric recipients.[9,18,32] A positive effect on the transplant outcome with a decreased incidence of acute GVHD and lower mortality with HLA matching might only be seen in the setting of pediatric recipients who receive cord blood with a larger cell dose compared to adults. A report from Eurocord of 171 adult recipients of single-unit CBT did not see a decrease in the probability of overall or relapse-free survival with the number of mismatched antigens.[33] A more recent collaborative study by the Center for International Blood and Marrow Transplant Research, the New York Blood Center National Cord Blood Program, and the Eurocord-Netcord registry with 514 adult recipients did not observe an increase in mortality after HLA-mismatched UCBT.[34]

Another potential cause of different findings in children and adults is differences in diagnosis. Adult recipients had a significantly greater proportion of patients with myeloid malignancy. The incidence of a graft-*versus*-leukemia effect is reportedly higher in myeloid malignancy.[35-37] The decreased risk of relapse with a significant graft-*versus*-leukemia effect in HLA-mismatched UCB recipients was also more prominent in adult recipients with acute myeloid leukemia in our study. Furthermore, there were differences in disease risk between children and adults. Only 36% of adults were in a standard-risk disease status at transplant, while this value was 50% in children. Although we had adjusted for the disease status at transplant, we cannot rule out the possibility that these differences influenced the results.

An increase in the total nucleated cell dose increased the neutrophil recovery rate in both children and adults, consistent with other reports.[18,31-33] A lower total nucleated cell dose was not associated with increased transplant-related or overall mortality in our cohort, thus, we did not see a combined effect of HLA disparity and total nucleated cell dose. This differs from the findings of a recent report from New York Cord Blood Bank.[18] In our cohort, a lower cell dose was associated with a slower recovery; however, the differences in the overall incidences of neutrophil recovery between cell dose groups were small, especially in the adult cohort. This may explain our finding that a lower total nucleated cell dose was not associated with increased mortality. Another probable reason for the different findings is that for our analyses we separated children and adults. A small percentage of older adults who received lower cell dose CB included in the subjects of previous studies may have affected increased mortality with lower cell doses. Lastly, TNC dose in adults is highly concentrated in a very small, low-dose area (nearly 70% lie in the range of 2.0-3.0 x 10$^7$/kg) which is a unique finding for adult recipients of single-unit cord blood in Japan. Therefore, differences in cell doses between the TNC dose groups is quite small, which is suspected to be one of the reasons for these findings. The results of our study support the current recommended cut-off TNC dose for cord blood search in Japan, which is 2.0 x 10$^7$/kg.

Although information is still limited because of the limited number of 6/6 and 5/6 CB adult recipients, the large number of adult recipients of 4/6 CB enabled us to analyze the association of outcomes with the type of HLA mismatches in this population. There was no effect of HLA mismatch type on overall mortality; therefore, there is no

preference recommendation for HLA mismatch types from our study. The increase in the number of HLA-DRB1 mismatch was associated with decreased mortality; however, it is important to note that HLA-DRB1 double mismatch was associated with increased transplant-related mortality.

This study included a large number of HLA-A, HLA-B, low-resolution and HLA-DRB1 high-resolution typed CB recipients, but there are limitations. UCB selection is mainly influenced by the availability of an acceptable cell dose, but is also influenced by many unmeasured factors that can affect the outcome. Although we adjusted for known risk factors and disparities between groups, we cannot rule out the influence of a potential selection bias. Another limitation involves the results for 3/6. Since, in current practice in Japan, HLA-DR typing for UCB unit selection is performed at low resolution, with a preference of up to two HLAantigen-mismatched UCB units, most (97%) of the HLA-A, HLA-B, low-resolution and HLA-DRB1 high-resolution 3/6 UCB in the present study were selected as one- or two-antigen-mismatched for theHLA-A, HLA-B, and HLA-DR low-resolution level. If we consider the effect of the current practice for UCB unit selection regarding 3/6 UCB, our conclusions should only apply to HLA-A, HLA-B, and HLA-DRB1 or HLA-A, HLA-B, and HLA-DR zero- to two-mismatched UCBT. Furthermore, we may have underestimated the impact of HLA-matching, since we did not have enough data to include low- or high-resolution information on HLA-C matching, which was recently reported to affect mortality.[38]

In conclusion, we found that the effects of HLA disparity on transplant outcome differed between children and adults. In children, an increased number of mismatched HLA loci correlated with an increased risk of mortality. These findings support the selection of a UCB unit with HLA 6/6 followed by 5/6, consistent with the recommendations from the US and Europe. In adults, there was no increase in mortality with an increase in the number of mismatched HLA loci. In this case, a UCB unit with up to 4/6 can be selected if transplant is urgently needed.

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***Authorship and Disclosures***

*Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.*

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# Single nucleotide polymorphisms and outcome risk in unrelated mismatched hematopoietic stem cell transplantation: an exploration study

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# Single nucleotide polymorphisms and outcome risk in unrelated mismatched hematopoietic stem cell transplantation: an exploration study

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**Genetic risk factors contribute to adverse outcome of hematopoietic stem cell transplantation (HSCT). Mismatching of the HLA complex most strongly determines outcomes, whereas non-HLA genetic polymorphisms are also having an impact. Although the majority of HSCTs are mismatched, only few studies have investigated the effects of non-HLA polymorphisms in the unrelated HSCT and HLA-mismatched setting. To understand these effects, we genotyped 41 previously studied single nucleotide polymorphisms (SNPs) in 2 independent, large cohorts of HSCT donor-recipient pairs (n = 460 and 462 pairs) from a homogeneous genetic background. The study population was chosen to pragmatically represent a large clinically homogeneous group (acute leukemia), allowing all degrees of HLA matching. The *TNF*-1031 donor-recipient genotype mismatch association with acute GVHD grade 4 was the only consistent association identified. Analysis of a subgroup of higher HLA matching showed consistent associations of the recipient *IL2*-330 GT genotype with risk of chronic GVHD, and the donor *CTLA4*-CT60 GG genotype with protection from acute GVHD. These associations are strong candidates for prediction of risk in a clinical setting. This study shows that non-HLA gene polymorphisms are of relevance for predicting HSCT outcome, even for HLA mismatched transplants. (*Blood.* 2012; 119(26):6365-6372)**

## Introduction

It is thought that a large proportion of risk for adverse outcomes after hematopoietic stem cell transplantation (HSCT) is genetic, attributed to HLA matching,[1] killer-immunoglobulin-like receptor matching,[2,3] minor histocompatibility antigens,[4,5] and non-HLA gene polymorphisms.[6]

Whereas the degree of HLA mismatching exerts the strongest genetic effect on risks, such as acute and chronic GVHD, relapse, and survival, non-HLA polymorphisms in immune response genes, such as cytokines, at least modify these risks, as shown in studies that have shown light on the pathobiology of HSCT,[7,8] and the relation of cytokine gene polymorphisms,[6,9,10] with gene expression and biologic effects.[11-15]

Non-HLA gene polymorphisms have been widely studied (a systematic search conducted revealed 192 studies over the last 2 decades). Most of these studies used a candidate gene approach, and only one study was a genome-wide association study.[5] To minimize genetic confounding, most of these studies used either fully or largely HLA-matched related or unrelated HSCT cohorts. Limited availability of study subjects in the past made consideration of demographic or clinical risk factors in study cohort selection difficult, despite the existence of these risks being well established in the literature (eg, patient and donor age,[16,17] female donor to male recipient,[18] diagnosis and staging, prior chemotherapy, conditioning regimen,[19] concurrent infections). Although more than 100 genetic markers in more than 60 candidate genes have been studied, consistency of results has been poor across studies, which has been attributed to differences in HSCT setting or stem cell source, ethnicity of the population, marker genotype distribution, and study quality and power. Only a limited number of associations underwent replication studies, and very few of these showed some consistency in different settings, such as polymorphisms in *TNF, IL10, IL6, CTLA4*.[6]

HLA mismatching is common in daily unrelated donor HSCT practice, most commonly because of nonavailability of an HLA-matched donor. In the Japan Marrow Donor Program (JMDP), less than 10% of HSCT have a 12 of 12 allele HLA match, and approximately 30% have an 8 of 8 allele HLA match. Despite this, only a very small number of studies have deliberately used populations that represent the full spectrum of HLA matching.

It is an important clinical question whether non-HLA polymorphisms have an impact on HSCT outcome in an unrelated HSCT population despite the competing effects of HLA mismatching.

The aim of this study was to identify genetic polymorphisms influencing HSCT outcome in an unrelated donor, HLA-mismatched setting, pragmatically choosing a large diagnostic group (acute leukemia) with additional selection and correction for the most relevant confounding variables (see "Population"). We applied a study design aiming to comply with recommendations for more

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**Table 1. Selected candidate SNP markers of this study**

| Target gene | SNP | Target gene | SNP |
|---|---|---|---|
| *CCL4* | rs2634508 | *NOD2* | rs1077861 |
| *CD86* | rs1129055 | | rs1861757 |
| *CTLA4* | rs231777 | | rs1861759 |
| | rs231775 (*CTLA4*–49) | | rs6500328 |
| | rs3087243 (*CTLA*-CT60) | | rs2111234 |
| *FAS* | rs1800682 (*FAS*-670) | | rs2111235 |
| *FCGR2A* | rs1801274 | | rs7203344 |
| *HLA-E* | rs1264457 (*HLA-E* R128G) | | rs17313265 |
| | rs1800795 | *TGFB1* | rs1800469 (*TGFB1*-509) |
| *HSP70/hom* | rs2075800 | | rs2241715 |
| *IFNg* | rs2069705 | | rs2241716 |
| *IL1A* | rs1800587 (*IL1A*-889) | | rs4803455 |
| *IL1B* | rs16944 (*IL1B*-511) | *TLR4* | rs12377632 |
| *IL2* | rs2069762 (*IL2*-330) | | rs1927907 |
| *IL10* | rs1800896 (*IL10*-1082) | *TNF* | rs361525 (*TNF*-238) |
| | rs1800871 (*IL10*-819) | | rs1799964 (*TNF*-1031) |
| | rs1800872 (*IL10*–592) | | rs1800629 (*TNF*-308) |
| *IL15RA* | rs2228059 (*IL15RA* N182T) | | rs1799724 (*TNF*-857) |
| *IL23R* | rs6687620 | *TNFRSF1B* | rs1061622 (*TNFR2* codon 196) |
| *MIF* | rs755622 | *VDR* | rs731236 |
| *MTHFR* | rs1801133 (MTHFR C677T) | | |

stringent genetic association study designs,[20-24] testing a panel of strong candidate SNP markers from previous studies. Key features include significance as well as effect size testing on 2 large, independent, clinically homogeneous study cohorts stemming from a population of homogeneous ethnic background.

## Methods

### Population

Donor and recipient HSCT pairs were selected from the JMDP registry of unrelated HSCT. This study was approved by the review boards of the JMDP and Tokai University Medical School, Isehara, Kanagawa, Japan. We chose pairs with a diagnosis of acute leukemia. These form the largest subgroup within HSCT. Cohorts represented 2 samplings of the same national pool, taken from 2 distinct timeframes (1993-2000, 2001-2005). Inclusion criteria were diagnosis (acute lymphoblastic leukemia; acute nonlymphoblastic leukemia), age (4-40 years), conditioning (myeloablative), and stem cell source (bone marrow). All transplants were T-cell replete and received GVHD prophylaxis with either cyclosporin A or tacrolimus with methotrexate and corticosteroids. Analysis of the source as well as the selected HSCT population showed that HLA mismatching, donor age, and GVHD prophylaxis regimen (cyclosporin A vs tacrolimus) were the only confounders remaining significant in multivariate analysis (data not shown here).

All donor-recipient pairs were HLA-typed retrospectively to allele level at 6 loci (HLA-A, HLA-B, HLA-C, HLA-DRB1, HLA-DQB1, and HLA-DPB1). The distribution of HLA matching of the confirmatory cohort was adjusted to that of the screening cohort by matching each sample of the screening cohort with a confirmatory cohort sample of the same HLA class or HLA class combination according to the previous literature[25,26] and our own analyses of risk matches/mismatches within this study population (data not shown). Supplemental Table 1 (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article) shows the demographic and clinical characteristics of the selected cohorts. There was no statistically significant difference between the cohorts in the baseline demographic criteria. Supplemental Table 2A and B specify the degree of HLA matching and mismatching. For reasons of comparison, we have used the National Marrow Donor Program/Center for International Blood and Marrow Transplant Research classification of HLA matching.[27] According to this classification, 357 HSCT pairs have an 8 of 8 (HLA A, B, C, DRB1) high-resolution allele match, 331 (35.9%) are partially matched (1 mismatch within these HLA loci), and 234 (25.4%) are mismatched (2 or more mismatches within these HLA loci). Considering the HLA DQ and DP loci also, only 78 HSCT pairs (8.5%) had a 12 of 12 allele match. In Japanese, HLA A, B, and C mismatches are associated with risk of acute GVHD. HLA C mismatches, however, have a protective effect on relapse (whereas HLA A, C, and B mismatches associate with a risk of death).[25,26,28] More recent research has focused on specific allele mismatches, rather than mismatches in loci, aiming to identify nonpermissive mismatches for acute GVHD[29] or protective mismatches against relapse,[30] as well as risk HLA haplotypes for GVHD.[31]

### Gene and SNP marker selection

Selection of candidate markers was based on a search of the published literature on genetic associations with HSCT outcomes. As the TaqMan SNP genotyping platform was used, selection was limited to markers for which standard assays were available for this system.

For some genetic loci, the same markers that were associated in other populations were nonpolymorphic in Japanese (*NOD2*, *TGFB1*). The HapMap database (www.hapmap.org) was used to identify haploTag SNP for these loci.

The SNP markers included in this study are detailed in Table 1; the assay details are available in supplemental Methods.

### Genotyping

TaqMan SNP genotyping assays (Applied Biosystems) were applied for 38 selected SNP according to the maker's instructions.

The *IL10* promoter SNPs rs1800872 (-592A/C), rs1800871 (-819T/C), and rs1800896 (-1082A/G) were genotyped by PCR-SSO using Luminex Multi-Analyte Profiling system (xMAP; Luminex). Details of both genotyping methods can be found in supplemental Methods.

### Statistical analysis

Genotype results were imported into SPSS Statistics Version 17.0 (SPSS Inc). Because little is known about effects of non-HLA polymorphisms in HLA-mismatched populations, we used 3 analytic approaches to identify significant associations: 2-sided Fisher exact test (95% confidence intervals [CIs]) with Bonferroni correction for significance testing, odds ratio (OR; 95% CIs) as a measure of effect size, and independent testing in a confirmatory cohort (without application of multiple testing correction).

**Table 2. Results of SNP genotyping on all donor samples**

| Gene | Marker | Discovery cohort (genotype and association) | Confirmatory cohort (genotype and association) |
|---|---|---|---|
| *CTLA4* | rs231775 | AA aGVHD* ($P$ = .0043, OR = 0.049,* CI = 0.028-0.083) | NS |
| | | GG aGVHD ($P$ = .0071, OR = 1.90, CI = 1.19-3.03) | |
| *CTLA4* | rs3087243 | GG aGVHD ($P$ = .0086, OR = 1.81, CI = 1.18-2.78) | NS |
| *CTLA4* | Haplotype | CAA aGVHD ($P$ = .0025, OR = 0.59, CI = 0.42-0.82) | NS |
| | | CGG aGVHD* ($P$ = .00057,* OR = 1.72, CI = 1.27-2.34) | |
| *FAS* | rs1800682 | CC aGVHD4* ($P$ = .023, OR = 0.21,* CI = 0.37-0.96) | NS |
| *IFNg* | rs2069705 | CC ext cGVHD ($P$ = .035, OR = 0.57, CI = 0.33-0.96) | NT |
| | | CC relapse ($P$ = .04, OR = 0.60, CI = 0.37-0.96) | |
| *IL10* | rs1800896 | AA survival* ($P$ = .001)* protective | NS |
| *IL10* | Haplotype | CCA survival ($P$ = .032) protective | NT |
| *MTHFR* | rs1801133 | CT cGVHD ($P$ = .03, OR = 0.63, CI = 0.42-0.96) | NT |
| *NOD2* | rs17313265 | CT survival ($P$ = .012) risk | NT |
| | | CC survival ($P$ = .008) protective | NT |
| *NOD2* | rs2111235 | TT aGVHD4* ($P$ = .016, OR = 0.33,* CI = 0.14-0.80) | NS |
| *NOD2* | rs6500328 | GG ext cGVHD* ($P$ = .011, OR = 0.17,* CI = 0.023-0.78) | NS |
| *TGFB1* | rs1800469 | CC aGVHD2-4 ($P$ = .035, OR = 1.69, CI = 1.09-2.61) | NT |
| | | CT aGVHD2-4 ($P$ = .036, OR = 0.66, CI = 0.45-0.96) | NT |
| *TGFB1* | rs2241715 | GG aGVHD2-4 ($P$ = .047, OR = 1.64, CI = 1.06-2.53) | NT |
| | | GT survival ($P$ = .03) protective | NT |
| | | GT ext cGVHD ($P$ = .032, OR = 0.57, CI = 0.34-0.94) | NT |
| | | GT aGVHD2-4 ($P$ = .037, OR = 0.67, CI = 0.46-0.98) | NT |
| *TNF* | rs1799964 | TT relapse ($P$ = .041, OR = 1.71, CI = 1.04-2.82) | NT |
| *TNF* | rs1799724 | CC survival ($P$ = .014) protective | NT |

$P$ values (2-sided Fisher exact test; survival, log rank test, Kaplan-Meier). Marker rs231777 had no individual association and is therefore not included in this table, but it was included into the confirmatory cohort as part of the CTLA4 haplotype.

aGVHD indicates acute GVHD; aGVHD4, acute GVHD grade 4; aGVHD2-4, acute GVHD grade 2-4; cGVHD, chronic GVHD; ext cGVHD, extensive chronic GVHD; mismatch, genotype mismatch between donor and recipient; NS, not significant; and NT, not tested.

*Withstanding Bonferroni multiple testing corrections or have OR $\leq$ 0.5 or $\geq$ 2.

Variables were the 3 individual genotypes, and mismatch between donor and recipient genotypes. Outcomes were acute GVHD (0-4), acute GVHD grades 2 to 4, acute GVHD grades 3 to 4, acute GVHD grade 4, chronic GVHD, extensive chronic GVHD, relapse, death (overall, at 100 d/1 y/3 y), and survival (as log-rank test in Kaplan-Meier analysis). For the screening cohort, we considered as significant a $P$ value of .05 with Bonferroni correction for the number of SNP markers tested. As the $P$ value is not a good surrogate marker for effect size, and often small in HSCT-outcome association studies, we decided to separately include associations showing ORs of less than or equal to 0.5 and $\geq$ 2.0 (this follows observations of ORs of significant markers in previous studies).

Screening and confirmatory cohort data were analyzed on the overall cohort in the first instance. To reduce confounding by HLA mismatching, we conducted identical analyses on a subgroup with a higher degree of HLA matching (8 of 8 allele matching at the HLA A, B, C, DRB1 loci, with additional exclusion of combined HLA-DQB1 and DPB1 mismatches; allowing for either a HLA-DQB1 or a HLA-DPB1 mismatch only), similar to previous reports from JMDP,[5] resulting in cohorts of 160 (discovery) and 166 (confirmatory) pairs.

For the screening cohort, we would genotype all 41 chosen SNP markers (Table 1) on both donor and recipient cohorts and conduct overall and subgroup analyses. Markers only that show a corrected $P$ value of less than .05 and/or an OR of less than or equal to 0.5 and more than or equal to 2.0 in either the overall or the subgroup analyses would be selected for confirmatory typing. If a marker showed an association that was persisting when applying Bonferroni correction, we tested other associations of the same marker in the confirmatory cohort, even if these would not reach the multiple testing thresholds, to capture borderline significance or effect size of genotypes, building on the strength of testing in an independent confirmatory cohort.

Given the high degree of linkage between the *CTLA4* as well as the *IL10* SNPs in the study, unambiguous haplotypes could be determined directly without recourse to computational methods.

As the distribution of acute GVHD degrees of severity was significantly different between the screening and confirmation cohort, all associations with acute GVHD as outcome were reanalyzed after randomizing the study population into 2 different cohorts (using an online based tool for random assignment: http://www1.assumption.edu/users/avadum/applets/RandAssign/GroupGen.html).

Multivariate analysis was performed on the combined cohorts using STATA Version 11.0. OR of acute GVHD for the selected SNP in multivariate analysis was estimated by a multivariate logistic regression analysis with the adjustment for recipient and donor ages, underlying diagnosis, the use of total body irradiation, antithymoglobulin, female donor into male transplant, GVHD prophylaxis (tacrolimus vs cyclosporin A), relapse, and HLA mismatch to address possible confounding.

## Results

### Screening cohort

***All transplants (n = 460 pairs).*** In the screening cohort, involving 460 bone marrow transplants performed between 1993 and 2000, 41 single nucleotide SNP markers were typed in both patient and donor cohorts. Of these, 6 markers were excluded from analysis, for technical (multiple clusters: rs1927907, rs4803455) and statistical reasons (minor allele frequency < 5%: rs1800795, rs6687620, rs361525, rs1800629). All 35 markers included in the analysis were in Hardy-Weinberg equilibrium (defined as $P > .05$, with statistical correction for the number of tested markers).

Thirteen markers, plus the *IL10* and *CTLA4* haplotypes, showed an association with an HSCT outcome in the donor screening cohort (Table 2). By significance testing applying Bonferroni correction, only the marker *IL10*-1082 and the *CTLA4* haplotype showed significant association, whereas 3 further markers were selected for confirmatory typing by their effect size (marker *CTLA4* rs231775 also shows relevant effect size individually; marker *CTLA4* rs231777, which showed no individual association, was

**Table 3. Significant results of SNP genotyping on all recipient samples**

| Gene | Marker | Discovery cohort (genotype and association) | Confirmatory cohort (genotype and association) |
|---|---|---|---|
| *CTLA4* | rs231775 | AA cGVHD ($P$ = .046, OR = 1.83, CI = 1.02-3.28) | NS |
| *CTLA4* | rs231777 | Mismatch aGVHD ($P$ = .004, OR = 1.91, CI = 1.24-2.96) | NS |
| *CTLA4* | haplotype | CAA cGVHD ($P$ = .011, OR = 1.5, CI = 1.11-2.03) | NS |
| | | CGG cGVHD* ($P$ = .0013,* OR = 0.62, CI = 0.47-0.83) | NS |
| | | CGG aGVHD2-4 ($P$ = .019, OR = 0.70, CI = 0.52-0.94) | NS |
| | | TAG aGVHD4* ($P$ = .0071, OR = 3.71,* CI = 1.56-8.86) | NS |
| *FAS* | rs1800682 | CC relapse ($P$ = .017, OR = 1.68, CI = 1.03-2.74) | NS |
| | | CT relapse* ($P$ = .0025, OR = 0.50,* CI = 0.33-0.78) | NS |
| | | CT aGVHD ($P$ = .009, OR = 1.79, CI = 1.15-2.77) | NS |
| | | TT cGVHD ($P$ = .024, OR = 1.75, CI = 1.03-2.82) | NS |
| | | TT ext cGVHD ($P$ = .014. OR = 1.74, CI = 1.03-2.94) | NS |
| *HLA-E* | rs1264457 | Mismatch survival ($P$ = .023) risk | NT |
| *IL1A* | rs1800578 | Mismatch aGVHD2-4 ($P$ = .026, OR = 1.69, CI = 1.11-2.56) | NT |
| *IL1B* | rs16944 | AA aGVHD ($P$ = .048, OR = 0.63, CI = 0.39-0.99) | NT |
| | | GG aGVHD ($P$ = .032, OR = 1.75, CI = 1.08-2.82) | NT |
| *IL15RA* | rs2228059 | AC survival ($P$ = .024) risk | NT |
| *IL2* | rs2069762 | GG aGVHD4* ($P$ = .0014,* OR = 4.51,* CI = 1.91-10.6) | NS |
| | | GT survival ($P$ = .0021) protective | NS |
| | | TT survival ($P$ = .0061) risk | NS |
| *NOD2* | rs17313265 | CC aGVHD2-4 ($P$ = .036, OR = 2.15, CI = 1.06-4.37) | NS |
| *TGFB1* | rs1800469 | Mismatch aGVHD2-4 ($P$ = .02, OR = 1.63, CI = 1.1-6.4) | NT |
| *TGFB1* | rs2241715 | Mismatch aGVHD2-4 ($P$ = .015, OR = 1.61, CI = 1.09-2.39) | NT |
| | | Mismatch cGVHD ($P$ = .035, OR = 1.58, CI = 1.04-2.41) | NT |
| *TGFB1* | rs2241716 | AA ext cGVHD* ($P$ = .0041, OR = 2.58,* CI = 1.36-4.87) | NS |
| *TNF* | rs1799964 | Mismatch aGVHD4*† ($P$ = .022, OR = 2.53,*† CI = 1.16-5.53) | Mismatch aGVHD4*† ($P$ = .0053, OR = 3.40,*† CI = 1.48-7.81) |
| | | CC aGVHD4* ($P$ = .041, OR = 4.92,* CI = 1.27-19.02) | CC aGVHD4 trend ($P$ = .06) |
| *TNF* | rs1799724 | CC survival ($P$ = .02) protective, | NT |
| | | CT survival ($P$ = .02) risk | NT |
| *TNFRSF1B* | rs1061622 | TT aGVHD4* ($P$ = .023, OR = 4.69,* CI = 1.1-20.11) | NS |

The marker rs3087243 was not associated individually with chronic GVHD (cGVHD) or acute GVHD (aGVHD) and is not listed here, but it was included in the confirmatory cohort forming part of the CTLA4 haplotype.

NS indicates not significant; and NT, not tested. For other abbreviations please see Table 2.

*Withstanding Bonferroni multiple testing corrections or have OR ≤ 0.5 or ≥ 2.

†Consistent associations.

included in the confirmatory cohort as part of the *CTLA4* haplotype, not listed in Table 2). The recipient cohort (Table 3) revealed 15 markers, plus the *CTLA4* haplotype, that were associated with a HSCT outcome. The *IL2*-330 SNP and the *CTLA4* haplotype revealed significant associations above the multiple testing thresholds, whereas 5 SNP markers had ORs ≤ 0.5 and ≥ 2.0.

***HLA-matched subgroup (n = 160 pairs).*** When analyzing the HLA-matched subgroups of these cohorts, 7 markers and the *CTLA4* and *IL10* haplotypes in the donor cohort (Table 4) showed outcome associations, of which 5 markers and the *CTLA4* haplotype were included for confirmatory typing. Only the *CTLA4* haplotype had a $P$ value significant when multiple testing correction was applied. In the HLA matched recipient subgroup, 3 markers showed an association with HSCT outcome, of which one was selected for the confirmation cohort by strength of OR (Table 5).

## Confirmatory cohort

***All transplants (n = 462 pairs).*** Seven markers for the donor cohort (*CTLA4*: rs231775, rs231777, rs3087243 [included for forming the *CTLA4* haplotype, only rs231775 and rs3087243 showed an association in the screening cohort]; *FAS*: rs1800682; *IL10*: rs1800896; *NOD2*: rs2111235, rs6500328) and 10 markers for the recipient cohort (*CTLA4*: rs231775, rs231777, rs3087243

**Table 4. Results of SNP genotyping on HLA-matched donor samples**

| Gene | Marker | Discovery cohort (genotype and association) | Confirmatory cohort (genotype and association) |
|---|---|---|---|
| *CTLA4* | rs231775 | GG aGVHD* ($P$ = .026, OR = 2.02,* CI = 1.09-3.75) | NS |
| *CTLA4* | rs3087243 | GG aGVHD ($P$ = .021, OR = 1.97, CI = 1.11-3.50) | NS |
| *CTLA4* | Haplotype | CAA aGVHD ($P$ = .012, OR = 0.55, CI = 0.35-0.87) | NS |
| | | CGG aGVHD* ($P$ = .00097,* OR = 2.06,* CI = 1.22-5.94) | NS |
| *IFNg* | rs2069705 | CC ext cGVHD* ($P$ = .036, OR = 0.42,* CI = 0.20-0.93) | NS |
| | | CT ext cGVHD* ($P$ = .017, OR = 2.69,* CI = 1.22-5.94) | NS |
| *IL10* | rs1800896 | AA aGVHD* ($P$ = .038, OR = 0.21,* CI = 0.04-0.96) | NS |
| *IL10* | Haplotype | CCG aGVHD* ($P$ = .027, OR = 4.70, CI = 1.08-20.54) | NS |
| *MTHFR* | rs1801133 | TT aGVHD ($P$ = .0016, OR = 12.13,* CI = 2.73-53.90) | NT |
| *NOD2* | rs17313265 | CT relapse* ($P$ = .013, OR = 2.68,* CI = 1.02-7.09) | NS |
| *TNF* | rs1799724 | CC survival ($P$ = .006) protective | NT |

NS indicates not significant; and NT, not tested. Explanation of other abbreviations found in Table 2.

*Withstanding Bonferroni multiple testing corrections or have OR ≤ 0.5 or ≥ 2.

**Table 5. Results of SNP genotyping on HLA-matched recipient samples**

| Gene | Marker | Discovery cohort (genotype and association) | Confirmatory cohort (genotype and association) |
|---|---|---|---|
| *FAS* | rs1800682 | CT aGVHD* ($P$ = .0024, OR = 0.39,* CI = 0.22-0.71) | NS |
| *IL1B* | rs16944 | AA aGVHD ($P$ = .043, OR = 0.51, CI = 0.27-0.97) | NT |
| *IL2* | rs2069762 | GT survival ($P$ = .037) protective | NS |
| | | GT cGVHD ($P$ = .039, OR = 1.97, CI = 1.05-3.71) | GT cGVHD*† ($P$ = .00041,*† OR = 3.24,*† CI = 1.69-6.20) |
| | | TT survival ($P$ = .039) risk | NS |

NS indicates not significant; and NT, not tested.
*Withstanding Bonferroni multiple testing corrections or have OR ≤ 0.5 or ≥ 2.
†Consistent associations.

[part of *CTLA4* haplotype, only rs231775 and rs231777 were associated in the screening cohort]; *FAS*: rs1800682; *IL2*: rs2069762; *NOD2*: 17313265; *TGFB1*: rs2241716; *TNF*: rs1799964; *TNFRSF1B*: rs1061622) were selected for typing in the confirmatory cohort. First, we were seeking to confirm associations from the screening cohorts that had significant $P$ values after multiple testing correction (high significance); then, associations that had ORs ≤ 0.5 or ≥ 2.0 (large effect size); and third, associations within these selected markers that were consistent in both screening and confirmatory cohort (independent cohort confirmation), regardless of multiple testing correction or effect size.

There were no consistent findings in the overall donor confirmatory cohort (Table 2). In the overall recipient confirmatory cohort (Table 3), the donor-recipient genotype mismatch of the *TNF*-1031 SNP (rs1799964) was consistently associated in both screening and confirmatory cohorts with a higher risk of severe acute GVHD (grade 4). The CC genotype of the same marker was associated with acute GVHD grade 4 in the screening cohort and just escaped significance level in the confirmatory cohort ($P$ = .06).

***HLA-matched subgroups (166 pairs).*** In the donor HLA-matched subgroup (Table 4), none of the markers typed in the confirmatory cohort showed any association. The HLA-matched recipient cohort (Table 5) revealed a consistent association between risk of chronic GVHD and the GT genotype of rs2069762 (*IL2*-330).

Table 6 summarizes the consistent associations of this study, composed of the *IL2*-330 and *TNF*-1031 SNP.

### Further analyses

To understand the mechanism of the associated genotype, we extended the analysis to all *IL2*-330 genotypes and chronic GVHD outcomes in the confirmatory cohort and found that GT also associated with extensive chronic GVHD ($P$ = .00022, OR = 5.18, 95% CI, 2.37-11.39). The TT genotype exerts a protective effect against extensive chronic GVHD ($P$ = .0029, OR = 0.3, 95% CI, 0.13-0.67). This finding is replicated when combining screening and confirmatory cohorts (GT and extensive chronic GVHD: $P$ = .00055, OR = 2.90, 95% CI, 1.74-5.08; TT and extensive chronic GVHD: $P$ = .001, OR = 0.40, 95% CI, 0.23-0.71), suggesting that the GG genotype is probably the higher risk genotype. We did not find a significant association with the GG genotype, which is probably because of limited statistical power of this low frequency genotype. Mirroring the analysis by MacMillan et al[32] in our combined cohorts, the G allele showed a trend with risk of extensive chronic GVHD ($P$ = .07), but not with acute GVHD.

The extended analysis of the *TNF*-1031 CC genotype in the confirmatory cohort showed that it was also associated with acute GVHD grade 2 to 4 ($P$ = .029, OR = 3.41, 95% CI, 1.99-5.82). The *TNF*-1031 donor-recipient genotype mismatch was found to be a risk factor for acute GVHD grade 2 to 4 ($P$ = .003, OR = 1.93, 95% CI, 1.13-3.30) and grade 3 or 4 ($P$ = .002, OR = 2.21, 95% CI, 1.13-3.80) in the confirmatory cohort.

The stratification we applied in "matching" the degree of HLA mismatch of the confirmatory cohort to that of the screening cohort may have introduced bias (significantly different distribution of acute GVHD grades; supplemental Table 1). To address this, we randomly assigned samples to 2 cohorts, resolving any significant difference between time frames, and acute GVHD as an outcome measure. Reanalysis of the data for acute GVHD outcomes showed that the genotype mismatch of the *TNF*-1031 SNP as a risk factor for acute GVHD grade 4 would still hold up as significant ($P$ = .005, OR = 3.26, 95% CI, 1.91-5.58; $P$ = .021, OR = 2.60, 95% CI, 1.52-4.45). The *CTLA4*-CT60 (rs3087243) SNP showed a consistent association of the GG genotype as protective against acute GVHD ($P$ = .022, OR = 0.46, 95% CI, 0.27-0.78; $P$ = .045, OR = 0.49, 95% CI, 0.29-0.83) in the random cohort analysis of the HLA-matched subgroup.

### Multivariate analyses

Multivariate analyses (Tables 7-9) were performed on the combined (screening and confirmatory) cohorts and showed that the *TNF*-1031 donor-recipient genotype mismatch (acute GVHD grade 4), the CC genotype (acute GVHD grade 4), and the *IL2*-330 GT genotype (chronic GVHD) are independent risk factors, whereas the *CTLA4*-CT60 GG genotype is independently protective against acute GVHD.

**Table 6. SNP markers showing significant association in recipient screening and cohorts**

| Marker | Genotype | Cohort | Outcome | $P$ | Total | Cases, all | Controls, all | Cases positive | Cases negative | Controls positive | Controls negative | OR | OR (95% CI) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *TNF*-1031 | Mismatch | Screening | aGVHD4 | .022 | 448 | 28 | 420 | 12 | 16 | 96 | 324 | 2.53 | 1.16-5.53 |
| rs1799964, recipients (all) | Mismatch | Confirmation | aGVHD4 | .0053 | 460 | 24 | 436 | 12 | 12 | 99 | 337 | 3.40 | 1.48-7.81 |
| *IL2*-330 | GT | Screening | cGVHD | .039 | 160 | 72 | 88 | 39 | 33 | 33 | 55 | 1.97 | 1.05-3.71 |
| rs2069762, recipients (HLA matched) | GT | Confirmation | cGVHD | .00041 | 166 | 75 | 92 | 40 | 35 | 23 | 68 | 3.24 | 1.70-6.20 |
| *CTLA4*-CT60 | GG | Random 1 | aGVHD | .022 | 159 | 58 | 101 | 20 | 38 | 54 | 47 | 0.46 | 0.27-0.78 |
| rs3087243, donors (HLA matched) | GG | Random 2 | aGVHD | .045 | 166 | 53 | 11 | 22 | 31 | 67 | 46 | 0.49 | 0.29-0.83 |

**Table 7. Multivariate analysis of the IL2–330 GT genotype as risk factor for chronic GVHD in the HLA-matched subgroup**

| | Univariate | | Multivariate | |
|---|---|---|---|---|
| Variable | OR (95% CI) | P | OR (95% CI) | P |
| Recipient age | 1.008 (0.99-1.03) | .481 | 1.008 (0.98-1.03) | .528 |
| Donor age | 1.024 (0.99-1.05) | .106 | 1.020 (0.99-1.05) | .195 |
| Female to male transplant | 0.900 (0.52-1.57) | .71 | 0.876 (0.48-1.60) | .664 |
| Diagnosis ANLL vs ALL | 1.087 (0.70-1.69) | .711 | 1.022 (0.63-1.67) | .929 |
| Total body irradiation | 1.419 (0.72-2.80) | .313 | 1.284 (0.62-2.67) | .502 |
| Cyclosporine vs tacrolimus | 1.024 (0.66-1.59) | .916 | 0.996 (0.61-1.62) | .987 |
| Relapse | 0.526 (0.32-0.86) | .011 | 0.573 (0.34-0.96) | .033 |
| Genotype GT | 2.507 (1.60-3.93) | .000066 | 2.273 (1.42-3.63) | .0006 |

The genotype is an independent risk factor.

## Discussion

This study has identified 3 consistent non-HLA SNP associations with HSCT outcome: the *TNF*-1031 donor-recipient genotype mismatch with severe GVHD (grade 4, in the overall cohort), the recipient *IL2*-330 GT genotype with risk of chronic GVHD, and the *CTLA4*-CT60 GG genotype protective against acute GVHD (grade 1-4; the latter 2 associations were found in the HLA-matched subgroup only).

TNF-α is a cytokine that has been associated with severity of acute GVHD in several previous genetic, gene expression, and animal model studies. Teshima et al have demonstrated in an animal model that TNF is essential in the development of acute GVHD.[13] Previous data from a Japanese population have shown that the *TNF* haplotype, including *TNF*-1031, was associated with severe GVHD,[33] and the *TNF*-1031C allele was associated with higher TNF expression.[34] A more recent study[35] describes the C allele as a risk factor for grade 3 or 4 acute GVHD. Therefore, an association of the *TNF*-1031 CC genotype with severe acute GVHD, as seen in this study, albeit showing only a trend in the confirmation cohort, would be biologically meaningful and replicate previous findings. However, the *TNF*-1031 CC genotype displays strong linkage disequilibrium with HLA, in particular with HLA-B61.[34] This may explain our finding of the strong association between donor-recipient genotype mismatch and acute GVHD grade 4 in the overall cohort only, but not in the HLA matched subgroup. Our study did not have the power to elucidate whether any particular *TNF*-1031 genotype mismatch combinations carry a higher risk. As the group affected with acute GVHD grade 4 is small (just $> 5\%$), further studies should confirm this result independently. The finding that genotype mismatch was also associated with grade 2 to 4 as well as grade 3 or 4 acute GVHD (which are larger groups) in the confirmatory cohort gives further indication that the genotype mismatch is probably a risk factor for acute GVHD. Nevertheless, the strength and consistency of this association mean that it is potentially a strong discriminator for prediction of the most severe form of acute GVHD (grade 4), which could be exploited in clinical practice.

The *IL2*-330 (rs2069762) SNP has an almost identical genotype distribution between white and Japanese populations (white: TT, 0.536; GT, 0.464; GG, 0; Japanese [this study]: TT, 0.450; GT, 0.440; GG, 0.110). The G allele is the known high-expressing allele, and high levels of IL2 have been described to correlate with severity of acute GVHD.[32,36] A previous study from North America on a cohort of similar time frame to our screening cohort[32] reported an association between the recipient *IL2*-330 G allele and acute GVHD as well as a trend toward risk of chronic GVHD. In our study, we found an association of the GT genotype with risk of chronic GVHD. More detailed analysis showed that the low-frequency GG genotype is probably the highest risk genotype for chronic GVHD, whereas GT associated with risk, and TT with protection. Our findings therefore confirm those of the previous study, even across different ethnic populations, qualifying this marker as a predictor of chronic GVHD risk.

The effect of the *CTLA4*-CT60 polymorphism on HSCT outcomes was studied previously, in settings of HLA matched sibling donors[37,38] and matched unrelated donors[39] in white populations. In HLA-matched sibling transplants, the donor G allele was associated with increase of relapse and worse survival, whereas the AA genotype was linked to risk of acute GVHD. The findings in matched unrelated donor HSCT were similar, with the donor AA genotype associating with severe acute GVHD (grade 3 or 4), but risk of G allele or GG genotype with relapse or survival was not observed. Our findings are in accordance with these results, identifying the GG genotype as protective against acute GVHD (remarkably, the screening cohort result indicated a risk of the GG genotype with acute GVHD [Table 4], a finding completely reversed by the randomization). We could not establish any risk of the GG genotype with relapse or survival, or the AA genotype with acute GVHD. This may be explained by the fact that, in the

**Table 8. Multivariate analysis of the CTLA4-CT60 GG genotype for acute GVHD (grade 1-4 vs no GVHD) in the HLA-matched subgroup, confirming this genotype as an independent risk factor**

| | Univariate | | Multivariate | |
|---|---|---|---|---|
| Variable | OR (95% CI) | P | OR (95% CI) | P |
| Recipient age | 1.017 (0.99-1.04) | .146 | 1.020 (0.99-1.05) | .121 |
| Donor age | 0.995 (0.97-1.03) | .763 | 0.997 (0.97-1.03) | .854 |
| Female to male transplant | 1.644 (0.93-2.89) | .085 | 1.630 (0.89-2.97) | .111 |
| Diagnosis ANLL vs ALL | 1.280 (0.81-2.03) | .296 | 1.129 (0.69-1.85) | .631 |
| Total body irradiation | 0.847 (0.43-1.68) | .634 | 0.916 (0.45-1.86) | .809 |
| Relapse | 1.255 (0.77-2.06) | .369 | 1.330 (0.80-2.24) | .273 |
| Genotype GG | 0.468 (0.29-0.75) | .002 | 0.497 (0.31-0.80) | .004 |

**Table 9. Multivariate analysis of TNF-1031 genotype mismatch and CC genotype as a risk factors* for acute GVHD grade 4 in the overall (HLA matched and mismatched) cohort**

| | Univariate | | Multivariate | |
|---|---|---|---|---|
| Variable | OR (95% CI) | P | OR (95% CI) | P |
| Recipient age | 0.978 (0.95-1.01) | .109 | 0.975 (0.94-1.01) | .112 |
| Donor age | 1.038 (1.00-1.08) | .044 | 1.033 (0.99-1.07) | .105 |
| Female to male transplant | 0.610 (0.27-1.38) | .235 | 0.582 (0.24-1.42) | .236 |
| Diagnosis ANLL vs ALL | 1.001 (0.57-1.76) | .996 | 1.148 (0.60-2.18) | .673 |
| Total body irradiation | 0.909 (0.40-2.07) | .819 | 0.992 (0.39-2.51) | .987 |
| Antithymoglobulin | 3.562 (0.99-12.73) | .051 | 2.246 (0.45-11.15) | .322 |
| Cyclosporine vs tacrolimus | 1.336 (0.75-2.37) | .321 | 1.516 (0.80-2.86) | .198 |
| Relapse | 0.115 (0.03-0.48) | .003 | 0.154 (0.04-0.65) | .011 |
| HLA match | 0.465 (0.24-0.92) | .027 | 0.765 (0.35-1.67) | .502 |
| Genotype CC | 4.336 (1.7-11.1) | .002 | 3.888 (1.39-10.90) | .010 |
| Genotype mismatch | 2.905 (1.65-5.1) | .00023 | 2.307 (1.18-4.52) | .015 |

*Both are independent risk factors, with competing effects from HLA matching and relapse.

Japanese population, the GG genotype is more prominent than in whites, whereas the AA genotype is more rare (HapMap data of genotypes: whites: AA, 0.208; AG, 0.513; GG, 0.283; Japanese: AA, 0.047; AG, 0.389; GG, 0.542). The risk of acute GVHD, relapse, or survival associated with this marker may therefore be lower in the Japanese population, compared with whites.

The results raise also some methodologic questions which are beyond the scope of this study: (1) By incorporating a measure of effect size into the statistical analysis, this study extends beyond previous approaches focusing on significance and correction for multiple testing. Our results suggest that this approach may be more sensitive; but because of limited power and small number of identified associations, no conclusions could be made about the impact on sensitivity and specificity, and statistical multiple testing burden. (2) Despite the effort to control variability of study population characteristics, reproducibility of associations remains low and appeared to be dependent on distribution of these characteristics among the cohorts. This may be the result of the overall small effect size of the associations, confounders in the study cohort, or both. A more comprehensive typing (full typing of all markers on both screening and confirmation cohort) and analysis would be required.

Clinical and population characteristics of study cohorts may explain some of the contradictory results observed in previous studies; therefore, careful design of study cohorts and control of confounders should receive more attention. The growing number of HSCTs may facilitate in the future the availability of larger, genetically and clinically more homogeneous study cohorts; however, the changing and expanding indications of HSCT are likely to prove a challenge.

In conclusion, this study demonstrates that non-HLA genetic association with HSCT outcomes does exist and can be detected, even in the HLA-mismatched setting. Such associations could be useful for application in future clinical practice in this clinically highly relevant population. These findings should be verified by larger studies also on populations of different ethnicities.

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## Authorship

Contribution: C.H. designed and coordinated the project, carried out the experiments and univariate data analyses, and wrote the manuscript; A.O. designed the study and the experiment and provided technical advice; M.O., H.I., A.R.G., and K.A. designed the study; P.G.M. designed the study and experiment and inferred the CTLA4 haplotypes; K.K., K.H., and T.Y. performed the IL-10 SNP genotyping and haplotype inference; H.N. gave statistical advice and performed multivariate analyses; and Y.M. designed the study and acted as liaison to JMDP, providing clinical datasets and DNA samples.

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