

Figure 3. Overall survival according to pretransplantation factors, age (A) and disease status at transplantation (B).

ATLL has an extremely poor prognosis, with projected 2- and 4-year survival rates of 16.7% and 5.0% for the acute type and 21.3% and 5.7% for the lymphoma type, respectively. [3] Neither intensified chemotherapy nor autologous stem cell transplantation have improved the prognosis. Encouraging results for allo-HSCT for ATLL from HLA-matched related donors have been reported by several groups; thus, allo-HSCT may improve the poor prognosis of ATLL. However, the number of patients in most reports has been too small to allow evaluation of the efficacy of allo-HSCT for ATLL. The present results were derived from a large number of patients who underwent transplantation (33 patients) performed through the JMDP. Longer follow-up is, of course, needed to confirm the curative potential of allo-HSCT for ATLL. However, the good survival rates noted here suggest that allo-HSCT is an effective treatment for ATLL, and that patients with ATLL will benefit from allo-HSCT through HTLV-I-neg-

ative unrelated donors, because the OS and PFS rates at 1 year after UBMT were 49.5% and 49.2%, respectively. Compared with the results for patients with non-Hodgkin's lymphoma in the National Marrow Donor Program, the incidence of grade III-IV acute GVHD in the present study was low (18% vs 30%). [26] The outcome in the present study appears to be favorable, possible due to the lower incidence of grade III-IV acute GVHD. This observation is compatible with previous studies showing a lower incidence of acute GVHD in Japanese patients compared with Western patients, which might reflect the less diverse genetic background of in the Japanese population. [27,28]

Frequency of relapse after transplantation differs between autologous and allo-HSCT for ATLL. The use of high-dose chemotherapy with autologous HSCT has been reported in only 9 patients, all of whom relapsed or died from transplantation-related mortality. [8] In contrast, the cumulative incidence of

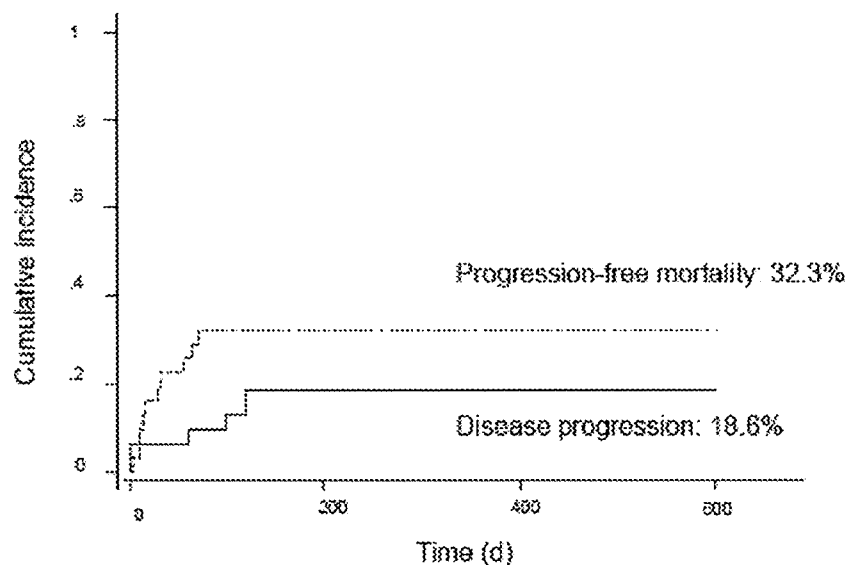
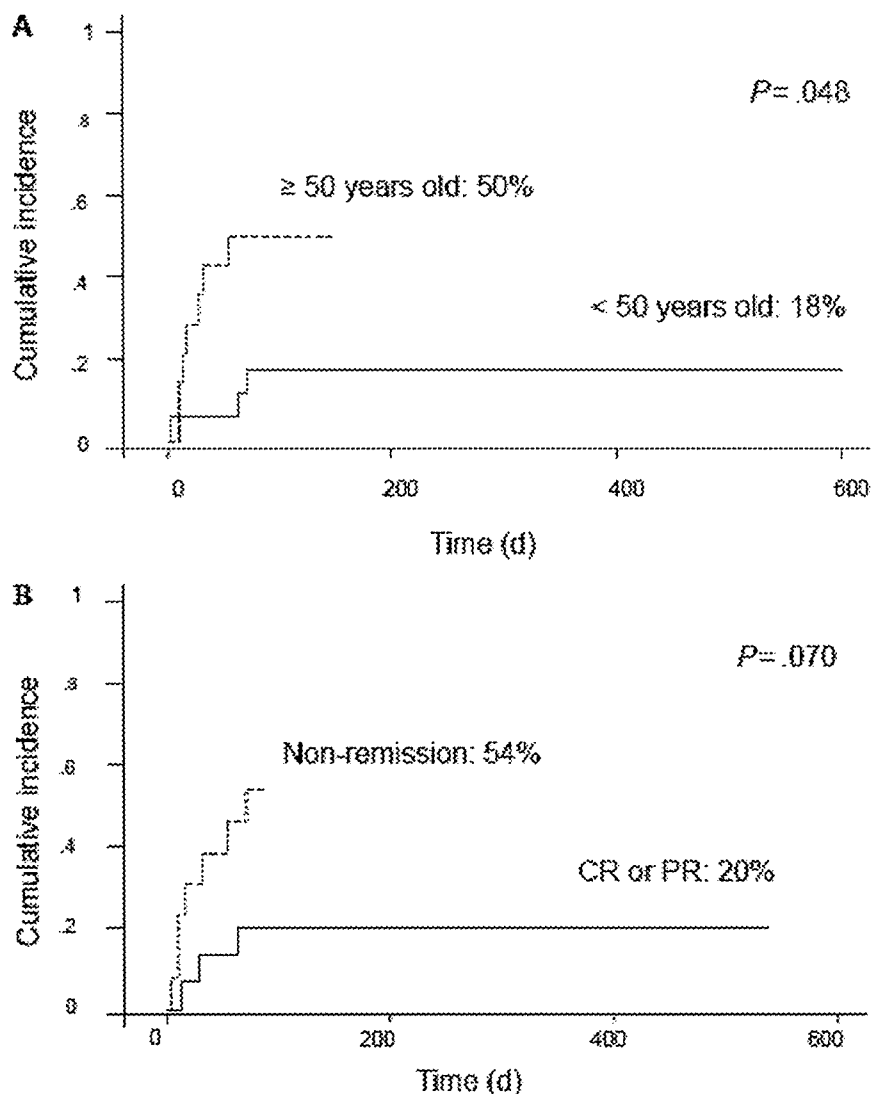


Figure 4. Cumulative incidence of disease progression (—) and progression-free mortality (---) after transplantation.

disease progression was lower after UBMt in this study. Interestingly, patients with ATLL displaying acute or chronic GVHD reportedly did not relapse. [9] In another report, patients with ATLL who relapsed after allo-HSCT reached CR after tapering or discontinuation of immunosuppressive agents and donor lymphocyte infusions. [10,11] Reactivation in tax-specific CD8-positive cytotoxic T lymphocytes (CTLs), which has been recently shown in patients with ATLL after allo-HSCT, may indicate a potential contribution of CTLs to anti-ATLL immunity and induction of a GvATLL effect. [29] These results strongly suggest that a GvATLL effect could work on some patients with ATLL to prevent relapse after allo-HSCT. In the present study, neither univariate nor multivariate analysis showed a survival benefit for acute GVHD. We were unable to analyze the relationship between chronic GVHD and relapse, because of the low number of patients with chronic GVHD. In fact, the number of patients may have been insufficient to confirm GvATLL in this study. On the other hand, the absence of benefit from GVHD in preventing relapse suggests that a GvATLL effect could occur in patients with ATLL after allo-HSCT without clinically obvious GVHD. [11]

Transplantation-related mortality was a significant problem in this study. Five patients (15%) died within 20 days, from infection in 3 patients and TMA in 2 patients. Nine patients (27%) died within 100 days, due to infection in 3 patients, TMA in 2 patients, and VOD in 1 patient. Patients with ATLL might have an increased risk of frequent opportunistic infection, because they have an associated T-cell immunodeficiency. Furthermore, ATLL is usually systemic in distribution, and the accumulated organ damages as a

result of repeated cytotoxic chemotherapy seen in patients before transplantation may have contributed to the onset of TMA. In univariate and multivariate analysis, recipient age ( $\geq 50$  years) and NR disease status at transplantation represented significant risk factors for OS. The multivariate analyses were limited by the small number of patients in each subgroup; however, patients displaying these risk factors tended to have a higher rate of treatment-related mortality than patients without these factors, and it can be assumed that these risk factors have a significant relationship with outcome clinically. In this study, mostly myeloablative conditioning regimens were used before transplantation. Given that conventional allo-HSCT is designed to eradicate tumor cells with myeloablative intensity using maximally tolerated doses of high-dose chemotherapy and radiotherapy, the desirable effects often may be offset by overwhelming toxicity in patients age  $\geq 50$  years. Moreover, the number of patients with ATLL who are eligible for allo-HSCT with myeloablative conditioning is limited, because the typical patient with ATLL has a relatively advanced age at presentation (about 60 years). To reduce treatment-related mortality, allo-HSCT with reduced-intensity conditioning offers a new treatment option for patients with ATLL who are ineligible for allo-HSCT with myeloablative conditioning due to advanced age or medical infirmity. [30,31] Okamura et al [32] reported on 16 patients age  $> 50$  years with ATLL who underwent allo-HSCT with reduced-intensity conditioning from HLA-matched donors and found that treatment-related mortality was acceptable and that allo-HSCT with reduced-intensity conditioning was a feasible treatment for ATLL. Given these findings, UBMt



**Figure 5.** Cumulative incidence of progression-free mortality grouped according to pretransplantation factors, age (A) and disease status at transplantation (B).

with reduced-intensity conditioning should be considered for elderly patients with ATLL.

Another concern related to allo-HSCT for ATLL involves the use of HTLV-1-positive carrier donors. About 2/3 of siblings of patients with ATLL are HTLV-I carriers. From the perspective of HTLV-I-positive donor risk, granulocyte colony-stimulating factor (G-CSF) can reportedly stimulate the proliferation of ATLL cells [33], and HTLV-I-positive donors may be at increased risk of developing ATLL due to the administration of G-CSF in the setting of allogeneic peripheral blood stem cell transplantation. From the perspective of patients with ATLL, allo-HSCT from an HTLV-I-positive donor may carry a risk of HTLV-I-associated disease after allo-HSCT [34] or a risk of promoting the future development of ATLL due to the new HTLV-I load on immunocom-

promised recipients [13,14]. On the other hand, to date there is no evidence in the JMDP or the literature that ATLL can develop from infected HTLV-I-negative donor cells due to the HTLV-I load of the recipient. The HTLV-I proviral load dramatically decreased to an undetectable level after transplantation, especially after transplantation from HTLV-I-negative donors. [18, 32] This decreased HTLV-I proviral load was observed after both myeloablative and reduced-intensity conditioning. Transplantation from an HTLV-I-positive donor is reportedly associated with a higher frequency of relapse compared with transplantation from an HTLV-I-negative donor. [11] Therefore, the uninfected normal donor T cells might overwhelm infected HTLV-I recipient T cells due to a GvATLL response and might act as an antiviral therapy. However, an HTLV-I-positive do-

nor might avoid clonal expansion of HTLV-I-infected T lymphocytes after allo-HSCT through the provision of cytotoxic T cells. Thus, it is currently difficult to determine whether an HTLV-I-positive or-negative donor should be selected. Longer follow-up is needed to resolve this issue. In the meantime, a prudent clinical attitude toward both HTLV-I-positive donors and recipients with ATLL is warranted.

In conclusion, allo-HSCT from an HTLV-I-negative unrelated donor appears to be a feasible alternative treatment for patients with ATLL for whom an HLA-matched related donor is unavailable. Further prospective controlled studies are needed to assess the efficacy of allo-HSCT for ATLL and to define the clinical indications of allo-HSCT for ATLL, taking into account donor selection, the conditioning regimen, and the prognostic factors identified in this study.

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#### APPENDIX: PARTICIPATING INSTITUTIONS

The following centers in Japan participated in this study: Hokkaido University Hospital, Sapporo University Hospital, Sapporo Hokuyu Hospital, Japanese Red Cross Asahikawa Hospital, Asahikawa Medical College Hospital, Hirosaki University Hospital, Tohoku University Hospital, Yamagata University Hospital, Akita University Hospital, Fukushima Medical College, National Cancer Center Central Hospital, Institute of Medical Science at the University of Tokyo, Toho University Hospital, Omori Hospital, Tokyo Metropolitan Komagome Hospital, Nihon University Hospital, Itabashi Hospital, Jikei University Hospital, Keio University Hospital, Tokyo Medical College Hospital, Tokyo Medical and Dental University Hospital, Tokyo University Hospital, Yokohama City University Hospital, Kanagawa Children's Medical Center, Kanagawa Cancer Center, Tokai University Hospital, St Marianna University Hospital, Chiba University Hospital, Chiba Children's Hospital, Matsudo Municipal Hospital, Kameda General Hospital, Saitama Children's Medical Center, Saitama Cancer

Center Hospital, Saitama Medical School Hospital, Ibaraki Children's Hospital, Jichi Medical School Hospital, Dokkyo University Hospital, Fukaya Red Cross Hospital, Saiseikai Maebashi Hospital, Gunma University Hospital, Niigata University Hospital, Niigata Cancer Center Hospital, Shinshu University Hospital, Saku Central Hospital, Hamamatsu University Hospital, Hamamatsu Medical Center, Shizuoka General Hospital, Shizuoka Children's Hospital, Japanese Red Cross Nagoya First Hospital, Nagoya Daini Red Cross Hospital, Meitetsu Hospital, Nagoya University Hospital, Nagoya Ekisaikai Hospital, National Nagoya Hospital, Aichi Medical School Hospital, Nagoya City University Hospital, Showa Hospital, Anjo Kousei Hospital, Fujita Health University Hospital, Mie University Hospital, Kanazawa University Hospital, Kanazawa Medical University Hospital, Toyama Prefectural Central Hospital, Fukui Medical School Hospital, Shiga University of Medical Science, Center for Adult Disease in Osaka, Kinki University Hospital, Osaka University Hospital, Osaka Medical Center and Research Institute for Maternal and Child Health, Matsushita Memorial Hospital, Hyogo College of Medicine Hospital, Hyogo Medical Center for Adults, Kobe City General Hospital, Kobe University Hospital, Kyoto University Hospital, Kyoto Prefectural University of Medicine Hospital, Social Insurance Kyoto Hospital, Tottori Prefectural Central Hospital, Tottori University Hospital, Hiroshima Red Cross Hospital and Atomic-Bomb Survivors Hospital, Yamaguchi University Hospital, Ehime Prefectural Central Hospital, Okayama National Hospital, Kurashiki Central Hospital, Kyushu University Hospital, Harasanshin General Hospital, Hamanomachi General Hospital, National Kyushu Cancer Center, St Mary's Hospital, Kokura Memorial Hospital, Saga Prefectural Hospital, Nagasaki University Hospital, Miyazaki Prefectural Hospital, Kumamoto National Hospital, Kumamoto University Hospital, Oita Medical University Hospital, Kagoshima University Hospital, and Imamura Bun-in Hospital.

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## Mapping of susceptibility and protective loci for acute GVHD in unrelated HLA-matched bone marrow transplantation donors and recipients using 155 microsatellite markers on chromosome 22

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**Abstract** Despite matching donors and recipients for the human leukocyte antigens (HLAs) expressed by the major histocompatibility genomic region of the short arm of

chromosome 6, several recipients still develop acute graft-versus-host disease (aGVHD) after bone marrow transplantation (BMT). This is possibly due to non-HLA gene polymorphisms, such as minor histocompatibility antigens (mHAs) and genes coding for cytokines. However, a detailed genetic background for aGVHD has not yet been established. To find novel susceptibility and/or protective loci for aGVHD, a whole genome-wide association study of donors and recipients needs to be performed. As the first step to such a study, we retrospectively analyzed polymorphisms of 155 microsatellite markers spread across the long arm of chromosome 22 in 70 pairs of HLA-matched unrelated BMT donors and recipients. We performed individual typing and then compared the markers' allele frequencies (1) between all the aGVHD (grades III and IV GVHD) and GVHD-free (grade 0 GVHD) groups in donors and recipients and (2) between the aGVHD and aGVHD-free groups in donor/recipient pairs that were matched and mismatched for the microsatellite marker's allele. Screening of the microsatellite markers revealed five loci with a significant difference between the aGVHD and GVHD-free groups and revealed eight loci on chromosome 22, where the microsatellite allele mismatched markers were associated with aGVHD. This screening analysis suggests that several aGVHD-associated susceptible and protective loci exist on chromosome 22, which may encompass novel gene regions that need to be elucidated for their role in aGVHD.

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**Keywords** Microsatellite · Bone marrow transplantation · Acute GVHD · Chromosome 22 · Non-HLA

## Introduction

The occurrence of acute graft-versus-host disease (aGVHD) is still a major cause of mortality in the bone marrow transplantation (BMT) recipients who are not related familiarly to donors. Despite successfully matching the human leukocyte antigen (HLA) alleles of donors and recipients for hematopoietic stem cell transplantation, a significant proportion of transplantation recipients develop aGVHD because of genetic differences attributed to minor histocompatibility antigens (mHa) (Chao 2004; Falkenburg et al. 2003), non-HLA genes coding for cytokines, and other molecules involved in the pathogenesis of aGVHD (Charron 2003; Kallianpur 2005; Dickinson and Charron 2005; Mullighan et al. 2004).

Genetic association studies of aGVHD can be performed at least in two ways: the candidate gene approach and genome-wide approach. The former approach is hypothesis-driven and dependent on the systematic knowledge of the aGVHD biological process. By using the candidate gene approach, single nucleotide polymorphisms (SNPs) were found within cytokine or cytokine receptor genes, which affect the aGVHD (Charron 2003; Kallianpur 2005; Dickinson and Charron 2005; Mullighan et al. 2004). However, aGVHD is a complex pathophysiological disease, and undoubtedly, a number of unknown genes contribute to or affect the GVHD mechanism. In this regard, the candidate gene approach would fail to find novel genes that are not already reported or thought to be immunoregulatory genes involved with aGVHD. In comparison, the genetic association studies using the genome-wide approach and genetic markers to test all possible variants systemically across the whole genome would be a more experimentally ideal approach to find novel genes involved with aGVHD. In addition, genomic matching by using SNP and/or microsatellite markers for finding compatibility of minor antigens in BMT may improve survival and other clinical outcomes.

Microsatellites and SNPs are two types of genetic markers that can be applied to genome-wide disease association studies, with each type of marker presenting certain advantages as well as inconveniences. Microsatellites are direct tandem-repeated sequences of DNA with a repeat size ranging from 2 to 6 bp. The number of repeats within a microsatellite sequence is usually less than 100. Because the microsatellite polymorphism is based on the differences in number of repeats, microsatellites are highly polymorphic with a high degree of heterozygosity. Polymorphic microsatellites are fewer in number than SNPs, but like SNPs, they are widely distributed across the human genome enabling efficient and accurate calculations of linkage disequilibrium (LD) between pairs of microsatellite loci separated by less than 100 kb of genomic sequence.

Indeed, we have already established and described a set of 27,039 microsatellite markers for the systematic analysis of the whole human genome and, together with SNP analysis, revealed at least seven potential susceptibility gene loci of rheumatoid arthritis (Tamiya et al. 2005). Therefore, the main advantage of using microsatellites as the primary or “first pass” genotyping method is that they allow for a genome association analysis to become an immediate and efficient reality.

To date, there are only a few association studies using microsatellite analysis to determine the potential clinical outcomes in hematopoietic stem cell transplantation, and these studies are limited mainly to the cytokine genes and the HLA region (Karabon et al. 2005; Li et al. 2004; Cullup et al. 2003; Nordlander et al. 2002; Witt et al. 1999). As a set of 27,039 microsatellite markers for the systematic analysis of the whole human genome has been established, we decided to use them in a genome-wide search of allele frequency differences to find and map novel susceptibility and/or protective loci for aGVHD. Although our ultimate goal is a complete genome-wide study, we have started our search for aGVHD susceptibility/protective loci within chromosome 22 (chr 22) for simplicity and economic convenience. A number of studies (Abecasis et al. 2001; Keicho et al. 2000; Oka et al. 1999; Ota et al. 1999; Li et al. 2004) suggest that association analysis using microsatellite markers as a first step of the genome-wide approach is a useful way to find candidate genes and specifically the mHa genes on chr 22 of BMT donors and recipients.

Human chr 22 is the second smallest of the autosomes comprising 1.6–1.8% of the genomic DNA (Dunham et al. 1999). There is no evidence to indicate the presence of any protein coding genes on the short arm of chr 22 (22p). In contrast, the long arm of the chr 22 (22q) is rich in genes compared with other chromosomes. In addition, alteration of gene dosage on the part of 22q is responsible for the etiology of 29 Mendelian disorders and a number of congenital abnormality disorders including cat eye syndrome and DiGeorge syndrome (McDermid and Morrow 2002). Linkage studies have shown an association of chr 22 loci to several disorders, such as schizophrenia, epilepsy, multiple sclerosis, and myopia (DeLisi et al. 2002; Berkovic et al. 2004; Liguori et al. 2004; Stambolian et al. 2004).

Interestingly, two recent reports have highlighted that there are many signal transducers and activators of transcription (STAT) and NF-kappaB-binding sites distributed across chr 22 (Martone et al. 2003; Hartman et al. 2005). STAT and NF-kappaB family members play an essential role in regulating the induction of genes involved in physiological processes, such as apoptosis, immunity, and inflammation, and they may also affect immunoregulatory genes relevant to the recognition and rejection of

foreign tissue. In addition, Gubarev et al. (1996) reported the localization of a gene encoding mHa to chr 22. On the basis of these reports and in an attempt to improve efficiency by screening chromosomal regions of high gene density, chr 22 is a very attractive target for genome-wide association research of GVHD and other immune-related diseases.

As the first step to our genome-wide study, we retrospectively genotyped 155 microsatellite markers on chr 22 in 70 HLA-matched unrelated BMT recipient and donor pairs and associated at least eight significant allele frequency differences with aGVHD. In accordance with our previous study using microsatellite markers to identify mHa (Li et al. 2004), we performed individual DNA typing to investigate the association between statistically significant donor/recipient microsatellite marker mismatches.

## Materials and methods

### Recipient and donor pairs

A total of 70 unrelated donor/recipient pairs after BMT who were treated through the Japan Marrow Donor Program and completely allele-matched for the HLA-A, HLA-B, HLA-C, HLA-DRB1, and HLA-DQB1 genes at the high resolution level were enrolled for this study after BMT (Sasazuki et al. 1998; Morishima et al. 2002). All 70 recipients underwent BMT from 1995 to 2000 for hematopoietic malignancy. None of the recipients received T-cell-depleted grafts. Patient, transplantation, and treatment information data are summarized in Table 1. All the donors and recipients provided informed consent for study, which was approved by the relevant institutional ethical committee.

### Diagnosis and evaluation of the acute GVHD study group

Acute GVHD was diagnosed clinically and classified into four groups according to standard criteria (grades 0, I, II, III, and IV; Glucksberg et al. 1974; Thomas et al. 1975). The 30 recipients who experienced grades III and IV were designated in this study as the aGVHD group. The 40 recipients who had not developed aGVHD (grade 0) were designated as the aGVHD-free group. The recipients with GVHD grades I or II were excluded from this analysis to differentiate more efficiently between the aGVHD and aGVHD-free groups.

### Microsatellite markers set

The association study was performed using 155 microsatellite markers spanning the long arm of chromosome 22.

**Table 1** Numbers and ratios for the major clinical characteristics

Clinical characteristics	Number or Ratio
Recipient's age (median year, range)	27.5, 1–50
Donor's age (median year, range)	33.9, 21–52
Recipient's sex (M/F)	38:32
Sex combination (recipient/donor)	M/M 29 M/F 19 F/F 13 F/M 9
Diagnosis	
Acute myeloid leukemia	28
Acute lymphoblastic leukemia	21
Chronic myeloid leukemia	21
Conditioning regimen	
CY+TBI	26
CY+CA+TBI	17
BU+CY+CA	2
BU+CY	7
CY+BU+TBI	3
CY+VP+TBI	4
BU+CY+TLI	1
LP+TBI	2
BU+VP+LP	1
CA+VP+TBI	1
CA+TBI	2
VP+TBI	1
BU+LP+TBI	1
CA+TBI	2
aGVHD frequency	
Grade 0	40
Grade III	20
Grade IV	10
GVHD prophylaxis	
CsA+MTX	64
FK+MTX	2
FK+PDR	1
CsA	1
CsA+MTX+PDR	1
CsA+MTX+FK	1

*M* Male, *F* female, *CY* cyclophosphamide, *TBI* total body irradiation, *CA* cytosine arabinoside, *BU* busulfan, *VP* etoposide, *TLI* total lymph node irradiation, *LP* melpharan, *CsA* cyclosporine A, *MTX* methotrexate, *FK* tacrolimus hydrate, *PDR* prednisolone

These markers were selected from Japan Biological Information Research Center (JBIRC) database (<http://jbirc.jbic.or.jp/gdbs/>). The markers covered the human genome from 15647099b (D22S0283i) to 49510061b (D22S0211i) on 22q with an average spacing of 200 kb.

### Microsatellite genotyping

Genomic DNA was isolated from the peripheral blood lymphocytes of patients and donors. The PCR procedure was performed in 10 µl reactions using fluorescent-dye conjugated PCR primers that were unilaterally labeled at



the 5'-end with the fluorescent reagent, 6-FAM (Applied Biosystems Japan, Tokyo, Japan). The PCR reaction mixture contained 10 ng of genomic DNA, 1  $\mu$ l of deoxyribonucleotide triphosphate (5 mM each), 1  $\mu$ l of 10 $\times$  buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM MgCl<sub>2</sub>), and 20 pmol of forward and reverse primers as well as 0.5 unit of Ampli Taq Gold DNA polymerase (Applied Biosystems Japan). After initial denaturation for 5 min at 96°C, amplification was carried out in an automated thermal cycler (Applied Biosystems Japan Co.) for 40 cycles of 1 min at 96°C, 45 s at 57°C, and 45 s at 72°C with a final extension of 7 min at 72°C. Each PCR product was diluted 1:40 with water. The samples containing 1  $\mu$ l of the diluted PCR product, 10  $\mu$ l Hi-Di formamide (Applied Biosystems Japan Co.) and 0.1  $\mu$ l GeneScan-500LIZ size standard (Applied Biosystems Japan) were denatured for 3 min at 95°C, separated on capillary gels using an ABI PRISM 3730 automated sequencer, and the electrophoretic runs were analyzed with GeneMapper software (Applied Biosystems Japan).

#### Statistical analysis

Microsatellite allele frequency was calculated by direct counting. The strength of association was expressed by odds ratio (O.R), which was calculated from 2 $\times$ 2 contingency tables. Statistical significance was examined by the Fisher's double-sided exact test and the  $m\times n$  contingency table. Univariate analysis was performed to determine the association between microsatellite mismatch and aGVHD incidence. The *P* value, except when comparing a mismatch, was corrected by multiplying the number of microsatellite alleles (corrected *P*<sub>c</sub> value). The *P*<sub>c</sub> value less than 0.05 was accepted as statistically significant, and the *P*<sub>c</sub> value between 0.05 and 0.1 was indicative of a trend.

#### Definition of a microsatellite mismatch

Donor/recipient pairs were classified as matched or mismatched at each microsatellite marker locus. Pairs were defined as mismatched only when one or more recipient alleles are not shared by the corresponding donor (direction aGVHD).

#### Results

The overall genotyping results for paired transplantation donors and recipients

A total of 155 microsatellite markers spanning the long arm of chromosome 22 were used to genotype 70 pairs of transplantation patients and donors. Of the 70 transplanta-

tion recipients, 30 (42.8%) developed aGVHD with GVHD grade III in 20 patients and GVHD grade IV in 10 patients. The genotyping results obtained for the 70 transplantation recipients and the 70 transplantation donors were then analyzed and compared between the aGVHD-free group (grade 0 aGVHD) and the severe aGVHD group (group III to IV aGVHD).

Of the 155 markers, there were three markers with significant allele frequency differences between all donors and all recipients (D22S0052i-385; D22S0099i-412; D22S0115i-225; data not shown). As these three markers have different allele distribution between all donors and all recipients, they were considered to be inappropriate markers for the comparison between the aGVHD-free and the aGVHD group and were therefore excluded from further analysis. The remaining 152 markers were retained for further analysis in this study because they showed no significantly different allele distribution between all donors and recipients (data not shown).

#### Recipient age and GVHD prophylaxis

The recipient age was not significantly higher in the patients with aGVHD than the aGVHD-free group (*P*=0.27 Student's *t* test). In regard to GVHD prophylaxis (Table 1), there was no significant association (*p*>0.07) of aGVHD factor risk between the patients in the total body irradiation (TBI) group and those in the non-TBI group.

#### Comparison of allele frequency differences between the aGVHD-free and the aGVHD group for the microsatellite polymorphisms in donors and recipients

The frequency differences for the microsatellite alleles between the aGVHD-free group and the aGVHD group were compared separately for the donors and recipients. The significant association (*P*<0.05) of markers with the occurrence of aGVHD was found for five markers (Table 2), with a significant difference (*P* and *P*<sub>c</sub><0.05) for two donor markers (D22S283 and D22S0141i) and for three recipient markers (D22S0021i, D22S0199i, D22S0222i). The comparison of individual allele frequencies of the microsatellite markers in the grade 0 (aGVHD-free) and grade III+IV (aGVHD) groups revealed the presence of possible risk (R) alleles (O.R>1) and protective (P) alleles (O.R<1; Table 2). In the donors, the allele D22S0141i-431 was increased significantly (*P*<sub>c</sub>=0.049) and the allele D22S283-132 was decreased significantly (*P*<sub>c</sub>=0.008) in aGVHD when compared to the aGVHD-free group. Both of the marker loci were in position 22q12.3. In the recipients, the frequency of the allele D22S0021i-348 was significantly increased (*P*<sub>c</sub>=0.035) and three alleles (D22S0021i-357, D22S0199i-444,

**Table 2** Statistically significant alleles associated with aGVHD grade in patients and donors, respectively

Marker	Position	No. of alleles	Significant allele	aGVHD grade (N=40)	aGVHD grade III+IV (N=30)	Odds ratio (95% confidence interval)	Protective (P) or at risk (R)	P value	Pc
Donor									
D22S283	22q12.3	12	132	35 (87.5%)	15 (50.0%)	0.14 (0.04–46)	P	0.0007	0.008
D22S0141i	22q12.3	7	431	4 (10.0%)	14 (46.7%)	7.87 (2.24–27.7)	R	0.007	0.049
Recipient									
D22S0021i	22q13.2	5	348	21 (52.5%)	25 (83.2%)	4.52 (1.44–14.2)	R	0.007	0.035
			357	38 (90.0%)	18 (67.7%)	0.16 (0.06–0.48)	P	0.004	0.020
D22S0199i	22q13.2	4	444	22 (55.0%)	7 (23.3%)	0.25 (0.09–0.72)	P	0.007	0.028
D22S0222i	22q13.3	7	258	17 (42.5%)	4 (13.3%)	0.21 (0.06–0.71)	P	0.007	0.049

and D22S0222i-258) were significantly decreased in aGVHD when compared to the aGVHD-free group.

In regard to the D22S0021i locus, we found both a risk allele (D22S0021i-348) and a protective allele (D22S0021i-357). On the basis of a genotype analysis, there was a significant association ( $P=0.001$ ) between the D22S0021i genotype and aGVHD occurrence (Table 3).

Comparison of differences between the aGVHD-free and the aGVHD groups for microsatellite alleles that were matched or mismatched in donors and recipients

As a further comparison between the aGVHD-free and the aGVHD groups, we determined the significant differences between the number of alleles of the aGVHD-free and the aGVHD groups that were matched and mismatched for the donor and recipient pairs. We estimated that there were eight significant marker mismatches for an association with the occurrence of aGVHD (Table 4). Of these eight markers, three (D22S0267i, D22S0220i, and D22S683) were more often mismatched in the severe GVHD group ( $O.R>1$ ), and therefore, these markers appear to be protective against the occurrence of severe aGVHD. As D22S0220i and D22S683 are located in a relatively close position to each other on 22q12.3 where they are 780 kb apart, we selected an additional five markers between D22S0220i and D22S683. As a result, three markers (Z67524,  $P=0.09$ ,  $O.R=0.35$ ; D22S0132i,  $P=0.07$ ,  $O.R=2.54$ ; D22S0075i,  $P=0.07$ ,  $O.R=0.03$  in order from the centromere to telomere) showed a tendency of association with aGVHD (Fig. 1).

**Table 3** Univariate analysis of D22S0021i genotype

Allele genotype	aGVHD grade 0 (N=40)	aGVHD grade III+IV (N=30)	P value
348/348	2	10	0.001
348/357	19	15	
357/357	17	3	

On the other hand, five markers (D22S0152i,  $P=0.0005$ ; D22S0145i,  $P=0.017$ ; Z66750,  $P=0.014$ ; D22S0085i,  $P=0.035$ ; D22S0197i,  $P=0.005$ ) were more often mismatched in the aGVHD-free group ( $O.R<1$ ), suggesting that they are significant susceptibility markers for aGVHD. Of these markers, D22S0152i and D22S0145i were located in a relatively close position to each other on 22q11.23 where they were 960 kb apart. We, therefore, genotyped an additional six markers (D22S0068i, D22S0186i, D22S0163i, D22S0169i, D22S0184i, and D22S1174) but found that none of them were significantly associated with aGVHD (data not shown).

Candidate genes within the aGVHD susceptibility regions

Table 5 lists the candidate susceptibility genes that are located within or near to the genomic susceptibility region which was identified by microsatellite genotyping. These genes are in the close vicinity of the significant microsatellite markers that were found within intron 3 of CACNG2, intron 3 of PEX26, intron 4 of KIAA0376, intron 7 of LARGE, and intron 8 of TOM1. Other genes, such as MYH9, EP300, TCF20, ARSA, FLJ31568, EMID1, APOL3, and FLJ44385, are located within 10 kb to 172 kb of the significantly associated microsatellite markers.

Genomic map of the association of microsatellite polymorphisms on 22q12.3 with the occurrence of aGVHD

The  $P$  values for comparing the matching of microsatellite marker alleles between those of the aGVHD-free group and the aGVHD group were determined and plotted as a  $P$  value plot against the physical location of the microsatellite markers and the known genes on 22q12.3. Figure 1 shows a  $P$  value plot and the gene map of one of the aGVHD susceptibility regions determined by the association analysis using the microsatellite markers from D22S0220i to D22S683 and beyond the border of 22q12.3 and 22q13.1. The figure shows that the genes TOM1, HMOX1, and

**Table 4** Correlation between matched mismatch donor–recipient pairs and aGVHD grade for each of the significant microsatellite markers on chromosome 22

Marker	Position	aGVHD grade 0		aGVHD grade III+IV		Odds ratio (95% CI)	Protective (P) or at risk (R)	P value
		Matched	Mismatched	Matched	Mismatched			
D22S0267i	22q11.21	38	2	23	7	5.78 (1.10–30.24)	P	0.028
D22S0152i	22q11.23	25	15	29	1	0.05 (0.01–0.41)	R	0.0005
D22S0145i	22q11.23	8	32	14	16	0.29 (0.10–0.82)	R	0.017
Z66750	22q12.1	28	12	28	2	0.17 (0.03–0.81)	R	0.014
D22S0085i	22q12.3	6	34	11	19	0.30 (0.10–0.96)	R	0.035
D22S0220i	22q12.3	17	23	4	26	4.80 (1.41–16.35)	P	0.008
D22S683	22q12.3	6	34	0	30	11.86 (0.64–219.35)	P	0.027
D22S0197i	22q13.33	16	24	22	8	0.24 (0.087–0.67)	R	0.005

MCM5 are in the region of the most significant *P* values and in close vicinity to the protective microsatellite marker D22S0220i.

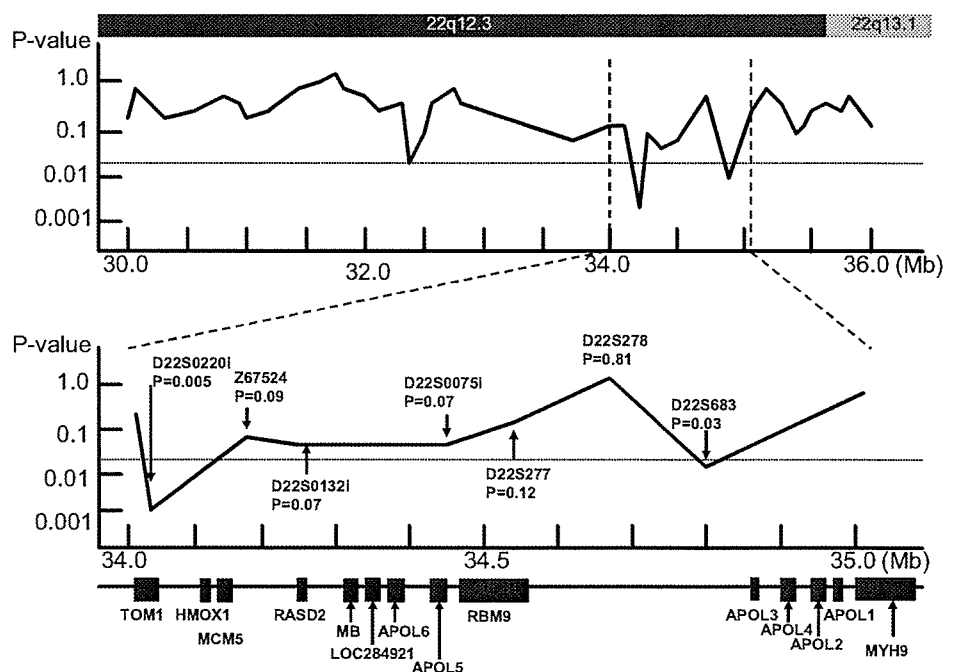
## Discussion

Of the 155 markers analyzed for differences between the aGVHD-free group and the aGVHD group and separately for the recipients and donors, only five markers on chr 22 (Table 2) were found to be significantly associated with aGVHD ( $P < 0.05$ ). Interestingly, of these five positive markers, the donor positive marker D22S283 was previously reported to be associated with schizophrenia (DeLisi et al. 2002), Sorsby's fundus dystrophy (Assink et al. 2000), and CDAGS (Mendoza-Londono et al. 2005).

Although the susceptibility genes on chr 22 for those diseases are still unknown, the positive microsatellite marker D22S283 is located within the SC2D4 schizophrenia susceptibility locus (NCBI GeneID 6379) and could be associated with neuropsychological impairment that may evolve with aGVHD (Sostak et al. 2003).

The other four positive markers, D22S0141i, D22S0021i, D22S0199i, and D22S0222i, which were associated with aGVHD (Table 2) had not been previously associated with any human disease. However, these markers are located in a region of human chr 22 that was previously associated with the presence of strong mucosal and T-cell immune response against HIV-1 (Kanari et al. 2005) and, therefore, that could also affect the aGVHD. The p300 gene, which is a transcriptional factor located 10 kb from D22S0021i, is believed to participate in the activities of hundreds of

**Fig. 1** aGVHD susceptibility gene mapping by association analysis using microsatellite markers on 22q12.3. *P* value (*y*-axis) was plotted against physical location of the microsatellite markers on 22q12.3 (*x*-axis), their distance (in Mb) in order from the centromere to the telomere. All markers were plotted according to their genetic map position taken from JBIRC database (<http://jbirc.jbic.or.jp/gdbs/>). The gene map at the bottom of the figure shows the representative genes that are indicated by black boxes on the locus near the two positive markers D22S0220i and D22S683 on 22q12.3. The dotted horizontal line shows the threshold for 5% significance



**Table 5** Candidate genes close to the positive microsatellite markers

Marker	Position	Distance	Neighboring gene	Description	Function
Donor allele					
D22S283	22q12.3	172 kb	MYH9	Myosin, heavy chain 9, nonmuscle	Cytoskelton
D22S0141i	22q12.3	Intron 3	CACNG2	Calcium channel, voltage dependent, gamma-2 subunit	Cell signaling
Recipient allele					
D22S0021i	22q13.2	10 kb	EP300	E1A binding protein, 300 KD	Transcription
D22S0199i	22q13.2	40 kb	TCF20	Transcription factor 20	Transcription
D22S0222i	22q13.33	45 kb	ARSA	Arylsulfatase A	Enzyme
Mismatching allele					
D22S0267i	22q11.21	Intron 3	PEX26	Peroxisome biogenesis factor 26	Protein degradation
D22S0152i	22q11.23	20 kb	FLJ31568	Hypothetical protein	Unknown
D22S0145i	22q11.23	Intron 4	KIAA0376	Hypothetical protein	Unknown
Z66750	22q12.1	10 kb	EMID1	BMI domain containing protein 1	Unknown
D22S0085i	22q12.3	Intron 7	LARGE	Acetyl glucosaminyltransferase-like protein	Unknown
D22S0220i	22q12.3	Intron 8	TOM1	Target of myb 1	Immuno regulation
D22S683	22q12.3	20 kb	APOL3	Apolipoprotein L-III	Lipid metabolism
D22S0197i	22q13.33	10 kb	FLJ44385	Hypothetical protein	Unknown

different genes (Vo and Goodman 2001). The p300 protein, together with the adenovirus serotype 5 E1A, has been reported to regulate the NKG2D ligand, NK cell lysis, and tumor rejection (Routes et al. 2005). In addition, p300-binding domains are known to interact with STAT1, 2, and 3, which play an important role in cytokine signal transduction (Pfitzner et al. 2004). Therefore, the p300 gene might be associated haplotypically with the D22S0021i marker, which has both a protective and risk allele for aGVHD (Table 3). These facts, together with our association results, strongly suggest that the loci at position 22q12–13 could affect the development of aGVHD. Whereas three of these positive markers are located 10 kb to 45 kb from any of the known genes, the positive microsatellite marker D22S0141i is located within intron 3 of the CACNG2 gene that encodes the calcium channel, voltage dependent, gamma-2 subunit (Table 5). This protein appears to interact with neural proteins (Black and Lennon 1999; Chen et al. 2000), and it might have a role in neurological complications arising from aGVHD (Sostak et al. 2003).

We found eight microsatellite markers that were significantly different between the aGVHD-free group and the aGVHD group when matched or mismatched between the recipient and donor groups. Three of the eight markers, D22S0267i, D22S0220i, and D22S683, are considered to be protective because they were more often mismatched in the severe aGVHD group (OR > 1), suggesting the existence of one or more protective candidate genes in close vicinity. Two of these markers, D22S0220i and D22S683, were approximately 780 kb apart (D22S0085i and D22S0220i) with another three markers (Z667524, D22S0132i, and D22S0075i) located between them that showed a positive trend ( $P < 0.1$ ) of association (Fig. 1). Interestingly, Gubarev

et al. (1996) reported the localization of a gene encoding mHa on 22q12.3 in close vicinity to our significant markers by using T-cell clone and linkage-analysis. This report, which used different methods from our genome-wide approach, therefore strongly supports our results.

The highly significant protective microsatellite marker D22S0220i is located within intron 8 of the gene TOM1. The specific function of this gene has not yet been determined, but Tom1 may be a negative regulator of interleukin-1 and tumor necrosis factor-induced signaling pathways (Yamakami and Yokosawa 2004), and, therefore, affect aGVHD. D22S0220i is also located near to the HMOX1 gene (NCBI Gene ID 3162) that encodes the heme oxygenase (decycling) 1 protein. This association is biologically significant because HMOX1 (alias HO-1) is known to be a protective protein with anti-inflammatory and antiapoptotic properties (Willis et al. 1996; Brouard et al. 2002). Moreover, induction of HMOX1 in recipient mice of a BMT model resulted in a reduction in aGVHD and improved survival (Gerbitz et al. 2004). Therefore, HMOX1 is an excellent protective candidate gene for further aGVHD association studies specifically at the level of gene SNP analysis.

Another potential protective microsatellite marker D22S683 is located ~172.2 kb from the MYH9 gene (MIM 160775) and the Epstein syndrome locus (MIM 153650). The MYH9 mutations are known to result in the autosomal dominant giant-platelet disorders such as the May–Hegglin anomaly, the Fechtner syndrome, and the Sebastian syndrome (Seri et al. 2000). In addition, the MYH9 or the motor protein non-muscle heavy chain II A has been associated with the chemokine receptor CXCR4 in the T cell (Rey et al. 2002) and with the modulation of T cell motility (Jacobelli et al.

2004). Considering that one of the alleles of the microsatellite marker D22S283 is located within 172.2 kb of the MYH9 gene of the transplantation donors that were positively associated with aGVHD, then it can be envisaged that a neighboring SNP may affect the donor T cell behavior in a protective role against the occurrence and/or maintenance of aGVHD.

The five ‘disease-negative’ markers shown in Table 4 were associated with a risk of aGVHD because they were more often mismatched in aGVHD grade 0 group ( $OR < 1$ ) than the aGVHD group. This result seems to be paradoxical when considering the concept of a minor antigen mismatch, but it suggests that some gene products might need to be mismatched to prevent the development of disease. For example, it has been reported that the killer cell immunoglobulin-like receptor ligand (KIR-ligand), when mismatched between the donor and recipient, is associated with improved survival after stem cell transplantation for acute myeloid leukemia (Ruggeri et al. 2002). In this regard, the product of an unknown gene located near the ‘disease-negative’ microsatellite markers, when mismatched between donor and recipient, might help to prevent the development of aGVHD in a way that is analogous to the unique KIR-ligand mismatch involved with the NK-KIR biological system in response to transplantation (Malmberg et al. 2005).

To identify the candidate genes that are located within close vicinity to the significant microsatellite markers, we searched the human genome sequence deposited at NCBI for locations and annotations of genes in both directions of the microsatellite markers (Table 5). Interestingly, many of the genes that we identified near the associated markers, such as MYH9, CACNG2, EMID1, LARGE, and TCF20, have proximal STAT1- and STAT2-binding sites. Many DNA binding sites for STAT1 and STAT2 have been identified distributed across chr 22 in interferon-treated cells (Hartman et al. 2005). The STAT family proteins mediate transcriptional responses to many cytokines and are a useful system for studying inducible gene regulation. In addition, APOL3, EMID1, and LARGE exhibit IFN-sensitive expression changes. Considering the complex roles of cytokines, such as IFN, in the aGVHD occurring phase after BMT, the cytokine inducible candidate genes may play an important role in aGVHD.

The results of our study are largely dependant upon the hypothesis that microsatellite markers in LD will reveal an association between polymorphisms and the functional risk conferred by the variants or relevant genes so that certain marker alleles will be over represented in the aGVHD donors or patients compared with the GVHD-free donors or recipients (Ohashi and Tokunaga 2003; Zapata et al. 2001). In this study, we used 155 microsatellite markers whose spacing average was about 200 kb on the basis of the

knowledge accumulated from a large number of recent data that the average length of LD between disease susceptible SNPs and nearby microsatellite alleles is  $\geq 100$  kb (Abecasis et al. 2001; Keicho et al. 2000; Oka et al. 1999; Ota et al. 1999). Although the LD pattern is variable between different regions of human genome depending on several factors such as allele frequency, mutation and recombination, and ethnic population, the 200 kb interval between markers is likely to be of sufficient distance for LD coverage of chr 22 in this study.

The multiple testing issues and the restricted sample size of our study limit the statistical power to find conclusive evidence of association particularly in the case of susceptibility genes with minor effects. It is statistically possible that at a probability level of less than 0.05 that 1 in 20 of our markers will represent false positives. We have analyzed 155 different microsatellite markers for association with aGVHD, and therefore, we could expect about eight false positive markers distributed randomly across the 40 Mb of the long arm of chr 22. Of the 13 microsatellite markers that were significantly different between the GVHD-free group and the GVHD severe group, the location of three of the markers, D22S0220i, D22S683, and D22S283, were relatively close to each other, which increases the probability that they represent a true association. Moreover, this GVHD susceptibility locus, from D22S0220i and D22S283, spans approximately 1 MB of genomic sequence and contains at least 14 candidate genes, including TOM1 and HMOX1 and MYH9, near the APOL1 to APOL6 gene cluster (Fig. 1).

In conclusion, we used 155 microsatellite markers distributed across the long arm of chr 22 and the ‘genome-wide approach’ in this genetic association study of aGVHD to identify and map potential aGVHD susceptibility and resistant regions on the basis of a small number of significant markers. It now remains to use the ‘candidate gene approach’ and investigate the SNPs and haplotypes of the candidate genes, such as TOM1, HMOX, MCM5, and MYH9, which are located closely to the most significant microsatellite markers.

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## ORIGINAL ARTICLE

# Significance of additional high-dose cytarabine in combination with cyclophosphamide plus total body irradiation regimen for allogeneic stem cell transplantation

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**The combination of cyclophosphamide (CY) and total body irradiation (TBI) has been used as a standard conditioning regimen for allogeneic transplantation. Several studies showed an advantage of adding high-dose cytarabine (HDCA) to this regimen. To clarify the significance of additional HDCA, we conducted a retrospective multicenter study and compared the clinical results of these two regimens. From June 1985 to March 2003, 219 patients with hematological malignancies underwent allogeneic transplantation after conditioning with CY + TBI 12Gy ( $n = 73$ ) or CA + CY + TBI 12Gy ( $n = 146$ ). Engraftment, overall survival, transplant-related mortality (TRM), relapse rate and incidence of graft-versus-host disease (GVHD) were compared according to risks and donors. Addition of HDCA had no impact on the relapse rate in all subgroups, and it was associated with lower TRM among standard-risk patients after related transplantation, and with higher TRM and worse survival among standard-risk patients after unrelated transplantation. The incidence of acute GVHD was not significantly different between the two regimens, and HDCA resulted in a higher incidence of chronic GVHD among standard-risk patients after related transplantation. In summary, addition of HDCA is not beneficial for high-risk patients, and is not recommended for standard-risk patients receiving unrelated transplantation.**

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**Keywords:** cytarabine; cyclophosphamide; conditioning; allogeneic transplantation; anti-leukemic activity

## Introduction

For allogeneic stem cell transplantation, the conditioning regimen is one of the most important factors. The combination of cyclophosphamide (CY) and total body irradiation (TBI) has been used as a standard conditioning regimen for myeloablative hematopoietic stem cell transplantation.<sup>1–4</sup> Intensification of the conditioning regimen using high-dose cytarabine (HDCA) has been investigated as possibly reducing disease relapse in hematological malignancies. Some studies are encouraging additional HDCA,<sup>5–11</sup> whereas others are reporting more toxicity using HDCA particularly on the heart and lung.<sup>12–16</sup> Our previous preliminary report did not show any significant differences between CY + TBI and CA + CY + TBI in a small cohort.<sup>17</sup>

To clarify the significance of additional HDCA, we conducted a retrospective multicenter study of 219 patients, and compared the clinical results of these two regimens. We confirmed that addition of HDCA neither did improve overall survival, nor reduce the relapse rate.

## Patients and methods

### *Patients, conditioning regimen and GVHD prophylaxis*

From June 1985 to March 2003, a total of 219 patients with various hematological malignancies from 13 institutes

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underwent allogeneic stem cell transplantation after conditioning with either CY + TBI ( $n = 73$ ) or CA + CY + TBI ( $n = 146$ ). CY was given at a dose of 60 mg/kg once daily intravenously (i.v.) on days -5 and -4 (total dose 120 mg/kg), CA at a dose of 2 g/m<sup>2</sup> twice daily i.v. over 3 h on day -6 and 2 g/m<sup>2</sup> once daily i.v. over 3 h on days -5 and -4 (total dose 8 g/m<sup>2</sup>) and TBI at a dose of 300 cGy fractions twice daily on days -2 and -1 (total dose 12 Gy). Seven institutions used only one regimen, either CY + TBI or CA + CY + TBI. The other six institutions used both regimens at the same time. There were no consistent indications for either regimen in any institution. Donors were HLA-fully-matched related donors or HLA-fully-matched unrelated donors. GVHD prophylaxis consisted of either cyclosporine (CsA) and short-term methotrexate (sMTX) or tacrolimus (FK) and sMTX.

**Statistical analysis**

Engraftment, overall survival, transplant-related mortality (TRM), relapse rate and incidence of graft-versus-host disease (GVHD) were compared between the two regimens in each subgroup, which was defined according to risk (standard or high) and donor (related or unrelated). TRM was defined as mortality owing to any cause other than relapse or disease progression. Standard-risk patients are defined as those with acute myeloblastic leukemia (AML) or acute lymphoblastic leukemia (ALL) in first complete remission, chronic myelogenous leukemia (CML) in first chronic phase, or myelodysplastic syndromes (MDS) as refractory anemia (RA) or refractory anemia with ringed sideroblasts (RARS). High-risk patients were those with AML or ALL in subsequent complete remission, in relapse or of induction failure, Philadelphia-chromosome-positive ALL, CML in subsequent chronic phase, accelerated phase or blastic phase or MDS as RAEB or overt leukemia with MDS. The  $\chi^2$  test and Fisher's exact test were used for comparison of the two groups. Overall survival was calculated using the Kaplan-Meier method and *P*-values were calculated using the log-rank test. Cumulative incidence curves for TRM and relapse, with or without death, were constructed, reflecting time to relapse and time to TRM as competing risks. *P*-values were calculated at the fixed point in time as reported by Klein *et al.*<sup>18</sup> Univariate and multivariate analyses were performed using the Cox proportional hazard regression model, and variables were selected using stepwise method. A two-sided *P*-value of less than 0.05 was considered significant. Data were analyzed as of March 2003.

**Results**

*Patient characteristics*

Patient characteristics of each subgroup are summarized in Table 1. One hundred and twenty-seven patients received transplantation from a related donor whereas 92 received from an unrelated donor. GVHD prophylaxis consisted of CsA + sMTX in 182 patients and FK + sMTX in 37 patients. FK was used in one patient after related transplantation in 1999, and in 36 patients after unrelated

**Table 1** Patient characteristics

Risk	Donor	Standard						High				
		Related			Unrelated			Related		Unrelated		
		CY + TBI(19)	CA + CY + TBI(71)	CY + TBI(24)	CA + CY + TBI(40)	CY + TBI(14)	CA + CY + TBI(23)	CY + TBI(16)	CA + CY + TBI(12)			
Conditioning												
Median age (range)	29 (20-50)	33 (16-53)	33 (18-54)	31 (17-50)	39 (24-51)	9 (16-44)	27 (15-48)	31 (16-50)				
Sex, F/M	6/13	25/46	8/16	15/25	3/11	8/15	3/13	6/6				
Diagnosis												
AML	5	26	2	4	5	6	3	3				
ALL	8	18	4	16	3	10	9	4				
CML	4	26	15	19	4	5	1	5				
MDS	2	1	3	1	2	2	3	0				
<i>P</i> -value <sup>a</sup>		0.25		0.09		0.31		0.41				
GVHD prophylaxis												
CsA + sMTX	18	71	10	26	14	23	11	9				
FK + sMTX	1	0	14	14	0	0	5	3				
<i>P</i> -value		0.48		0.12		—		1.0				

Abbreviations: ALL = acute lymphoblastic leukemia; AML = acute myeloblastic leukemia; CA = cytarabine; CML = chronic myelogenous leukemia; CY = cyclophosphamide; CsA = cyclosporine; FK = tacrolimus; MDS = myelodysplastic syndromes; sMTX = short-term methotrexate; TBI = total body irradiation.  
<sup>a</sup>Myeloid malignancy vs lymphoid malignancy.

transplantation since 1996. All stem cell sources were from bone marrow except for three patients who received peripheral blood stem cell transplantation from a related donor. Diagnosis and GVHD prophylaxis did not differ significantly between conditioning regimens in each subgroup. The median follow-up period of survivors was 979 days (range 31–4704 days).

**Engraftment**

All evaluable patients achieved sustained engraftment (an absolute neutrophil count of  $>0.5 \times 10^9/l$  for three consecutive days) in both regimens.

**Overall survival**

Overall survival did not differ significantly in any patient between the two regimens (58 vs 56% at 3 years,  $P=0.90$ ) (Figure 1a). Addition of HDCA resulted in significantly worse survival among standard-risk patients after unrelated transplantation (45 vs 81% at 3 years,  $P=0.02$ ) (Figure 1b), whereas it resulted in comparable survival among standard-risk patients after related transplantation (80 vs 60% at 3 years,  $P=0.27$ ).

No significant differences were observed among high-risk patients (40 vs 40% at 3 years,  $P=0.48$  among patients

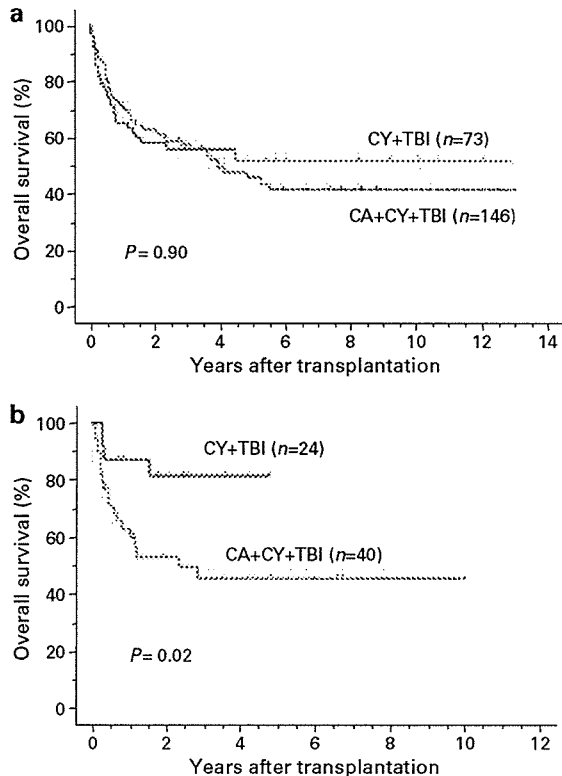
after related transplantation; and 11 vs 28% at 3 years,  $P=0.93$  among patients after unrelated transplantation).

**TRM and hazard analysis for TRM**

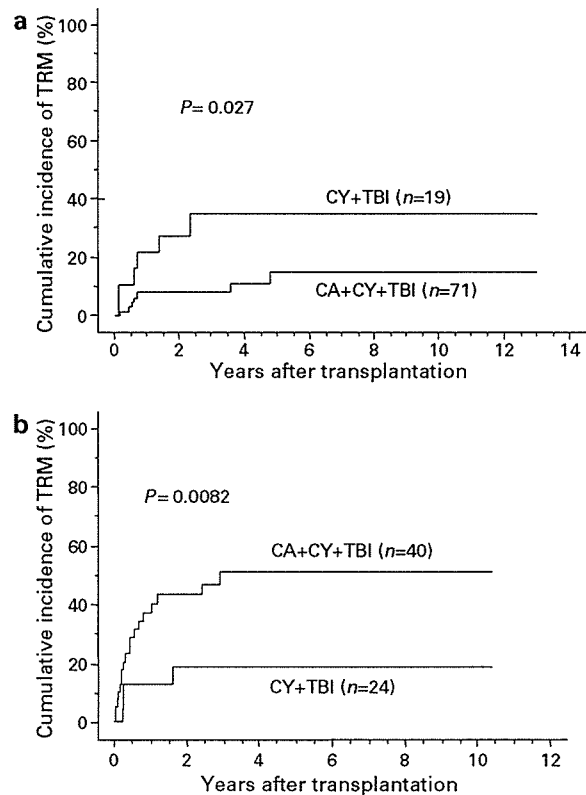
TRM did not differ significantly in any patient between the two regimens (28 vs 32% at 3 years,  $P=0.56$ ). Addition of HDCA was associated with significantly lower TRM among standard-risk patients after related transplantation (7.8 vs 35% at 3 years,  $P=0.027$ ) (Figure 2a), whereas it resulted in higher TRM among standard-risk patients after unrelated transplantation (51 vs 19% at 3 years,  $P=0.0082$ ) (Figure 2b).

No significant differences were observed among high-risk patients (22 vs 16% at 3 years,  $P=0.65$  among patients after related transplantation; and 69 vs 58% at 3 years,  $P=0.64$  among patients after unrelated transplantation).

Univariate analysis among standard-risk patients after related transplantation showed that addition of HDCA, female patients, age over 40 and GVHD prophylaxis with CsA + sMTX were significant factors affecting TRM. Addition of HDCA remained a significant factor on multivariate analysis (relative risk = 0.18; confidence interval, 0.052–0.63) (Table 2a). Univariate analysis among standard-risk patients, after unrelated transplantation, showed that addition of HDCA and GVHD prophylaxis



**Figure 1** Overall survival. (a) No significant differences were observed between CA + CY + TBI and CY + TBI ( $P=0.90$ ) in all patients. (b) CA + CY + TBI resulted in significantly worse survival than CY + TBI among patients who received transplantation from unrelated donors ( $P=0.02$ ).



**Figure 2** Cumulative incidence of TRM in patients with standard-risk disease. (a) CA + CY + TBI resulted in significantly lower TRM than CY + TBI among patients who received transplantation from related donors ( $P=0.027$ ). (b) CA + CY + TBI resulted in significantly higher TRM than CY + TBI among patients who received transplantation from unrelated donors ( $P=0.0082$ ).

**Table 2** Prognostic factors affecting TRM

Variables	Unfavorable factors	Univariate		Multivariate <sup>a</sup>	
		Hazard ratio (CI)	P-value	Hazard ratio (CI)	P-value
<i>(a) Related standard risk</i>					
Conditioning	CA + CY	0.32 (0.11–0.94)	0.038	0.18 (0.052–0.63)	0.0070
Sex	Female	3.3 (1.1–10)	0.039	7.0 (2.0–25)	0.0030
Female to male	Yes	0.95 (0.29–3.1)	0.94		
Disease	Other than CML (CP)	1.4 (0.44–4.7)	0.55		
Age	> 40	3.8 (1.2–12)	0.020	8.4 (2.4–30)	0.0010
GVHD prophylaxis	CsA + sMTX	0.12 (0.016–0.98)	0.047	0.53 (0.051–5.5)	0.59
Transplant year	~1996	1.9 (0.50–7.0)	0.35		
<i>(b) Unrelated standard risk</i>					
Conditioning	CA + CY	3.2 (1.1–9.3)	0.038	2.7 (0.90–8.1)	0.078
Sex	Female	0.70 (0.27–1.8)	0.45		
Female to male	Yes	0.74 (0.22–2.5)	0.63		
Disease	Other than CML (CP)	1.1 (0.46–2.5)	0.88		
Age	> 40	1.1 (0.41–2.7)	0.93		
GVHD prophylaxis	CsA + sMTX	2.6 (1.0–6.6)	0.048	2.2 (0.84–5.6)	0.11
Transplant year	~1996	0.88 (0.29–2.6)	0.82		

Abbreviations: CA = cytarabine; CI = confidence interval; CML = chronic myelogenous leukemia; CsA = cyclosporine; CY = cyclophosphamide; sMTX = methotrexate.

<sup>a</sup>Final model.

of CsA + sMTX were significant factors influencing TRM. On multivariate analysis, addition of HDCA was associated with a trend for increased TRM (relative risk = 2.7; CI, 0.90–8.1) (Table 2b).

#### Relapse rate

Relapse rate did not differ between the two regimens (20 vs 13% at 3 years,  $P=0.23$ ). Addition of HDCA was not associated with any significant differences as to relapse rate in any subgroups (18 vs 5.6% at 3 years,  $P=0.085$  among standard-risk patients after related transplantation; 2.8 vs 0% at 3 years,  $P=0.31$  among standard-risk patients after unrelated transplantation; 51 vs 47% at 3 years,  $P=0.81$  among high-risk patients after related transplantation; and 17 vs 13% at 3 years,  $P=0.81$  among high-risk patients after unrelated transplantation).

#### Graft-versus-host disease

Results are summarized in Table 3. The incidence of grade II–IV acute GVHD did not differ between the two regimens in any subgroup. Addition of HDCA was associated with a significantly higher incidence of chronic limited and extensive GVHD among standard-risk patients after related transplantation (40/69 vs 5/19,  $P=0.029$ ).

#### Discussion

We examined a total of 219 patients, which is the largest series in the literature. Aurer and Gale<sup>19</sup> reviewed modified conditioning regimens in 1991, and failed to detect any major improvements in the overall survival with any of the new regimen. Although intensification of the conditioning regimen with HDCA is one of the approaches designed to improve outcome, particularly for high-risk hematological malignancies,<sup>20–24</sup> our retrospective analysis did not show

**Table 3** Incidence of acute and chronic GVHD

Risk	Standard		High	
	Related	Unrelated	Related	Unrelated
<i>Donor</i>				
<i>Acute GVHD (II–IV)</i>				
CY + TBI	6/19	6/24	3/11	8/14
CA + CY + TBI	9/71	11/40	6/22	6/11
P-value	0.11	1.0	1.0	0.78
<i>Chronic GVHD</i>				
CY + TBI	5/19	11/21	7/10	3/6
CA + CY + TBI	40/69	15/34	7/19	6/8
P-value	0.029	0.75	0.13	0.58

Abbreviations: CA = cytarabine; CY = cyclophosphamide; GVHD = graft-versus-host disease; TBI = total body irradiation.

any improvement in overall survival in any subgroups. In addition, no significant reduction in relapse rate was observed in any subgroups, suggesting that anti-leukemic activity may not be intensified by HDCA.

Many of the previous studies reported the superior anti-leukemic activity of HDCA for high-risk disease. Champlin *et al.*,<sup>9</sup> for example, showed that HDCA had good anti-leukemic activity before transplantation. Riddell *et al.*<sup>21</sup> reported a low relapse rate of 14% with the higher dose of CA (36 g/m<sup>2</sup>), but an accurate relapse rate could not be fully evaluated because the day 100 TRM was as high as 50%. Mineishi *et al.*<sup>22</sup> reported a lower relapse rate of 11% after related transplantation compared to the 51% in our study. However, of 55 patients, 18 patients with AML/ALL with cytogenetic abnormalities in first remission were classified as high risk in their study. The difference in the definition of high-risk patients may be one reason for the lower relapse rate. In addition, the higher dose of CA (18 g/m<sup>2</sup>) in their study may explain the lower relapse rate. Jillella *et al.*<sup>10</sup> also reported a similar outcome, but almost three-quarters of the patients had standard-risk disease. Woods

*et al.*<sup>6</sup> and Minami *et al.*<sup>17</sup> demonstrated a high relapse rate of 50–75% even with HDCA after related transplantation for high-risk disease. The dose effect of HDCA on anti-leukemic activity should be explored, but it may be offset by the increased toxicity reported in many earlier studies.

Interestingly, however, addition of HDCA was associated with lower TRM among standard-risk patients after related transplantation, and with higher TRM among standard-risk patients after unrelated transplantation. Thus, we performed multivariate analyses to clarify the factors affecting TRM, and confirmed that addition of HDCA still remained as a prognostic factor. Although the effects of the differences in unevaluable factors, such as supportive care, in each institute cannot be fully excluded, additional HDCA may play a role in the reduction of TRM after related transplantation. In contrast, a trend for increased TRM with HDCA after unrelated transplantation is reasonable. TRM is reported to be higher after unrelated than after related transplantation,<sup>25,26</sup> and intensification of the conditioning regimen increases TRM after unrelated transplantation.<sup>27</sup>

Intensity of conditioning is reported to modify the incidence of both acute and chronic GVHD,<sup>28</sup> but its effect on chronic GVHD is controversial.<sup>29</sup> Addition of HDCA was further associated with a significant increase in chronic GVHD among patients with standard-risk disease after related transplantation, but it was not associated with acute GVHD. Thus, other factors such as management of immunosuppression may also have affected the incidence of chronic GVHD in our series.

In summary, addition of HDCA is not beneficial for patients with high-risk disease. It is not recommended for patients with standard-risk disease who will receive transplantation from unrelated donors because of increased TRM and decreased survival. It may be beneficial for patients with standard-risk disease who will receive transplantation from a related donor. Although the number of patients in this subgroup is somewhat small, such differences could not have emerged without underlying facts. Therefore, further studies are warranted to verify our results in this subgroup.

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