付表：解析結果

症例数	全体	中国	日本	パキスタン	フィリピン	台湾
HLA 一致血縁者	810	203	564	7	1	35
1 座不一致血縁	120	16	104	0	0	0
2 座不一致血縁	281	78	201	0	0	2
その他の血縁	76	36	39	1	0	0
syngeneic	12	5	0	0	0	7
非血縁	2155	158	1968	0	2	27

II-IV （%）	全体	中国	日本	パキスタン	フィリピン	台湾
HLA 一致血縁者	29.0	15.8	33.2	14.3	0.0	42.9
1 座不一致血縁	36.7	18.8	39.4	–	–	–
2 座不一致血縁	30.2	25.6	31.3	–	–	100.0
その他の血縁	28.9	22.2	35.9	0.0	–	–
非血縁	35.2	23.4	36.1	–	0.0	40.7

III-IV （%）	全体	中国	日本	パキスタン	フィリピン	台湾
HLA 一致血縁者	10.0	6.9	10.8	0.0	0.0	17.1
1 座不一致血縁	20.0	18.8	20.2	–	–	–
2 座不一致血縁	16.0	11.5	16.9	–	–	100.0
その他の血縁	9.2	11.1	7.7	0.0	–	–
非血縁	12.0	10.1	12.2	–	0.0	7.4

別紙４ 研究成果の刊行に関する一覧表

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Harkensee C, Morishima Y, et al.	Single nucleotide polymorpisms and outcome risk in unrelated mismatched hematopoietic stem cell transplantation.	Blood	119 (26)	6365-72	2012
Atsuta Y, Morishima Y, Takanashi M, et al.	Comparison of unrelated cord blood transplantation and HLA mismatched unrelated bone marrow transplantation for adults with leukemia.	Biol. Blood Marrow Transplant	18 (5)	780-7.	2012
Espinoza JL, Takami A, et al.	Human microRNA-1245 down-regulates the NKG2D receptor in natural killer cells and impairs NKG2D-mediated functions.	Haematologica	97 (9)	1295-303.	2012
Espinoza JL, Takami A, et al.	Recipient PTPN22 -1123 C/C Genotype Predicts Acute Graft-versus-Host Disease after HLA Fully Matched Unrelated Bone Marrow Transplantation for Hematologic Malignancies.	Biol. Blood Marrow Transplant.	19 (2)	240-6.	2013

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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Comparison of Unrelated Cord Blood Transplantation and HLA-Mismatched Unrelated Bone Marrow Transplantation for Adults with Leukemia

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Recent advances in unrelated cord blood transplantation (UCBT) and high-resolution typing of human leukocyte antigen (HLA) from an unrelated donor have increased choices in alternative donor/stem cell source selection. We assessed HLA-mismatched locus-specific comparison of the outcomes of 351 single-unit UCB and 1,028 unrelated bone marrow (UBM) adult recipients 16 years old or older at the time of transplantation who received first stem cell transplantation with myeloablative conditioning for acute leukemia or myelodysplastic syndromes. With adjusted analyses, HLA 0 to 2 mismatched UCBT showed similar overall mortality (relative risk [RR] = 0.85, 95% confidence interval [CI], 0.68-1.06; $P = .149$) compared with that of single-HLA-DRB1-mismatched UBMT. UCBT showed inferior neutrophil recovery (RR = 0.50, 95% CI, 0.42-0.60; $P < .001$), lower risk of acute graft-versus-host disease (RR = 0.55, 95% CI, 0.42-0.72; $P < .001$), and lower risk of transplantation-related mortality (RR = 0.68, 95% CI, 0.50-0.92; $P = .011$) compared with single-HLA-DRB1-mismatched UBMT. No significant difference was observed for risk of relapse (RR = 1.28, 95% CI, 0.93-1.76; $P = .125$). HLA 0 to 2 antigen-mismatched UCBT is a reasonable second alternative donor/stem cell source with a survival outcome similar to that of single-HLA-DRB1-mismatched or other 7 of 8 UBMT.

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KEY WORDS: Unrelated cord blood transplantation, HLA-mismatched unrelated bone marrow transplantation

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INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) is a widely used, curative treatment for hematologic malignancies. When available, a human leukocyte antigen (HLA)-identical sibling is the donor of choice. However, only about 30% of candidates eligible for allogeneic HSCT will have such a donor. In addition, older patients with older siblings have more difficulty finding such a donor capable of stem cell donation. High-resolution donor-recipient HLA matching has contributed to the success of unrelated donor marrow transplantation, and the current first recommended alternative donor after an HLA-matched sibling for HSCT is an HLA-A, -B, -C, and -DRB1 8 of 8-allele-matched unrelated donor [1-4]. However, there are still a significant number of patients for which finding an HLA 8 of 8-matched unrelated donor is difficult and for whom a second alternative donor/stem cell source should be found.

The effect of HLA mismatches after bone marrow transplantation from unrelated donors (UBMT) has been well studied, and single mismatched UBM donors are usually selected as a second alternative donor/stem cell source [1-4]. Lee al. [3] showed that a single mismatch, antigen-level, or high-resolution, at HLA-A, -B, -C, or -DRB1 loci was associated with higher mortality and decreased survival. However, the reduction in survival may be acceptable in comparison with the survival rates for currently available alternative treatments. Analyses from the Japan Marrow Donor Program (JMDP) showed better survival in HLA class II mismatched recipients; thus, single-DRB1-mismatched UBM donor is currently a second alternative in Japan [1,2,5].

Recent advances in unrelated cord blood transplantation (UCBT) have provided patients with increased choices for a second alternative donor/stem cell source [6]. Clinical comparison studies of cord blood transplantation and HLA-A, -B, and -DRB1 6 of 6 allele-matched bone marrow transplantation for leukemia from unrelated donors in adult recipients showed comparable results [7-9]. More recently, promising outcomes of UCBT were shown compared with HLA-A, -B, -C, and -DRB1 8 of 8 allele-matched UBMT, the current first alternative donor/stem cell source [10-12].

The aim of this study was to determine the utility of UCBT as a second-alternative donor source in adult patients with acute leukemia or myelodysplastic syndromes. It is common today to perform high-resolution typing of HLA for donor selection of unrelated donors; thus, we performed mismatched-allele-specific analyses for comparison of HLA-mismatched UBMT and UCBT in terms of overall survival (OS) and other HSCT outcomes, setting single-DRB1-mismatched UBMT, the current second alternative, as the reference.

PATIENTS AND METHODS

Collection of Data and Data Source

The recipients' clinical data were provided by the Japan Cord Blood Bank Network (JCBBN) and the JMDP [13]. Peripheral blood stem cell donation from unrelated donors was not permitted in Japan during the study period. All 11 cord blood banks in Japan are affiliated with JCBBN. Both JCBBN and JMDP collect recipients' clinical information at 100 days posttransplantation. Patients' information on survival, disease status, and long-term complications including chronic graft-versus-host (cGVHD) disease and second malignancies is renewed annually using follow-up forms. This study was approved by the institutional review board of Nagoya University Graduate School of Medicine.

Patients

The subjects were adult patients of at least 16 years of age with acute myeloid leukemia, acute lymphoblastic leukemia, and myelodysplastic syndromes, who were recipients of first UBMT or UCBT with myeloablative conditioning. All patients in the UCBT cohort received a single-unit CB. Transplantation years were between 1996 and 2005 for UBMT and between 2000 and 2005 for UCBT to avoid the first 3 years of a pioneering period (1993-1995 for UBMT and 1997-1999 for UCBT). There were no statistically significant differences between UBMT in 1996-1999 and UBMT in 2000-2005 in probabilities of OS (41% versus 44%, at 3 years posttransplantation; $P = .86$) and in relapse-free survival (RFS) (40% versus 40%, at 3 years posttransplantation; $P = .93$).

Among 2,253 UBMT recipients with complete HLA high-resolution data, the following recipients with HLA -A, -B, -C, and -DRB1 8 of 8 allele match (n = 1,079) and more than three mismatches (5 of 8 allele match [n = 117], 4 of 8 allele match [n = 24], 3 of 8 allele match [n = 4], 2 of 8 allele match [n = 1]) were excluded. There were no statistically significant differences in risk of mortality or treatment failure (RFS) associated with single high-resolution (allele) versus single low-resolution (antigen) mismatches (data not shown), so in the analyses, allele and antigen mismatches were considered equivalent. HLA matching of cord blood was performed using low-resolution molecular typing methods for HLA-A and -B, and high-resolution molecular typing for HLA-DRB1. Of 557 recipients of CB with complete HLA data, 105 recipients with three mismatches and nine recipients with four mismatches were excluded. A total of 1,028 UBMT recipients (248 HLA class II locus mismatched, 424 HLA class I locus mismatched, and 356 HLA 2 loci mismatched) and 351 UCBT recipients (20 HLA-A, -B, low-resolution and -DRB1 matched, 87

locus mismatched, and 244 2 loci mismatched) were the subjects for analyses. Both host-versus-graft and graft-versus-host directions were accounted for in terms of HLA mismatch.

HLA Typing

Alleles at the HLA-A, -B, -C, and -DRB1 with unrelated bone marrow donor-recipient pairs and for HLA-DRB1 for unrelated cord blood donor-recipient pairs were identified by the methods described previously [1,5,14]. Serologic or antigen-level typing was performed with a standard two-stage complement-dependent test of microcytotoxicity or low-resolution DNA-based typing usually by collapsing the four-digit typing result back to its first two digits in part.

Definitions

The primary outcome of the analyses was OS, defined as time from transplantation to death from any cause. A number of secondary endpoints were also analyzed. Neutrophil recovery was defined by an absolute neutrophil count of at least 500 cells per cubic millimeter for three consecutive points; platelet recovery was defined by a count of at least 50,000 platelets per cubic millimeter without transfusion support. Diagnosis and clinical grading of acute GVHD (aGVHD) were performed according to the established criteria [15,16]. Relapse was defined as a recurrence of underlying hematologic malignant diseases. Transplantation-related death was defined as death during a continuous remission. RFS was defined as survival in a state of 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 for categoric variables. Cumulative incidence curves were used in a competing-risks setting to calculate the probability of aGVHD and cGHVD, relapse, and transplantation-related mortality (TRM) [17]. Gray's test was used for group comparison of cumulative incidences [18]. Adjusted comparison of the groups on OS and RFS was performed with the use of the Cox proportional-hazards regression model [19]. For other outcomes with competing risks, Fine and Gray's proportional-hazards model for subdistribution of a competing risk was used [20]. For neutrophil and platelet recovery, death before neutrophil or platelet recovery was the competing event; for GVHD, death without GVHD and relapse were the competing events; for relapse, death without relapse was the competing event; and, for TRM, relapse was the competing event [21]. Adjusted probabilities of OS and RFS were estimated using the Cox proportional-hazards regression model, with consideration of other significant clinical variables in the final multivariate models. The variables considered were the patient's age at transplantation, patient's sex, donor-patient sex mismatch, donor-patient ABO mismatch, diagnosis, disease status at conditioning, the conditioning regimen, and the type of prophylaxis against GVHD. Factors differing in distribution between CB and BM recipients and factors known to influence outcomes were included in the final models. Variables with more than two categories were dichotomized for the final multivariate model. Variables were dichotomized as follows: patient age >40 or <40 years at transplantation, recipient's sex, sex-mismatched donor-patient pair versus sex-matched pair, donor-recipient ABO major mismatch versus others for ABO matching, advanced versus standard (first and second complete remission of acute myeloid leukemia, first complete remission of acute lymphoblastic leukemia, or refractory anemia or refractory anemia with ring sidoblasts of myelodysplastic syndromes) risk of the disease, cyclophosphamide, and total-body irradiation (TBI) or busulfan and cyclophosphamide or others for conditioning regimen, and cyclosporine-based versus tacrolimus-based prophylaxis against GVHD. No significant interactions were identified between each variable and HLA disparity/stem cell source groups. All P values were two-sided.

RESULTS

Patient Characteristics

Table 1 shows characteristics of patients, their disease, and transplantation regimens. Proportions of females, sex-mismatched donor-recipient pairs, and ABO mismatched donor recipient pairs were larger in cord blood recipients ($P < .001$, $P < .001$, and $P < .001$, respectively). UCB recipients were older than recipients of UBM (median age, 37 years versus 34 years; $P < .001$). A preparative regimen with TBI and cyclophosphamide was used in the majority of patients in all groups, and cytosine arabinoside was supplemented for CB recipients in addition to TBI and cyclophosphamide in about half the recipients with cyclophosphamide and TBI. For GVHD prophylaxis, tacrolimus and short-term methotrexate was used preferentially in BM recipients (61% of DRB1-one-mismatched BM recipients), while cyclosporine A and short-term methotrexate was used preferentially in CB recipients (61%). The median follow-up period for survivors was 2.1 years (range, 0.1-6.2) for CB recipients and 5.5 (range, 0.3-11.6) years for BM recipients.

Table 1. Patient, Disease, and Transplantation Characteristics According to Stem Cell Source and Number of Mismatched Loci

	Bone Marrow Transplant			
	Class II One Locus Mismatch	Class I One Locus Mismatch	Two Loci Mismatch	Cord Blood Transplantation
	N (%)	N (%)	N (%)	N (%)
Number of transplantations	248	424	356	351
Patient age at transplantation				
Median (range)	36 (16-60)	34 (16-67)	34 (16-59)	37 (16-58)
Patient sex				
Male	151 (61)	241 (57)	210 (59)	162 (46)
Female	97 (39)	183 (43)	146 (41)	189 (54)
Sex matching				
Matched	145 (58)	268 (63)	217 (61)	170 (48)
Male to female	52 (21)	82 (19)	73 (21)	97 (28)
Female to male	50 (20)	71 (17)	64 (18)	84 (24)
Unknown	1 (<1)	3 (1)	2 (1)	0 (0)
Diagnosis				
AML	135 (54)	204 (48)	172 (48)	193 (55)
ALL	78 (31)	149 (35)	135 (38)	113 (32)
MDS	35 (14)	71 (17)	49 (14)	45 (13)
Disease status				
Standard	124 (50)	214 (50)	168 (47)	147 (42)
Advanced	114 (46)	195 (46)	169 (47)	174 (50)
Unknown	10 (4)	15 (4)	19 (5)	30 (9)
ABO matching				
Matched	119 (48)	184 (43)	153 (43)	114 (32)
Minor mismatch	53 (21)	108 (25)	85 (24)	99 (28)
Major mismatch	67 (27)	116 (27)	97 (27)	73 (21)
Bidirectional	8 (3)	12 (3)	14 (4)	64 (18)
Unknown	1 (<1)	4 (1)	7 (2)	1 (<1)
HLA-mismatched number and direction				
Matched				20 (6)
One locus mismatched				87 (25)
HVG direction	16 (6)	38 (9)		8 (9)
GVH direction	17 (7)	30 (7)		8 (9)
Both directions	215 (87)	356 (84)		71 (82)
Two loci mismatched				244 (70)
Two HVG direction			4 (1)	2 (1)
One HVG direction and one GVH direction			6 (2)	4 (2)
Two GVH direction			4 (1)	3 (1)
One both directions and one HVG direction			42 (12)	40 (16)
One both directions and one GVH direction			29 (8)	28 (11)
Two both directions			271 (76)	167 (68)
No. of nucleated cells infused ($\times 10^7$/kg)				
Median	25.0	24.5	23	2.46
Range	2.40-59.8	2.10-97.5	1.5-66.0	1.41-6.01
Preparative regimen				
CY + TBI	94 (38)	168 (40)	151 (42)	109 (31)
CY + CA + TBI	46 (19)	78 (18)	74 (21)	124 (35)
CY + BU + TBI	20 (8)	39 (9)	27 (8)	15 (4)
Other TBI regimen	45 (18)	70 (17)	61 (17)	80 (23)
BU + CY	34 (14)	54 (13)	30 (8)	21 (6)
Other non-TBI regimen	9 (4)	15 (4)	13 (4)	2 (1)
GVHD prophylaxisis				
Cycrosporine A + sMTX	87 (35)	221 (52)	150 (42)	213 (61)
Cyclosporine A ± other	1 (<1)	5 (1)	5 (1)	24 (7)
Tacrolimus + sMTX	152 (61)	191 (45)	193 (54)	76 (22)
Tacrolimus ± other	8 (3)	5 (1)	6 (2)	35 (10)
Others	0 (0)	2 (<1)	2 (<1)	3 (1)

ALL indicates acute lymphoblastic leukemia; AML, acute myelogenous leukemia; BU, oral busulfan; CA, citarabine; CY, cyclophosphamide; GVH, graft-versus-host; HVG, host-versus-graft; MDS, myelodysplastic syndomes; sMTX, short-term methotrexate.

Outcome

OS and RFS

OS and RFS for CB recipients were similar when compared with that of single-HLA-DRB1-mismatched BM recipients (relative risk [RR] = 0.85, 95% confidence interval [CI], 0.68-1.06; $P = .149$ for OS and RR = 0.97, 95% CI, 0.92-1.35; $P = .747$) (Table 2).

The adjusted probabilities of survival at 3 years posttransplantation of CB recipients (47%) were not different from those of single HLA-DRB1 mismatched BM recipients (41%; $P = .19$) or single HLA class I-mismatched BM recipients (47%; $P = .96$), but superior to those of 6 of 8 BM recipients (38%; $P = .014$) (Figure 1A). Figure 1B shows adjusted RFS curves (42% for CB recipients, 36% for single HLA-DRB1-mismatched BM, 44% for single HLA class I-mismatched BM, and 36% for 6 of 8 BM recipients, at 3 years posttransplant) (P values of comparison between CB and single HLA-DRB1-mismatched BM, CB, and single HLA

Table 2. Multivariate Analyses of Overall Survival, Relapse-Free Survival, Relapse, and Transplant-Related Mortality

	Degree of HLA Mismatch	N	Overall Survival			Relapse-Free Survival			Relapse			Transplant-Related Mortality		
			RR	(95% CI)	P value	RR	(95% CI)	P value	RR	(95% CI)	P value	RR	(95% CI)	P value
Bone marrow transplant	Single DRB1 (7/8)	248	1.00			1.00			1.00			1.00		
	Single A or B (7/8)	137	0.84	(0.64-1.11)	.216	0.82	(0.63-1.08)	.158	0.65	(0.41-1.01)	.056	1.07	(0.77-1.49)	.698
	Single C (7/8)	287	0.89	(0.72-1.12)	.324	0.86	(0.69-1.07)	.170	0.60	(0.41-0.87)	.007	1.13	(0.86-1.48)	.391
	C + DRB1 (6/8)	144	0.97	(0.74-1.27)	.831	0.95	(0.73-1.24)	.726	0.76	(0.49-1.17)	.208	1.10	(0.78-1.55)	.600
	A/B + C (6/8)	122	1.22	(0.94-1.59)	.143	1.15	(0.88-1.49)	.300	0.70	(0.44-1.10)	.12	1.42	(1.03-1.96)	.032
	Other two loci (6/8)	90	1.25	(0.92-1.68)	.146	1.13	(0.84-1.53)	.409	0.60	(0.35-1.02)	.061	1.48	(1.03-2.13)	.035
Cord blood transplant		351	0.85	(0.68-1.06)	.149	0.97	(0.92-1.35)	.747	1.28	(0.93-1.76)	.125	0.68	(0.50-0.92)	.011

RR indicates relative risk; CI, confidence interval.
Adjusted by patient age at transplantation >40 versus ≤40, patient sex, donor-patient sex mismatch versus matched, ABO major mismatch versus others, advanced versus standard disease status at transplantation, cyclophosphamide and total-body irradiation or busulfan and cyclophosphamide for conditioning versus other conditioning regimen, and cyclosporine-based versus tacrolimus-based prophylaxis against graft-versus-host disease.

class I-mismatched BM, and CB and 6 of 8 BM recipients were 0.80, 0.12, and 0.43, respectively).

Relapse and TRM

There was no significant increase of relapse rates among CB recipients when compared with DRB1 single-mismatched BM recipients (RR = 1.28, 95% CI, 0.93-1.76; $P = .125$). The risk of TRM was lower in CB recipients compared with that of single HLA-DRB1-mismatched BM recipients (RR = 0.68, 95% CI, 0.50-0.92; $P = .011$) (Table 2). The risk of TRM was also lower in CB recipients when compared with 6 of 8 BM recipients (RR = 0.52, 95% CI, 0.39-0.68; $P < .001$).

Hematologic recovery

Neutrophil and platelet recovery was inferior in CB recipients, as shown in Table 3 (RR = 0.50, 95% CI, 0.42-0.60; $P < .001$ for neutrophil recovery, RR = 0.52, 95% CI, 0.42-0.63; $P < .001$ for platelet recovery).

Acute GVHD and chronic GVHD

The risk of grade 2 to 4 or severe (grades 3-4) aGVHD was lower in CB recipients than that of single HLA-DRB1-mismatched BM recipients (RR = 0.55, 95% CI, 0.42-0.72; $P < .001$ for grade 2 to 4 aGVHD and RR = 0.43, 95% CI, 0.27-0.58; $P < .001$ for severe aGVHD) (Table 4). Unadjusted cumulative incidence of severe aGVHD was 9% for CB, 19% for single HLA-DRB1-mismatched BM, 18% for single HLA

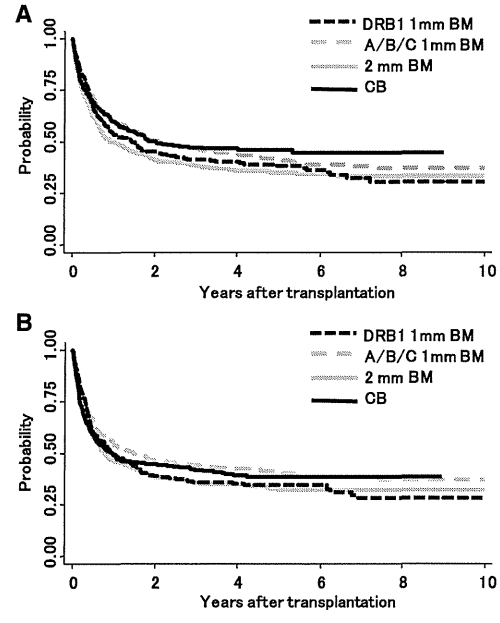

Figure 1. Adjusted probabilities of OS (A) and RFS (B). The adjusted 3-year probabilities of OS for unrelated cord blood recipients, single-HLA-DRB1-mismatched unrelated bone marrow (UBM) recipients, single-HLA-class-I-mismatched UBM, and 6 of 8 UBM recipients were 47%, 41%, 47%, and 38%, respectively (A). The adjusted 3-year probabilities of RFS were 42%, 36%, 44%, and 36%, respectively (B).

Table 3. Multivariate Analyses of Neutrophil and Platelet Recovery

			Neutrophil Recovery			Platelet Recovery		
	Degree of HLA Mismatch	N	RR	(95% CI)	*P* value	RR	(95% CI)	*P* value
Bone marrow transplantation	Single DRB1 (7/8)	248	1.00			1.00		
	Single A or B (7/8)	137	1.31	(1.04-1.65)	.021	1.31	(1.01-1.70)	.039
	Single C (7/8)	287	1.19	(0.98-1.43)	.069	0.98	(0.79-1.21)	.840
	C + DRB1 (6/8)	144	0.96	(0.77-1.20)	.735	0.79	(0.62-1.02)	.065
	A/B + C (6/8)	122	1.14	(0.89-1.45)	.307	0.84	(0.63-1.13)	.255
	Other two loci (6/8)	90	0.89	(0.68-1.14)	.346	0.80	(0.58-1.10)	.174
Cord blood transplantation		351	0.50	(0.42-0.60)	<.001	0.52	(0.42-0.63)	<.001

RR indicates relative risk; CI, confidence interval.

Adjusted by patient age at transplantation >40 versus <40, patient sex, donor-patient sex mismatch versus matched, ABO major mismatch versus others, advanced versus standard disease status at transplant, cyclophosphamide, and total-body irradiation or busulfan and cyclophosphamide for conditioning versus other conditioning regimen, and cyclosporine-based versus tacrolimus-based prophylaxis against graft-versus-host disease.

class I-mismatched BM, and 22% for 6 of 8 BM at 100 days posttransplantation ($P < .001$ between CB and single HLA-DRB1-mismatched BM) (Figure 2A).

Among recipients who survived at least 100 days posttransplantaton, the risk of developing cGVHD and extensive-type cGVHD was not significantly increased in all HLA disparity groups of CB recipients when compared with that of HLA-DRB1-allele/antigen-mismatched BM recipients (RR = 1.36, 95% CI, 0.99-1.88; $P = .057$ for cGVHD, and RR = 0.86, 95% CI, 0.55-1.34; $P = .500$ for extensive-type cGVHD). The unadjusted cumulative incidence of extensive-type cGVHD was 17% for CB recipients, 20% for single HLA-DRB1-mismatched BM, 25% for single HLA class I-mismatched BM, and 30% for 6 of 8 BM recipients at year posttransplantation ($P = .34$ between CB and single HLA-DRB1-mismatched BM) (Figure 2B).

DISCUSSION

Our main objective was to compare OS after transplantation of UCBT and single-HLA-mismatched UBMT and to provide useful data for selection of an appropriate donor and graft source in second stem cell source/donor selection for adults with hematologic malignancy. To the best of our knowledge, this is the first study to involve mismatched allele/antigen-specific analyses including CB for the process of donor selection. Our results suggest that 0 to 2 HLA-mismatched UCB is a reasonable second alternative of choice for adult patients with leukemia, with similar survival to that of single DRB1-mismatched or other 7 of 8 UBM recipients, the current first choice for second alternative donor/stem cells.

Neutrophil and platelet recovery was slower in CB recipients than BM recipients, consistent with the results of previous reports [7-10,12]. This is the major limitation of the use of UCB, and several strategies have been studied to reduce the neutropenic period, such as screening for patients' pretransplantation anti-HLA antibodies and their specificity, transplantation of 2 UCB units if a single UCB unit with an adequate cell dose is not available, or direct infusion of UCB into bone marrow [22-26].

Despite higher HLA disparity at the antigen level (69% 2 antigen mismatch, 25% antigen mismatch, and 6% matched), UCB recipients showed lower incidence of severe aGVHD than single DRB1-mismatched UBM recipients, consistent with other reports that compared UCB with single-mismatched UBM (7 of 8) [8,11,12]. In our study, tacrolimus and short-term methotrexate were used preferentially in BM recipients, whereas cyclosporine A was used in 68% of CB recipients. Prior studies have shown reduced severe aGVHD with tacrolimus, and this difference may have underscored the improved aGVHD control of UCB over mismatched BM in unadjusted analyses [27,28]. It is likely that decreased risk of grade 2 to 4 aGVHD in UCB recipients contributed to decreased risk of TRM among UCB recipients.

Increasing the number of HLA mismatches from 7 of 8 to 6 of 8 was associated with an approximately 10% reduction in survival in UBM recipients, which was quite similar to the results from the National Marrow Donor Program [3]. Because we eliminated data from the first 3 pioneering years of unrelated BMT, most of the bone marrow recipients and donors were allele-typed for at least HLA-A, -B, and -DRB1 before transplantation. Survival outcomes of single class I mismatch were not significantly different from those of single class II mismatch in the current analyses. We believe that allele typing of HLA-A, -B, and -DRB1 before transplantation led to better selection of the donor compared with that in the first several years of UBMT. This study includes a large number of fully typed BM and CB recipients, but there are limitations. The choice of stem cell source is influenced by many unmeasured factors that can affect outcome. It is also influenced by the availability of acceptable HLA disparity for unrelated donors and mainly cell dose for cord blood units. Although we have adjusted for known risk factors and disparities between groups, we cannot rule out the influence of potential selection bias, which can only be excluded in a randomized controlled trial. Transplantation years

Table 4. Multivariate Analyses of Acute (Grades 2 to 4 and Grades 3 to 4), Chronic, and Extensive-Type Chronic Graft-versus-Host Disease

	Degree of HLA Mismatch	N	Grade 2-4 acute GVHD			Grade 3-4 acute GVHD			Chronic GVHD			Extensive cGVHD		
			RR	(95% CI)	*P*-value	RR	(95% CI)	*P*-value	N	RR	(95% CI)	*P*-value	RR	(95% CI)	*P* value
Bone marrow transplantation	Single DRB1 (7/8)	248	1.00			1.00			199	1.00			1.00		
	Single A or B (7/8)	137	0.76	(0.55-1.06)	.103	0.91	(0.56-1.47)	.698	111	0.91	(0.61-1.36)	.646	0.89	(0.52-1.50)	.651
	Single C (7/8)	287	0.93	(0.72-1.20)	.584	0.91	(0.61-1.35)	.635	227	1.56	(1.15-2.10)	.004	1.79	(1.22-2.63)	.003
	C + DRB1 (6/8)	144	0.85	(0.60-1.18)	.320	0.88	(0.54-1.44)	.610	109	1.44	(1.01-2.05)	.041	1.47	(0.93-2.32)	.097
	A/B + C (6/8)	122	1.40	(1.04-1.90)	.028	1.90	(1.25-2.87)	.003	87	1.64	(1.14-2.34)	.007	2.26	(1.46-3.50)	<.001
	Other two loci (6/8)	90	0.88	(0.60-1.28)	.501	0.65	(0.34-1.22)	.183	60	1.35	(0.86-2.12)	.191	1.15	(0.62-2.13)	.652
Cord blood transplantation		351	0.55	(0.42-0.72)	<.001	0.43	(0.27-0.58)	<.001	252	1.36	(0.99-1.88)	.057	0.86	(0.55-1.34)	.500

GVHD indicates graft-versus-host disease; cGVHD, chronic graft-versus-host disease.
Adjusted by patient age at transplantation >40 versus <40, patient sex, donor-patient sex mismatch versus matched, ABO major mismatch versus others, advanced versus standard disease status at transplantation, cyclophosphamide, and total-body irradiation or busulfan and cyclophosphamide for conditioning versus other conditioning regimen, and cyclosporine-based versus tacrolimus-based prophylaxis against graft-versus-host disease.

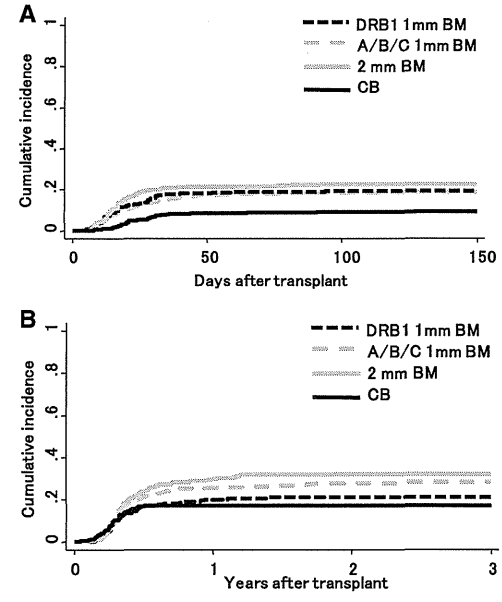

Figure 2. Cumulative incidence of grade 3 to 4 aGVHD (A) and extensive-type cGVHD (B). The cumulative incidences of grade 3 to 4 aGVHD at 100 days posttransplantation for unrelated cord blood recipients, single HLA-DRB1-mismatched unrelated bone marrow (UBM) recipients, and single HLA class I-mismatched UBM were 9%, 19%, 18%, and 22% (A). The cumulative incidences of extensive-type cGVHD at 1-year posttransplantation were 17%, 20%, 25%, and 30% (B).

of UBM recipients included from 1996 and 1999, for which there were no significant outcome differences between UBMT performed in 1996 to 1999 and after 2000. In these periods, there were advances including in supportive care and nutritional management, introduction of new antifungal agents, and more frequent use of tacrolimus, which may have affected transplantation outcomes [27-32].

In conclusion, we suggest that 0 or 2 HLA-mismatched UCB is a comparable second alternative for adult patients with leukemia in the absence of the first alternative, an 8 of 8 UBM donor, with survival similar to that of single DRB1-mismatched or other 7 of 8 UBM recipients. UCB may be preferred over single mismatched UBM when a transplantation is needed urgently, considering the short time needed for UCBT.

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AUTHORSHIP STATEMENT

Contributions: Y.A., Y.M., R.S., and S. Kato designed the study, and wrote the article; Y.A. analyzed results and created the figures; T.N.I., H.A, and M. Takanashi reviewed and cleaned the Japan Cord Blood Bank Network data, and reviewed the results; S. Taniguchi, S. Takahashi, S. Kai., H.S., Y. Kouzai., N.K., T.M., T.F., and Y. Kodera submitted and cleaned the data; M. Tsuchida, K.K., T.K., and Y.M. reviewed and cleaned the Japan Marrow Donor Program data, and reviewed the results.

SUPPLEMENTARY DATA

Supplementary data related to this article can be found online at doi:10.1016/j.bbmt.2011.10.008.

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Human microRNA-1245 down-regulates the NKG2D receptor in natural killer cells and impairs NKG2D-mediated functions

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The online version of this article has a Supplementary Appendix.

ABSTRACT

Background

NKG2D is an activating receptor expressed by natural killer and T cells, which have crucial functions in tumor and microbial immunosurveillance. Several cytokines have been identified as modulators of NKG2D receptor expression. However, little is known about *NKG2D* gene regulation. In this study, we found that microRNA 1245 attenuated the expression of NKG2D in natural killer cells.

Design and Methods

We investigated the potential interactions between the 3'-untranslated region of the *NKG2D* gene and microRNA as well as their functional roles in the regulation of NKG2D expression and cytotoxicity in natural killer cells.

Results

Transforming growth factor-β1, a major negative regulator of NKG2D expression, post-transcriptionally up-regulated mature microRNA-1245 expression, thus down-regulating NKG2D expression and impairing NKG2D-mediated immune responses in natural killer cells. Conversely, microRNA-1245 down-regulation significantly increased the expression of NKG2D expression in natural killer cells, resulting in more efficient NKG2D-mediated cytotoxicity.

Conclusions

These results reveal a novel NKG2D regulatory pathway mediated by microRNA-1245, which may represent one of the mechanisms used by transforming growth factor-β1 to attenuate NKG2D expression in natural killer cells.

Key words: NKG2D, microRNA-1245, TGF-β1.

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Introduction

NKG2D is an activating receptor expressed on natural killer (NK) cells which play a pivotal role in tumor immunosurveillance.[1-4] NKG2D is a member of the type II C-type lectin-like family of transmembrane proteins that function as both activating and co-stimulatory receptors and is constitutively expressed on most NK cells, as well on γδ T cells and some subsets of $CD4^+$ and $CD8^+$ T cells.[5] The NKG2D receptor recognizes multiple and structurally different ligands, including the MHC class I-chain related proteins (MICA and MICB) and the UL-16 binding proteins (ULBP 1-6).[6] These ligands are either absent or poorly expressed in normal tissues but are up-regulated in response to cellular stresses such as microbial infections and transformation.[1,5] Extensive research during the last few years has demonstrated the cytokine network that regulates the cell surface expression of the NKG2D receptor; however, little is known about the mechanisms that control expression of the *NKG2D* gene.

This study focused on the potential interactions between the 3'-untranslated region (3'UTR) of the *NKG2D* gene and microRNA. microRNA are endogenous, single-stranded RNA that modulate gene expression by binding to complementary sites in the 3'UTR of the target gene's mRNA. These 17-22 base oligonucleotides mediate gene regulation by either directly inducing mRNA degradation or by decreasing translational efficiency.[7,8] The data presented here identify microRNA (miR)-1245 as a novel negative regulator of NKG2D, and may clarify one of the mechanisms used by transforming growth factor-β1 (TGF-β1) to attenuate NKG2D expression.

Designs and Methods

Natural killer cell preparation and cell culture

Peripheral blood mononuclear cells were isolated from blood samples from healthy Japanese volunteers using a Lymphoprep (Pharmacia Biotech, Uppsala, Sweden) and the NK cell fraction was purified using the untouched NK isolation kit (Invitrogen). For some experiments NK cells were obtained by culturing the peripheral blood mononuclear cells from healthy donors in the presence of irradiated K562-mb15-41BBL cells in RPMI 1640 containing 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin and 100 IU/mL interleukin-2 for 10 days, as described previously.[9] These cultured peripheral blood mononuclear cells contained >95% $CD3^-CD56^+CD16^+$ NK cells and are referred to as "cultured NK cells". Details on the cell lines used in this study are given in the *Online Supplementary Design and Methods.*

Flow cytometry

CD3, CD56, CD16, CD160, MICA/B (BD Bioscience), NKG2D, NKp30, NKp44 and NKp46 (Beckman Coulter, Shizuoka, Japan) were detected by staining the cells with appropriate fluorescein isothiocyanate- or phycoerythrin-labeled monoclonal antibodies. ULBP ligand was detected by indirect staining using anti-ULBP1, ULBP2 and ULBP3 monoclonal antibodies (R&D Biosystems), followed by staining with fluorescein isothiocyanate-labeled anti-mouse IgG (BD Bioscience). Data acquisition and flow cytometry analyses were carried out on a BD FACS Calibur instrument using the CellQuest software package.

Quantitation of NKG2D mRNA levels

Total RNA was extracted from NK cells using Isogen (Nippon Gene, Tokyo, Japan) following the manufacturer's instructions. Complementary DNA (cDNA) synthesis was carried out using a QuantiTect Reverse Transcription kit (Qiagen Inc. Hilden Germany). Amplification of cDNA was monitored using a QuantiFast SYBR green PCR kit (Qiagen) on a StepOne plus instrument (Applied Biosystems). Predesigned specific primers for NKG2D (assay name Hs_KLRK1_1_SG, Qiagen) and a *GAPDH* primer kit (Search LC, Heidelberg, Germany) were used for mRNA quantification in each sample. The amount of *NKG2D* mRNA relative to *GAPDH* mRNA was calculated by the comparative CT method using the relative expression function included in the StepOne v2.2 software package (Applied Biosystems).

Measurement of microRNA

To detect mature miR-1245, total RNA was extracted using the Isogen LS reagent (Nippon gene) and reverse transcription was performed using a TaqMan microRNA RT kit following the manufacturer's recommendations (Applied Biosystems). The resultant cDNA was amplified using the TaqMan microRNA assay (hs-miR-1245, assay ID002823) with the TaqMan Universal PCR master mix II no UNG (Applied Biosystems). The polymerase chain reaction (PCR) and cycling parameters were set following the manufacturer's recommendations with minor modifications as follows: a 10 μL PCR contained 4.5 μL of diluted cDNA product, 1X TaqMan Universal master mix and 1X of the TaqMan microRNA assay or 1X of the U6b-specific TaqMan probe (hs-miR-U6B assay ID001093). The reactions were incubated in 96-well plates at 95°C for 10 min, followed by 44 cycles of 95°C for 15s and 58°C for 1 min. All reactions were run in duplicate in a StepOne plus RT-PCR system (Applied Biosystems). The data were analyzed with the StepOne v2.2 software package (Applied Biosystems). The relative quantities of mature miR-1245 were calculated using the comparative CT method after normalization to the expression of U6b, as reported by others.[10,11]

Exosome precipitation from human plasma and microRNA detection in exosomes

Serum exosomes were isolated from healthy donors and from ten patients with hematologic malignancies before starting chemotherapy. All patients gave their written informed consent to participate in molecular studies of this nature according to the Declaration of Helsinki. The patients and methods are described in detail in the *Online Supplementary Design and Methods.*

Reporter gene assays

The reporter gene assays were performed by constructing luciferase vectors containing wild-type or mutant 3' UTR fragments of the *NKG2D* gene. Further details are given in the *Online Supplementary Design and Methods.*

Natural killer cell transduction with exogenous microRNA-1245

Fresh NK cells were transduced by lentiviral delivery of a human miR-1245 precursor microRNA overexpression construct (PMIRH1245PA-1-SBI) or a negative control construct vector (pCDH-CMV-MCS-EF1-copGFP) designated hereafter as miR-1245-vector and NC-vector, respectively, following the manufacturer's recommendations (Systems Biosciences, Mountain View, CA, USA). Further details are given in the *Online Supplementary Design and Methods.*

Establishment of cell lines over-expressing microRNA-1245

NK cell sub-lines over-expressing miR-1245 derived from NK-92 and KHYG-1 cells were established by lentiviral delivery of human miR-1245 precursor microRNA overexpression construct. The