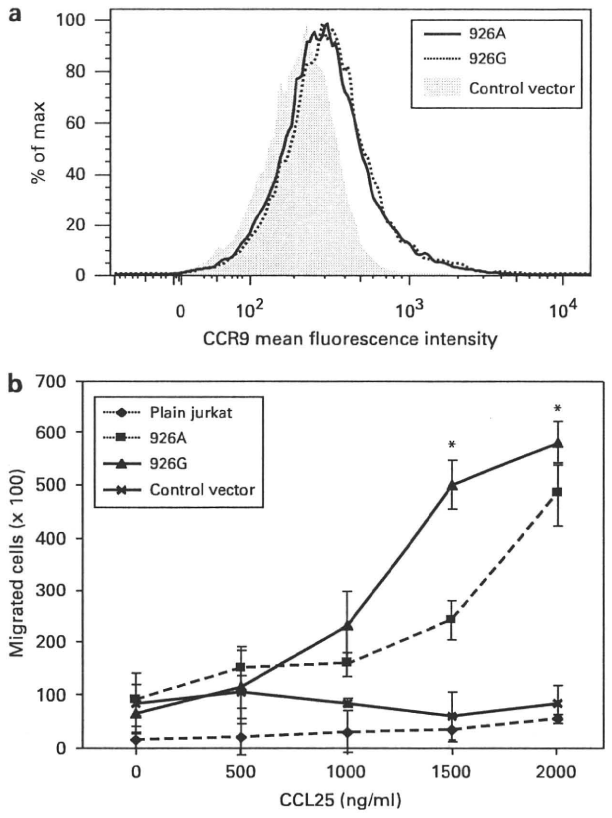

Figure 2 Chemotaxis assays with *CCR9*-polymorphism-transfected Jurkat cells: (**a**) Flow cytometric analysis of CCR9 expression. Transfected Jurkat cells were stained with phycoerythrin (PE)-labeled monoclonal anti-CCR9. Control staining with control-transfected Jurkat cells is also shown (shadow); (**b**) Jurkat cells transfected with cDNAs encoding *CCR9* migrated in response to CCL25. After puromycin selection, 1×10^6 transfected cells and the same number of Jurkat cells were added to porous Transwell tissue culture inserts and placed in wells containing various concentrations of CCL25. After 90-min of incubation, cells migrating through the membranes into the lower wells were counted. Results are expressed as cells migrating per 10^6 input cells. Assays were carried out in triplicate and error bars represent s.d. $^*P<0.05$

may be candidates for future therapeutic targets that alter the quality and incidence of GVHD.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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ORIGINAL ARTICLE

Cytochrome P450 genetic polymorphisms influence the serum concentration of calcineurin inhibitors in allogeneic hematopoietic SCT recipients

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Calcineurin inhibitors are necessary as immunosuppressants during hematopoietic SCT (HSCT) to prevent alloreactivity, but have unfortunate toxicities. So, we investigated the association of gene polymorphisms with the initial calcineurin inhibitor concentration and the subsequent drug dose from day 1 to day 28 among patients who underwent HSCT at a single institution. We analyzed 58 serial cases of Japanese patients receiving GVHD prophylaxis with CsA (21 cases) or tacrolimus (37 cases). We investigated eight single-nucleotide polymorphisms: rs4244285 (*CYP2C19*), rs15524, rs4646450, rs3800959, rs776746 (*CYP3A5*), rs1128503, rs2032582 and rs1045642 (*MDR1*). The CsA concentration was significantly higher when the genotype of *CYP3A5* rs15524 was T/T ($P=0.044$) or rs776746 was G/G ($P=0.027$). The *CYP3A5* rs776746 and rs4646450 genotypes were also associated with tacrolimus concentration ($P=0.013$ and $P=0.0058$, respectively). The dosage of tacrolimus was remarkably reduced from day -1 to day 28 when the patient had the *CYP3A5* rs4646450 C/C and/or rs776746 G/G genotype ($P=0.0010$ and $P=0.0021$, respectively). In this study, we show that genetic variation has a predictable effect on the pharmacological responses to calcineurin inhibitors in HSCT patients.

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Keywords: calcineurin inhibitor; polymorphism; CYP3A5; MDR1

Introduction

The successful outcome of HSCT has been greatly enhanced in the last 40 years not only by improvements in techniques and by progressive understanding of HLA histocompatibility and typing, but also by the development of effective immunosuppressive agents. CsA and tacrolimus have been critical for the improved outcome of both solid organ transplantation and HSCT that has been observed over the last three decades. Although the patients are given CsA and tacrolimus routinely as prophylaxis of GVHD, the agents have a narrow therapeutic window and a spectrum of undesirable side effects that often accompany their administration. These drugs can be both nephrotoxic and neurotoxic (insomnia, tremor and headache), findings consistent with the ubiquitous expression of calcineurin. Other side effects that limit their usefulness for prolonged therapy include hirsutism, hyperglycemia, hypertension, hyperkalemia and glucose intolerance.[1,2]

Clinically calcineurin inhibitors are often administered using the same dose based on the patient's body weight, even though the blood concentration of these agents shows a wide spread in different cases. Although individual differences in drug response can result from the effects of age, sex, disease or drug interactions, genetic factors can also influence both the efficacy of a drug and the likelihood of an adverse reaction.[3] The variability in CsA and tacrolimus disposition has been attributed to interindividual differences in the expression of the metabolizing enzymes cytochrome P450 (CYP) 3A4 and 3A5, and in the expression of the drug transporter P-glycoprotein (encoded by the ABCB1 gene, formerly known as the multidrug resistance 1 gene (*MDR1*)). As these genes have functional polymorphisms affecting drug metabolism, there have been many pharmacogenetics studies.[4–9] Because this type of study helps us predict the patient's biological response to a drug, we can avoid the side effects and provide suitable therapy for each individual.

There are many reports investigating the association of these gene polymorphisms and calcineurin inhibitors in organ transplant recipients, however, there are only a few studies looking at how this association influences the clinical features in HSCT recipients. In order to predict calcineurin inhibitor-related complications, we investigated the association between the initial concentration of these drugs, and cytochrome P450 and P-glycoprotein gene polymorphisms. Because the continuing dose of calcineurin inhibitor is often reduced in HSCT recipients during the first 4 weeks in order to maintain target blood concentrations,

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we also examined the relationship between the drug dose ratio of the day 28 dose compared with the starting dose and gene polymorphisms.

Materials and methods

Study patients, sample collection and drug administration

We analyzed 58 serial cases of adult Japanese patients who underwent HSCT in Tokai University School of Medicine from March 2006 to May 2009. Patient characteristics are shown in Table 1. GVHD prophylaxis was administered, with 21 cases receiving CsA (starting dose of 1.5 mg/kg, twice daily by 3-h i.v. infusion from day −1), and 37 cases receiving tacrolimus (starting dose of 0.02 mg/kg, by continuous i.v. infusion from day −1) and a short course of MTX (15 mg per day i.v. on day 1 and 10 mg per day i.v. on days 3 and 6). Because there were no patients with obesity whose body mass indices were in excess of 30 kg/m^2, we did not adjust the drug dosage according to patient's obesity. All patients were administered omeprazole (20 mg per day), itraconazole (200 mg per day), ciprofloxacin (400 mg per day) and acyclovir (500 mg per day) from 7 days before transplantation.

The initial drug concentration was investigated on day 2 or day 3, and subsequent drug concentrations were monitored three times a week. Blood samples from patients receiving CsA were collected before daily drug administration. The drug dosage was adjusted according to the target serum concentration. The target concentration was 250–300 ng/mL for CsA (at the trough) and 10–15 ng/mL for tacrolimus. If the drug concentration was outside the target range, we increased or decreased the daily drug dose by 20%. All subjects provided informed consent to participate in this study, as approved by the ethical committee of Tokai University School of Medicine.

Table 1 Patient characteristics

	CsA (n = 21)	*Tacrolimus (n = 37)*
Age (years), median (range)	48 (27–58)	42 (18–59)
Gender		
Male/Female	12/9	23/11
Underlying disease		
AML	6	11
ALL	9	8
Lymphoma	3	4
AA	1	5
MDS	1	4
CML	1	4
MM	0	1
Stem cell donor		
Related	14	5
Unrelated	7	32
Conditioning regimen		
Myeloablative	15	28
Reduced intensity	6	9
Acute GVHD[a]		
Grades 0–I	10	23
Grades II–IV	10	13

Abbreviations: AA = aplastic anemia; MDS = myelodysplastic syndrome; MM = multiple myeloma.
[a]One patient from each group died from primary graft failure.

Analysis of gene polymorphisms

Genomic DNA was purified from peripheral blood or BM obtained before HSCT. We studied single-nucleotide polymorphisms (SNPs) in genes encoding CYP2C19, CYP3A5 and MDR1 based on the following criteria: (1) known functional consequences on enzyme activity and drug metabolism and (2) a minor allele frequency higher than 5% in the Japanese population. The selection was based on publicly available databases (dbSNP, International HapMap project) and on Medline publications for selected SNPs. Finally we examined the *CYP2C19* SNP (rs4244285),[10,11] *CYP3A5* SNPs (rs15524, rs4646450, rs3800959 and rs776746)[4,12,13] and *MDR1* SNPs (rs1128503, rs2032582 and rs1045642)[12] by PCR based on suitable methods for each SNP (Table 2 and Supplementary Table 1).

Statistical analysis

Statistical analyses were performed using *t*-tests for polymorphisms and drug concentrations. Renal dysfunction and acute GVHD rates were estimated by the Kaplan–Meier method and *P*-values were calculated using the log-rank test. A significance level of $P<0.05$ was used for all analyses.

Results

Initial drug concentration

Mean initial CsA and tacrolimus concentrations were significantly influenced by the *CYP3A5* genetic polymorphisms. The CsA concentration was significantly higher when the *CYP3A5* rs15524 genotype was T/T (251.1 ± 92.8 and 145.7 ± 36.7 ng/mL for T/T and T/C + C/C, respectively; mean ± 95% confidence interval (CI)]; $P=0.044$; Figure 1a) or the *CYP3A5* rs776746 genotype was G/G (250.8 ± 69.9 and 138.6 ± 30.8 ng/mL for G/G and A/G + A/A, respectively; mean ± 95% CI; $P=0.027$; Figure 1b). There

Table 2 SNP rs number and frequency

Gene	*rs No.*	*Minor allele frequency*	
		CEU	*JPT*
CYP2C19	4244285	0.155	0.284
CYP3A5	15524	0.067	0.273
	4646450	0.175	0.273
	3800959	0	0.216
	776746	0.058	0.25
MDR1	1128503	0.392	0.422
	2032582	0.469	0.448
	1045642	0.429	0.459

Abbreviations: CEU = Utah residents with Northern and Western European ancestry from the Centre d'Etude du Polymorphisme Humain (CEPH) collection; JPT = Japanese in Tokyo, Japan; SNP = single-nucleotide polymorphism.

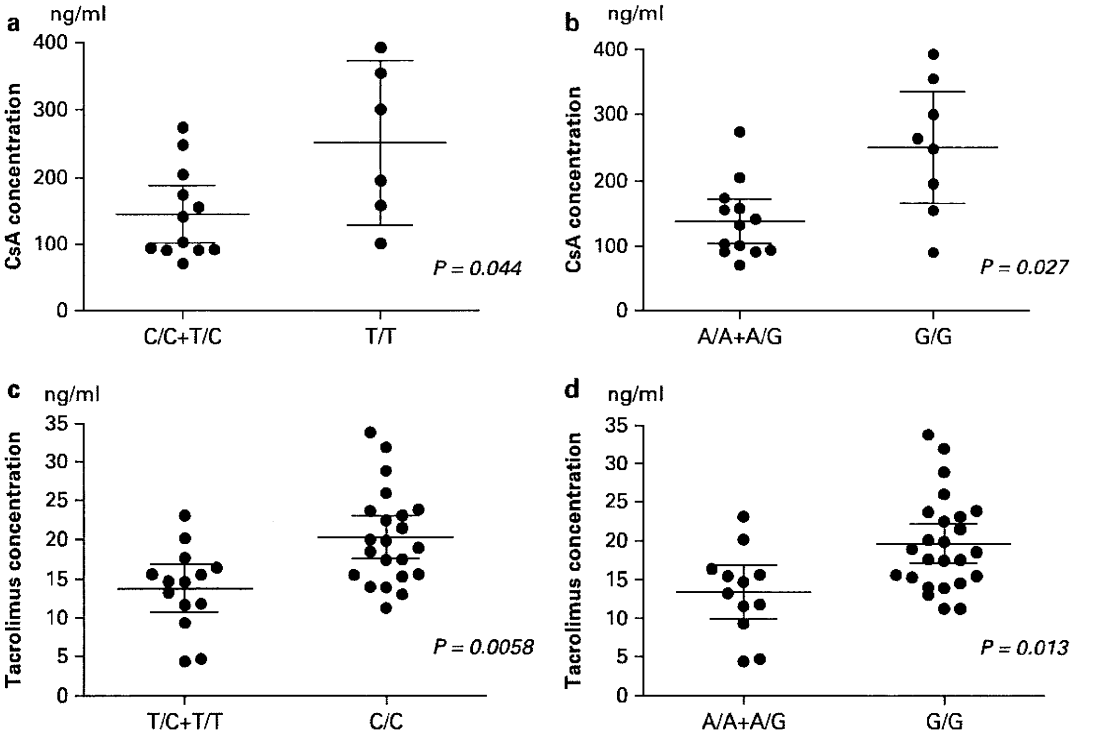

Figure 1 Initial concentration of calcineurin inhibitor and genetic polymorphisms. The initial concentration of CsA was significantly increased in patients with the *CYP3A5* rs15524 T/T genotype (**a**) or the *CYP3A5* rs776746 G/G genotype (**b**) ($P=0.044$, $P=0.027$, respectively). The initial concentration of tacrolimus was also significantly increased in patients with the *CYP3A5* rs4646450 C/C genotype (**c**) or the *CYP3A5* rs776746 G/G genotype (**d**) ($P=0.0058$, $P=0.013$, respectively).

were no significant associations between the initial trough level of CsA and the polymorphisms of *CYP2C19*, *MDR1* and the other investigated *CYP3A5* SNPs.

The tacrolimus concentration was also remarkably influenced by *CYP3A5* SNPs. When the patient had the C/C genotype for *CYP3A5* rs4646450, the tacrolimus concentration was significantly higher than in patients with the T/C or T/T genotype (20.3 ± 2.5 and 13.8 ± 2.7 ng/mL for C/C and T/C+T/T, respectively; mean ± 95% CI; $P=0.0058$; Figure 1c). The *CYP3A5* rs776746 genotype also had a significant association with the serum tacrolimus concentration. When the patient had a G/G genotype, the tacrolimus concentration was higher than in patients who had the A/G or A/A genotypes (19.7 ± 2.4 and 13.4 ± 3.1 ng/mL for G/G and A/G+A/A, respectively; mean ± 95% CI; $P=0.013$; Figure 1d). There were no significant associations between the tacrolimus concentration and the polymorphisms of *CYP2C19*, *MDR1* and other investigated *CYP3A5* SNPs.

Drug dose ratio of day -1 to day 28

To explore the predictive role of the genetic polymorphisms on dose adjustment of the drugs, we compared the initial drug dose with the day 28 drug dose and investigated the drug dose ratio (day 28 dose per day -1 dose) in each patient. The dosage of tacrolimus was remarkably reduced from day -1 to day 28 to maintain the target concentration, when the patient had the *CYP3A5* rs4646450 C/C genotype (39.8 ± 8.4 and $86.1\pm25.5\%$ for C/C and T/C+T/T, respectively; mean ± 95% CI; $P=0.0010$; Figure 2a) and/or the *CYP3A5* rs776746 G/G genotype (41.6 ± 8.2 and $90.5\pm29.4\%$ for G/G and A/G+A/A, respectively; mean ± 95% CI; $P=0.0021$, Figure 2b). There was no association of the drug dose ratio with the *CYP2C19*, *MDR1* or other *CYP3A5* polymorphisms.

Transplantation-related complications and genetic polymorphisms

As renal dysfunction is a common side effect of calcineurin inhibitor treatment, we analyzed the relationship between genetic polymorphisms and renal failure over the 12 months after HSCT. Patients with a creatinine level more than two times over the upper normal limit (men, >2.2 mg per 100 mL; female, >1.6 mg per 100 mL) were diagnosed as having renal failure. Among the CsA and tacrolimus patients, 9/22 (40.9%) and 15/37 (40.5%) developed renal failure, respectively. There were no significant associations between the frequency of renal failure and the genetic polymorphisms. We also investigated these polymorphisms and acute GVHD. There was also no significant association between developing acute GVHD and these genetic polymorphisms.

Discussion

The main aim of genetic association studies involving pharmacogenetics is to avoid treatment toxicity and enhance drug effects.[3,14,15] To date, no studies have been

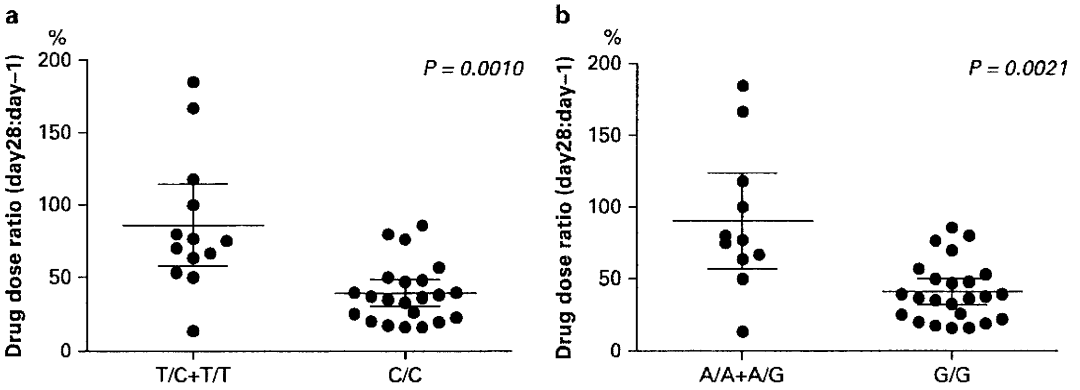

Figure 2 Tacrolimus drug dose ratio (day 28:day -1). The tacrolimus dose ratio comparing day 28 dose to day −1 dose was significantly different according to the *CYP3A5* rs4646450 genotype (**a**) or the *CYP3A5* rs776746 genotype (**b**) ($P=0.001$, $P=0.0021$, respectively).

reported in the field of SCT showing a relationship between complications and genetic polymorphisms of drug metabolism genes. In this study, we have demonstrated that calcineurin inhibitors have a wide range of serum concentrations because of the individual patient's genomic background. On the other hand, we have described a lack of association between the patient's genetic polymorphisms and transplantation-related complications. As we could adjust the drug dosage according to the patient's serum calcineurin inhibitor concentration, the drug monitoring strategy should be effective to reduce the frequency of complications even in the specific genetic backgrounds. In fact, the drug dose ratio results from day -1 to day 28 indicate that a patient who has the *CYP3A5* rs4646450 C/C and/or rs776746 G/G genotype could receive a reduced initial dose of tacrolimus. Thus, patients with the specific genotype tended to receive a reduced dosage, therefore avoiding the drug side effects. Because this consecutive case study included patients with a wide variety of clinical backgrounds, there was no association between clinical outcomes and genetic background. However, strong associations were seen between the drug concentration and genetic background. These results might provide useful information to avoid drug-induced toxicity.

Although calcineurin inhibitors are metabolized mainly by CYP2C and CYP3A, at the same time, these enzymes are also associated with the metabolism of several other drugs.[4,16,17] Because HSCT patients usually need several drugs at the same time to prevent infections and HSCT complications, we must consider all the drug interactions influenced by calcineurin inhibitor metabolism. There are some studies describing a clinically significant drug interaction with calcineurin inhibitors in renal transplants and HSCT recipients.[18–20] In order to reduce the impact of other drug interactions, our study compared the initial concentration of calcineurin inhibitor, because at that time the patients were uniformly administered omeprazole, itraconazole, ciprofloxacin and acyclovir. In our study, the conditioning regimen also did not affect the initial concentration of the calcineurin inhibitor. Therefore, during the early time of HSCT, the concentration of calcineurin inhibitor was influenced by the patient's genetic background rather than drug interactions.

Carriers of a reduced-function *CYP3A5* allele showed significantly higher levels of calcineurin inhibitor in this study. The most well-known functional polymorphism in this study was *CYP3A5* rs776746, which has reduced CYP3A5 enzyme activity when the subject has the G/G genotype.[4] However, our results showed that the SNP rs4646450 within *CYP3A5* also had a significant association, although there have been no reports of the function of the rs4646450 SNP. One genome-wide association study using 550 000 SNPs investigating the maintenance dose of warfarin reported that it was the *CYP3A5* rs4646450 SNP and not rs776746 that had the best association with stabilized warfarin dose.[13] From these results, *CYP3A5* rs4646450 might be associated with common mechanism affecting both warfarin and tacrolimus metabolism.

In conclusion, we have shown that genetic variation has an effect on the pharmacological response to calcineurin inhibitors in HSCT patients. These results are predictable and can therefore help to avoid drug toxicity in the future. Because this study was a serial case study from single institution, the clinical backgrounds of patients were heterogeneous. Moreover, this study was dealing with modest samples and homogeneous genetic background population. In order to define the genetic background influence on clinical outcome of HSCT, it is necessary to investigate further case-controlled study.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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ORIGINAL ARTICLE

Cytotoxic T-lymphocyte antigen 4 haplotype correlates with relapse and survival after allogeneic hematopoietic SCT

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CTLA-4 is a negative regulator of activated T cells and the association of CTLA-4 polymorphisms with autoimmune diseases and transplant outcome has been reported. We evaluated the effect of donor CTLA-4 polymorphisms on outcome after allogeneic hematopoietic SCT (HSCT). We analyzed 147 Japanese HLA-matched sibling recipients and their donors who had undergone allogeneic HSCT. Genotyping of three single-nucleotide polymorphisms in CTLA-4 (−318, +49, CT60) was performed using TaqMan-PCR. According to the international HapMap database, only these three CTLA-4 haplotypes, classified as C-G-G, C-A-A and T-A-G, are present in the Japanese population. In this study, percentage expression of the C-G-G, C-A-A and T-A-G haplotypes was 59.5, 30.6 and 9.9%, respectively. Recipients of the C-A-A haplotype donor showed a significantly lower risk of relapse (HR: 0.54, 95% CI: 0.30–0.97, $P=0.040$) and a trend toward higher OS (HR: 0.61, 95% CI: 0.36–1.0, $P=0.054$) than did recipients of a donor without the C-A-A haplotype. The presence or absence of the C-A-A haplotype did not affect GVHD or non-relapse mortality. As the presence of the C-A-A haplotype reduced relapse risk and improved survival after allogeneic HSCT, this CTLA-4 haplotype may provide useful information for donor selection.

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Keywords: cytotoxic T-lymphocyte antigen 4; single-nucleotide polymorphism; haplotype; Japanese population; allogeneic hematopoietic SCT

Introduction

Allogeneic hematopoietic SCT (HSCT) has been established as an effective treatment for patients with hematological malignancies. GVHD caused by donor-derived T cells is one of the most common causes of morbidity and mortality after allogeneic HSCT.[1] However, donor-derived T cells also mediate a GVL effect, which assists in the eradication of tumor cells.[2] Control of alloimmune reactions and separation of the potent GVL effect from severe GVHD are therefore important for a successful outcome after allogeneic HSCT. Although optimal HLA matching between patients and donors is critical for the prevention of severe GVHD, this can still develop after HSCT from an HLA-identical sibling donor due to non-HLA gene polymorphisms.[3,4] Thus, the association of polymorphisms in genes encoding mHA,[5,6] cytokines,[7,8] chemokines[9] and drug-metabolizing enzymes[10] with transplant outcomes has been reported.

CTLA-4 is a receptor expressed on the surface of activated T cells, and is a homolog of CD28 that is responsible for T-cell activation. Although both CTLA-4 and CD28 bind the two ligands B7.1 (CD80) and B7.2 (CD86) expressed on APCs, CTLA-4 binds B7 molecules with higher affinity and avidity than CD28. CTLA-4 gene polymorphisms correlate with autoimmune diseases such as systemic lupus erythematosus,[11–13] type 1 diabetes mellitus[14,15] and Graves' disease.[16] In addition, recent studies have shown an association of the CTLA-4 polymorphisms (−318 (rs5742909), +49 (rs231775) and CT60 (rs3087243)) with outcome after allogeneic HSCT.[17–19] We therefore focused our study on these three polymorphisms, and analyzed the impact of donor genotypes and haplotypes in the Japanese population on outcome after HLA-identical sibling HSCT.

Patients and methods

Patients

The study population included adult Japanese patients who received hematopoietic stem cells from an HLA-identical sibling donor for the treatment of hematological

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Table 1 Patient characteristics

No. of patients	147
Median age in years (range)	39 (15–62)
Sex (M/F)	81/66
Disease	
AML	49
ALL	28
CML	39
Myelodysplastic syndrome	17
Malignant lymphoma	6
Multiple myeloma	5
Others	3
Disease risk	
Standard[a]	81 (55%)
High[b]	66 (45%)
Graft source	
BM	110 (75%)
Peripheral blood	37 (25%)
Preconditioning	
Myeloablative	128 (87%)
Reduced-intensity	19 (13%)
Acute GVHD	
0	82 (56%)
I	37 (25%)
II	19 (13%)
III-IV	9 (6%)
Chronic GVHD	
None	74 (53%)
Limited	11 (8%)
Extensive	54 (39%)
Relapse at 5 years	40%
Non-relapse mortality at 5 years	21%
OS at 5 years	50%

[a]Acute leukemia in first CR, CML in first chronic phase and myeloid displastic syndrome with IPSS score of 1.0 or lower.
[b]More advanced status than standard-risk disease.

malignancies at the Nagoya University Hospital and the Japanese Red Cross Nagoya First Hospital between 1987 and 2006. A total of 147 recipient-donor pairs were selected according to the following criteria: (1) DNA samples and clinical data were available; (2) an unmanipulated graft was transplanted; and (3) short-term MTX and CsA were used as GVHD prophylaxis. MTX was administered i.v. on day +1 (10 mg/m^2) and on days +3 and +6 (7 mg/m^2 each day). CsA was administered by i.v. infusion at a dose of 3.0 mg per kg at beginning on day −1.

Patient characteristics are summarized in Table 1. A total of 81 patients (55%) were classified as having standard-risk disease defined as acute leukemia in first CR, CML in first chronic phase and myelodysplastic syndrome with an international prognostic scoring system (IPSS) score of 1.0 or lower, whereas 66 patients (45%) had high-risk disease defined as disease of more advanced status than standard risk disease. Graft source was BM for 110 patients (75%) and peripheral blood for 37 patients (25%). Conditioning was myeloablative for 128 patients (87%) and reduced-intensity for 19 patients (13%).

Informed consent was obtained from all patients and donors. The study was approved by the ethics committees at the Nagoya University Hospital, the Japanese Red Cross Nagoya First Hospital and the Tokai University School of Medicine.

CTLA-4 genotyping

Genomic DNA was obtained from donor peripheral blood or BM using the QIAamp DNA Blood Mini Kit (QIAGEN sciences, Germantown, MD, USA). The TaqMan PCR method was used to determine the three single-nucleotide polymorphism (SNP) genotypes of CTLA-4: −318 (rs5742909), +49 (rs231775) and CT60 (rs3087243). The respective primers and probes used for the TaqMan PCR were: −318 C/T, forward 5′-AAATGAATTGGACTGG ATGGT-3′ and reverse 5′-TTACGAGAAAGGAAGCC GTG-3′, probe 5′-GTCTCCACTTAGTTATCCAGATCC T[C/T]AAAGTGACATGAAGCTTCAGTTTC-3′; +49 A/G, forward 5′-GCTCTACTTCCTGAAGACCT-3′ and reverse 5′-AGTCTCACTCACCTTTGCAG-3′, probe 5′-G CACAAGGCTCAGCTGAACCTGGCT[A/G]CCAGGA CCTGGCCCTGCACTCTCCT-3′; CT60 A/G, forward 5′-ATCTGTGGTGGTCGTTTTCC-3′ and reverse 5′-CC ATGACAACTGTAATGCCTGT-3′, probe 5′-TCTTCAC CACTATTTGGGATATAAC[A/G]TGGGTTAACACAG ACATAGCAGTCC3′.

PCR reactions were performed in a 10-μL reaction volume containing 1 × TaqMan Universal Master Mix (Applied Biosystems, Tokyo, Japan), 1 μmol of each primer, 1 μL of each probe and 1 μL of genomic DNA. Thermal cycle conditions were 95 °C for 10 min, 40 cycles of 92 °C for 15 s and 60 °C for 1 min. All PCR and endpoint fluorescent readings were analyzed using an ABI7900 sequence detection system (Applied Biosystems).

Statistical analysis

OS was calculated from the date of transplantation to the date of death using the Kaplan–Meier method, and *P*-values were calculated using a log-rank test. EFS was calculated from the date of transplantation to the date of death or relapse, whichever occurred first, and *P*-values were calculated using a log-rank test. Non-relapse mortality (NRM) was defined as mortality due to any cause other than relapse or disease progression. Cumulative incidences of NRM and relapse were estimated using Gray's test, with relapse and NRM, respectively, as a competing risk.

Acute GVHD was diagnosed and graded according to consensus criteria.[20] Chronic GVHD was evaluated in patients who survived beyond day +100, and was categorized as limited or extensive.[21] A multivariate Cox model was created for analysis of grade II-IV acute GVHD, grade III-IV acute GVHD, chronic GVHD, OS, NRM, relapse and EFS using stepwise selection at a significance level of 5%. Age, sex, disease risk, conditioning regimen and graft source were used as covariates, and those variables with a *P*-value of less than 0.2 in the univariate analysis were entered into the stepwise selection method. Hazard ratios of the CTLA-4 haplotype CAA were adjusted using these models. Analysis was carried out using STATA (StataCorp. 2007; Stata Statistical Software: Release 10.0. Special Edition. Stata Corporation, College Station, TX, USA). *P*-values of less than 0.05 were

regarded as statistically significant, and *P*-values between 0.05 and 0.1 as suggesting a trend.

Results

Frequencies of CTLA-4 genotypes and haplotypes

Frequencies at which the three CTLA-4 SNPs were expressed in the 147 donors are listed in Table 2. The SNPs −318 (rs5742909), +49 (rs231775) and CT60 (rs3087243) were included in one haplotype block that was constructed using the international HapMap database. The haplotype analysis revealed only three haplotypes in the Japanese population: −318*C/+49*G/CT60*G (C-G-G), −318*C/+49*A/CT60*A (C-A-A) and −318*T/+49*A/CT60*G (T-A-G). In this cohort, the frequencies of the haplotype C-G-G, C-A-A and T-A-G were 59.5, 30.6 and 9.9%, respectively. All of the donors were distributed among the six haplotype combinations (Table 3).

Effect of the CTLA-4 haplotype C-A-A on transplant outcome

It has been shown that the donor −318 C allele is associated with a lower risk of relapse[19] and that the donor CT60 AA genotype is associated with a lower risk of relapse and a higher OS.[17] We therefore focused our analysis on the C-A-A haplotype, and examined the association between the C-A-A haplotype and the outcome after allogeneic HSCT.

The incidence of grade II-IV acute GVHD was 19% for all patients (Table 1). There was no significant difference between the cumulative incidences of grade II-IV acute GVHD in patients who received stem cells from a donor with the C-A-A haplotype (21%) or from a donor without the C-A-A haplotype (17%) ($P=0.66$) (Figure 1a).

Of 147 patients, 139 could be evaluated for chronic GVHD, and the incidence of chronic GVHD was 47% (Table 1). The incidence of chronic GVHD was not significantly different in the presence or absence of the C-A-A haplotype (51 vs 47%, $P=0.81$) (Figure 1b). Recipients of donors with the C-A-A haplotype showed a significantly lower incidence of relapse (28 vs 45%, $P=0.049$) and a higher OS (58 vs 36%, $P=0.033$) than recipients of donors without the C-A-A haplotype (Figures 2a and b). However, there was no significant difference in NRM between recipients of donors with or without the C-A-A haplotype (17% for both) ($P=0.87$).

Multivariate analyses showed that age >40 years was a risk factor for chronic GVHD, NRM, OS and EFS; that high-risk disease was a risk factor for relapse, OS and EFS; and that reduced intensity conditioning was a risk factor for chronic GVHD and relapse; and that PBSCT was a risk factor for acute and chronic GVHD. The hazard ratios of the C-A-A haplotype, adjusted by these factors, are listed in Table 4. The C-A-A haplotype was significantly associated with a lower relapse rate (HR: 0.54, 95%, CI: 0.30–0.97, $P=0.040$). Additionally, the group with the C-A-A haplotype exhibited trends toward higher OS (HR: 0.61, 95%, CI: 0.36–1.0, $P=0.054$) and EFS (HR: 0.67, 95%CI: 0.41–1.1, $P=0.1$), compared with the group without the C-A-A haplotype. The presence or absence of the C-A-A haplotype did not affect the incidence of acute or chronic GVHD or NRM.

Table 2 Frequency of CTLA-4 genotypes

Polymorphism	n *(%)*
No. of donors	147
−318	
CC	121 (82.4)
CT	23 (15.6)
TT	3 (2.0)
+49	
GG	51 (34.7)
AG	73 (49.7)
AA	23 (15.6)
CT60	
GG	69 (46.9)
AG	66 (44.9)
AA	12 (8.2)

Table 3 Frequencies of the CTLA-4 haplotype

Haplotype	*−318*	*+49*	*CT60*	n *(%)*
C-G-G/C-G-G	CC	GG	GG	51 (34.7)
C-G-G/C-A-A	CC	AG	AG	58 (39.5)
C-G-G/T-A-G	CT	AG	GG	15 (10.2)
C-A-A/C-A-A	CC	AA	AA	12 (8.2)
C-A-A/T-A-G	CT	AA	AG	8 (5.4)
T-A-G/T-A-G	TT	AA	GG	3 (2.0)

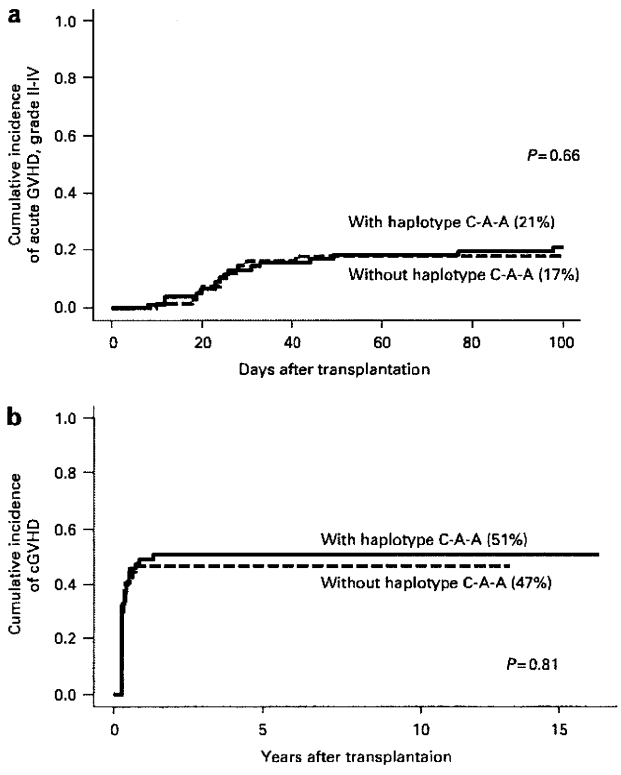

Figure 1 Association of (**a**) the cumulative incidence of grade II-IV acute GVHD and (**b**) chronic GVHD in recipients of donors with (solid line) and without (dotted line) the CTLA-4 C-A-A haplotype.

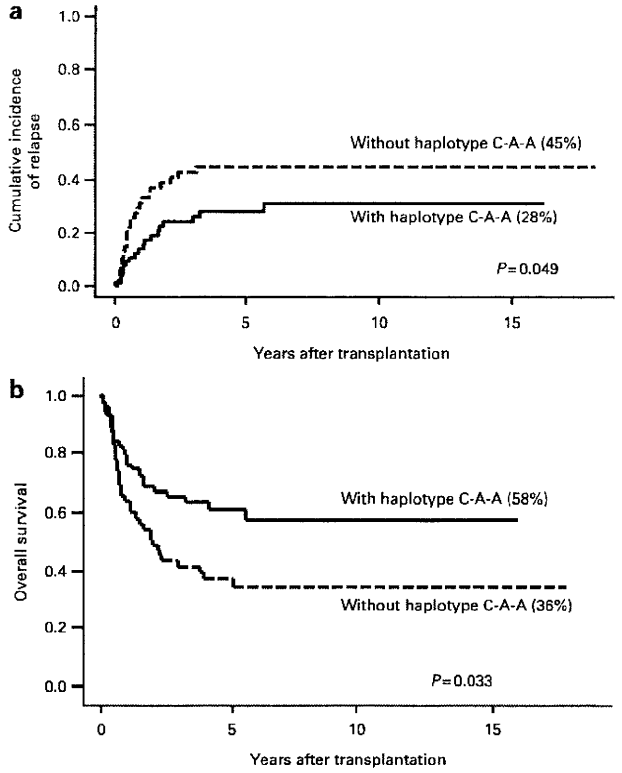

Figure 2 The impact of donors with (solid line) or without (dotted line) the CTLA-4 haplotype C-A-A on the incidence of relapse (**a**) and the OS (**b**) in recipient.

Table 4 Effect of donor CTLA-4 haplotype on transplant outcome

Events	*Factors*	*Multivariate*[a]	
		Hazard ratio (CI)	P-*value*
Acute GVHD Grade II-IV	PBSCT	3.4 (1.6–7.1)	0.001
	Haplotype C-A-A[b]	1.1 (0.53–2.4)	0.77
Acute GVHD Grade III-IV	PBSCT	6.1 (1.5–24)	0.011
	Haplotype C-A-A[b]	1.5 (0.38–6.0)	0.56
Chronic GVHD	Age > 40	1.7 (1.0–2.8)	0.05
	PBSCT	2.0 (1.1–3.5)	0.027
	RIC	0.28 (0.11–0.70)	0.0067
	Haplotype C-A-A[b]	1.0 (0.63–1.7)	0.92
Non-relapse mortality	Age > 40	2.4 (1.0–5.8)	0.042
	Haplotype C-A-A[b]	0.90 (0.39–2.0)	0.79
Relapse	High risk	2.6 (1.3–4.9)	0.005
	RIC	2.3 (1.2–4.5)	0.014
	Haplotype C-A-A[b]	0.54 (0.30–0.97)	0.04
OS	High risk	1.8 (1.1–3.1)	0.025
	Age > 40	1.9 (1.1–3.3)	0.013
	Haplotype C-A-A[b]	0.61 (0.36–1.00)	0.054
EFS	High risk	2.0 (1.3–3.4)	0.004
	Age > 40	1.6 (1.0–2.7)	0.046
	Haplotype C-A-A[b]	0.67 (0.41–1.1)	0.1

Abbreviation: RIC = reduced intensity conditioning.
[a]Age, sex, disease risk, conditioning regimen, graft source were used as covariates.
[b]Adjusted by significant factors.

Discussion

Our results highlight the impact of the donor CTLA-4 haplotype−318*C/+49*A/CT60*A (C-A-A) on outcome after allogeneic HSCT from an HLA-identical sibling.

It has been shown that the donor −318 C allele is associated with a lower risk of relapse[19] and that the donor CT60 AA genotype correlates with a lower risk of relapse and a higher OS.[17] Therefore, we focused on the C-A-A haplotype from three different haplotypes in the Japanese population, and examined the association between the C-A-A haplotype and the outcome after allogeneic HSCT. The presence of the CTLA-4 C-A-A haplotype exhibited a significantly lower incidence of relapse and a trend toward of a higher survival rate compared with the absence of the haplotype C-A-A, suggesting that the C-A-A haplotype might suppress the inhibitory function of CTLA-4 on tumor-reactive T cells and enhance the GVL effect.

The mechanism by which the C-A-A haplotype exerts its positive effects is still unclear. Several studies addressing the functional consequences of CTLA-4 SNP-318 and CT60 have been reported. The SNP−318 is located at the CTLA-4 promoter region, and the association of these alleles with promoter activity has been examined. Previous studies showed that the −318 C allele correlates with a lower promoter activity and a lower CTLA-4 expression than those observed with the −318 T allele.[22,23] The *CTLA-4* gene is composed of four exons and has two isoforms: a full-length isoform (flCTLA-4) and a soluble form (sCTLA-4) that lacks exon 3, which encodes the transmembrane domain. Serum levels of sCTLA-4 increase in patients with various autoimmune diseases[24,25] and sCTLA-4 has the potential to bind to CD80/CD86,[26,27] suggesting that sCTLA-4 blocks the interaction of flCTLA-4 with CD80/CD86 and thereby enhances T-cell activation. It has been reported that the CT60 A allele is associated with a higher level of the sCTLA-4 mRNA than the CT60 G allele.[17,28] These results indicate that the −318 C allele and the CT60 A allele contribute to the reduction in CTLA-4 inhibitory function and to T-cell activation. However, association of the +49 A allele with a higher expression of CTLA-4 and augmentation of CTLA-4 inhibitory function has been reported.[16,29] Therefore, further investigation is required to elucidate the effect of the C-A-A haplotype on the anti-tumor activity of donor-derived T cells.

Although the C-A-A haplotype was associated with a low incidence of relapse, in this study it did not affect the incidence of GVHD, suggesting that the C-A-A haplotype may have the potential to separate GVL from GVHD responses. However, it might be because of our small cohort, as Perez-Garcia *et al.*[17] demonstrated that the donor CT60 AA genotype was associated with an increased risk of grade II-IV acute GVHD in a large cohort of 536 donors. All of the patients in our study were Japanese, and many of them (75%) had received BM as a stem-cell graft. It is known that Japanese patients have a lower risk of developing acute GVHD,[30] and that BMT is associated with a decrease in the development of acute GVHD.[31]

Thus, the ethnic population or the stem-cell source might also affect the association between CTLA-4 polymorphisms and the development of acute GVHD.

In summary, the presence of the CTLA-4 C-A-A haplotype reduced the risk of relapse and improved survival after allogeneic HSCT. Therefore, knowledge of the CTLA-4 haplotype may provide useful information for donor selection. The exact effect of the CTLA-4 C-A-A haplotype on transplant outcome should be determined in different cohorts with a substantially larger number of subjects.

Conflict of interest

The authors declare no conflict of interest.

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