Table 2 Distribution of the *MTHFR* C677T polymorphism in patients and donors

	Genotype		
	CC	CT	TT
Patients, *n* (%)			
Total	56 (35)	83 (52)	20 (13)
Acute myeloid leukemia	17 (35)	25 (52)	6 (13)
Acute lymphoblastic leukemia	9 (32)	14 (50)	5 (18)
Chronic myeloid leukemia	14 (36)	20 (51)	5 (13)
Myelodysplastic syndrome	9 (56)	6 (38)	1 (6)
Severe aplastic anemia	3 (20)	10 (67)	2 (13)
Others	4 (30)	8 (61)	1 (8)
Donors, *n* (%)	48 (30)	98 (62)	13 (8)

higher than that in patients with other diseases; however, the distribution pattern of the C677T genotypes in patients with myelodysplastic syndrome was not significantly different from that in healthy volunteers ($P = 0.104$).

3.2 Acute GVHD

Acute GVHD developed in 64 (41%) of 156 evaluable patients. The severity of GVHD was grade I in 38 patients (24%), grade II in 17 patients (11%), grade III in 6 patients (4%), and grade IV in 3 patients (2%). In a univariate analysis, significant associations with a lower incidence of grade I–IV acute GVHD were observed in the patients with a non-malignant disease and the patients with the *MTHFR* 677TT genotype (Table 3). In a multivariate analysis, both a non-malignant disease and the patient *MTHFR* 677TT genotype were significantly associated with lower incidence of grade I–IV acute GVHD. When we performed a multivariate analysis using the patients who received myeloablative regimen ($n = 139$), significant associations with a lower incidence of grade I–IV acute GVHD were observed in the patients with *MTHFR* 677TT genotype ($P = 0.049$) and the patients with a non-malignant disease ($P = 0.035$). In the subset of patients with malignant disease alone ($n = 143$), significant associations with a lower incidence of grade I–IV acute GVHD were observed in the patients with *MTHFR* 677TT genotype ($P = 0.050$) and the patients with a standard disease ($P = 0.011$).

In a univariate analysis, a significant association with a lower incidence of grade II–IV acute GVHD was observed with the use of bone marrow for transplantation. A trend toward a lower incidence of grade II–IV acute GVHD was found for higher patient age, years of transplantation and the use of non-myeloablative regimen. The patient *MTHFR* 677TT genotype was not significantly associated with lower incidence of grade II–IV acute GVHD ($P = 0.11$). In a multivariate analysis, only use of bone marrow was significantly associated with lower incidence of grade II–IV acute GVHD. When we performed a multivariate analysis using the patients who received myeloablative regimen (n = 139), a trend toward a lower incidence of grade II–IV acute GVHD was observed for the use of bone marrow ($P = 0.088$). In the subset of patients with malignant disease alone ($n = 143$), only use of bone marrow was significantly associated with a lower incidence of grade II–IV acute GVHD ($P = 0.011$).

We analyzed the incidence of acute GVHD in relation to the patient *MTHFR* genotype using the Kaplan–Meier method. The incidence of grade I–IV acute GVHD in the patients with the *MTHFR* 677TT genotype was significantly lower than in those with the *MTHFR* 677CC/CT genotype (19 vs. 45%; $P = 0.035$) (Fig. 1a). There was a trend toward a lower incidence of grade II–IV acute GVHD in patients with the 677TT genotype compared with the 677CC/CT genotype (5 vs. 24%; $P = 0.077$) (Fig. 1b).

There was no significant association between the incidence of grade I–IV or grade II–IV acute GVHD and the donor *MTHFR* C677T genotypes in a multivariate analysis (Table 3). We additionally analyzed the incidence of acute GVHD in relation to the donor *MTHFR* genotype using the Kaplan–Meier method. There was no significant association between the incidence of grade I–IV acute GVHD and the *MTHFR* 677TT genotype in the donor (677TT genotype vs. 677CC/CT genotypes, 25 vs. 43%; $P = 0.33$) (Fig. 1c). Grade II–IV acute GVHD was not developed in any patients who were transplanted from the donor with *MTHFR* 677TT genotype.

3.3 Other outcome

Of the 147 evaluable patients, 75 (51%) developed chronic GVHD, including 16 (11%) with a limited type and 59 (40%) with an extensive type. No association was detected between the incidence of chronic GVHD and the *MTHFR* C677T genotypes in the patient or donor or any other factors.

Of all 159 evaluable patients, 28 (18%) were dead without relapse at the time of the survey. In a multivariate analysis, only higher patient age was significantly associated with higher TRM (Table 3). We further assessed the incidence and severity of mucositis/stomatitis and abnormality of liver function based on the peak level within 14 days after transplantation. There was no significant association between mucositis or liver dysfunction and the *MTHFR* genotype in the patient or donor.

Of all 143 evaluable patients with malignant disease, 39 (27%) relapsed after transplantation. In a multivariate analysis, only the advanced disease was significantly associated with higher relapse rate (Table 3).

Table 3 Univariate and multivariate analyses of risk factors for transplant outcome

Outcome and significant factor	Univariate analysis	Multivariate analysis	
	P	Relative risk (95% CI)	*P*
Acute GVHD (I–IV)			
Non-malignant disease	0.037	0.22 (0.05–0.89)	0.034
Patient MTHFR 677TT (vs. CC/CT)	0.045	0.35 (0.13–0.95)	0.040
Acute GVHD (II–IV)			
Higher patient age[a]	0.052		NS
Years of transplantation[a]	0.063		NS
Non-myeloablative regimen	0.086		NS
Bone marrow	0.001	0.32 (0.11–0.91)	0.032
Treatment-related mortality			
Higher patient age[a]	0.0007	1.07 (1.02–1.11)	0.003
Acute GVHD (I–IV)	0.002		NS
Acute GVHD (II–IV)	<0.0001		NS
Patient MTHFR 677CC (vs. CT/TT)	0.081		NS
Relapse rate			
Higher patient age[a]	0.050		NS
Advanced disease	0.002	2.74 (1.37–5.47)	0.004
Relapse-free survival			
Higher patient age[a]	0.0003	1.04 (1.02–1.07)	0.001
Advanced disease	0.002		NS
Bone marrow	0.087		NS
Acute GVHD (I–IV)	0.056		NS
Acute GVHD (II-IV)	0.012		NS
Overall survival			
Higher patient age[a]	<0.0001	1.05 (1.03–1.08)	0.0002
Advanced disease	0.023		NS
Non-malignant disease	0.089		NS
Bone marrow	0.067		NS
Acute GVHD (I–IV)	0.002		NS
Acute GVHD (II–IV)	0.001		NS

CI indicates confidence interval, *GVHD* graft-versus-host disease, *MTHFR* 5,10-methylenetetrahydrofolate reductase, *NS* not significant

[a] Continuous variable

Of the 143 evaluable patients with a malignant disease, 79 (55%) were alive without relapse at the time of survey. In a multivariate analysis, only higher patient age was significantly associated with lower RFS (Table 3). Similar to the results with RFS, only higher patient age was significantly associated with lower OS (Table 3).

4 Discussion

To evaluate the impact of the *MTHFR* C677T polymorphism on the outcome of allogeneic HSCT, we determined the frequencies of the *MTHFR* C677T genotypes in patients with a hematological disease and their HLA-identical sibling donors. The frequencies of CC, CT, and TT genotypes in the healthy volunteers were 30, 62, and 8%, respectively, which was compatible with the reported distribution in the Japanese population [23]. The frequencies of these genotypes in the studied patients with a hematological disease were 35, 52, and 13%, respectively. There was no significant difference in distribution patterns of the CC, CT, and TT genotypes between donors and all patients. In a previous meta-analysis, the *MTHFR* 677TT genotype was shown to correspond to a reduced risk of acute lymphoblastic leukemia [24], whereas in our study, the 677TT genotype was not significantly associated with a reduction of the risk for acute lymphoblastic leukemia.

The present study demonstrated that the *MTHFR* 677TT genotype in adult patients is significantly associated with a lower incidence of grade I–IV acute GVHD after HSCT from HLA-identical sibling donors in patients receiving prophylactic MTX. There was no significant association between grade II–IV acute GVHD and the C677T polymorphism in patients according to multivariate analysis, but the Kaplan–Meier analysis showed a trend toward a lower incidence of grade II–IV acute GVHD in the patients

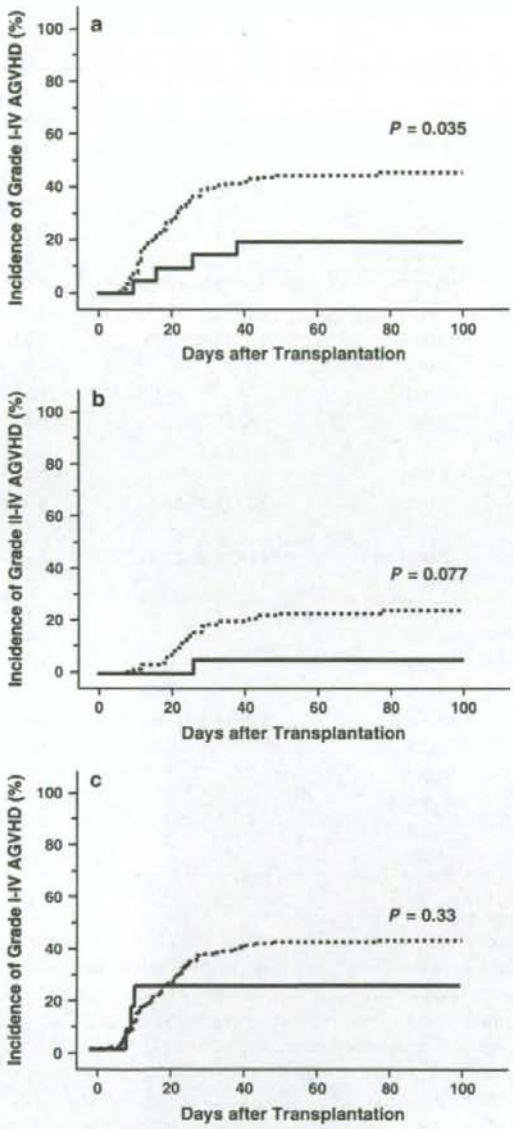

Fig. 1 Impact of patient *MTHFR* genotype on the incidence of acute GVHD. **a** The incidences of grade I–IV acute GVHD in the *MTHFR* 677TT patients (*solid line*; $n = 20$) and *MTHFR* 677CC/CT patients (*dotted line*; $n = 136$) were analyzed by the Kaplan–Meier method. **b** The incidences of grade II–IV acute GVHD in the *MTHFR* 677TT patients (*solid line*; $n = 20$) and in *MTHFR* 677CC/CT patients (*dotted line*; $n = 136$) were analyzed by the Kaplan–Meier method. **c** The incidences of grade I–IV acute GVHD in the patients who were transplanted from the donor with the *MTHFR* 677TT genotype (*solid line*; $n = 13$) and *MTHFR* 677CC/CT genotypes (*dotted line*; $n = 146$) were analyzed by the Kaplan–Meier method

with the 677TT genotype than the patients with the 677CC/CT genotypes (5 vs. 24%; $P = 0.077$). Of the 156 evaluable patients, only 26 (17%) patients developed grade II–IV acute GVHD. Because the incidence of severe (grade II–IV) acute GVHD is low in the Japanese population [25], further analysis in a larger study population is warranted to determine whether *MTHFR* C677T polymorphism is associated with development of grade II–IV acute GVHD.

MTX inhibits the intracellular activity of dihydrofolate reductase and other folate enzymes including MTHFR, a key enzyme in folate metabolism [26]. Although the hematopoietic cells are of donor origin after HSCT, the majority of systemic MTHFR activity remains that of the host tissues. In this study we apparently demonstrated the lower risk of acute GVHD in the *MTHFR* 677TT patients receiving prophylactic MTX for GVHD. This is consistent with the idea that patient *MTHFR* genotypes play a vital role in the overall availability of folate necessary for tissue repair and donor lymphocyte growth and activity [15].

In contrast to our findings, another study demonstrated that the *MTHFR* C677T polymorphism in the donor but not patient is associated with a higher incidence of acute GVHD [16]. They showed a significant association between the *MTHFR* 677CT or TT genotype in HLA-matched related donors and a decreased incidence of acute and chronic GVHD. The authors discussed the possibility that alloreactive T cells with low MTHFR enzyme activity could be negatively selected, because 677TT-genotyped lymphocytic cells show rapid growth in vitro and are more sensitive to MTX [27, 28]. The differences between their and our findings may be due to their inclusion of patients (~27%) who had MTX dose reductions due to severe mucositis or hepatic dysfunction and/or differences between Caucasians and Japanese.

Our study could not detect a significant association between C677T polymorphism and mucositis, liver dysfunction or TRM. However, several studies have shown that the polymorphism of MTHFR is associated with an increased risk of MTX toxicity in patients with acute leukemia [29, 30], ovarian cancer [31], breast cancer [32], or HSCT recipients [33–35]. Ulrich et al. measured the oral mucositis index score [36] in patients with chronic myelogenous leukemia that received HSCT with MTX for the prophylaxis of GVHD [33]. They found that patients with lower MTHFR activity (677TT genotype) had a higher mean oral mucositis index during days 1–18 and a slower recovery of platelet counts than the patients with a 677CC genotype. They later increased the size of the study cohort and confirmed that the *MTHFR* C677T genotype is a strong predictor of oral mucositis [34]. In addition, Kim et al. [35] investigated liver function in MTX-treated patients receiving HSCT from an HLA-identical sibling donor.

They found increased peak bilirubin and aspartic transaminase levels in patients with the 677TT genotype. Further analysis of the influence of the *MTHFR* C677T polymorphism focusing on treatment-related toxicities is of considerable interest.

It is expected that determination of genetic polymorphisms in the recipient and/or donor will allow for better prediction of the development of HSCT-related complications. Ours and other's studies suggest a decreased risk of GVHD and other treatment-related toxicities in patients with the *MTHFR* 677TT genotype who received MTX as prophylaxis for GVHD. However, how *MTHFR* polymorphisms affect the biological mechanisms of MTX remains unclear despite the fact that the molecular and cellular effects of MTX on cancer cells have been well characterized [37]. Accordingly, it is not yet known whether there is an association between low MTHFR activity and a decreased risk of acute GVHD or higher risk of TRM in patients treated without MTX [38]. Further studies are needed to confirm that *MTHFR* C677T polymorphism can predict the outcome of HSCT using prophylactic MTX to prevent GVHD.

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Identification of human minor histocompatibility antigens based on genetic association with highly parallel genotyping of pooled DNA

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Identification of human minor histocompatibility antigens based on genetic association with highly parallel genotyping of pooled DNA

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Minor histocompatibility (H) antigens are the molecular targets of allo-immunity responsible both for the development of antitumor effects and for graft-versus-host disease (GVHD) in allogeneic hematopoietic stem cell transplantation (allo-HSCT). However, despite their potential clinical use, our knowledge of human minor H antigens is largely limited by the lack of efficient methods of their characterization. Here we report a robust and efficient method of minor H gene discovery that combines whole genome association scans (WGASs) with cytotoxic T-lymphocyte (CTL) assays, in which the genetic loci of minor H genes recognized by the CTL clones are precisely identified using pooled-DNA analysis of immortalized lymphoblastoid cell lines with/without susceptibility to those CTLs. Using this method, we have successfully mapped 2 loci: one previously characterized (*HMSD* encoding ACC-6), and one novel. The novel minor H antigen encoded by *BCL2A1* was identified within a 26 kb linkage disequilibrium block on chromosome 15q25, which had been directly mapped by WGAS. The pool size required to identify these regions was no more than 100 individuals. Thus, once CTL clones are generated, this method should substantially facilitate discovery of minor H antigens applicable to targeted allo-immune therapies and also contribute to our understanding of human allo-immunity. (Blood. 2008;111: 3286-3294)

Introduction

Currently, allogeneic hematopoietic stem cell transplantation (allo-HSCT) has been established as one of the most effective therapeutic options for hematopoietic malignancies[1] and is also implicated as a promising approach for some solid cancers.[2] Its major therapeutic benefits are obtained from allo-immunity directed against patients' tumor cells (graft-versus-tumor [GVT] effects). However, the same kind of allo-immune reactions can also be directed against normal host tissues resulting in graft-versus-host disease (GVHD). In HLA-matched transplants, both GVT and GVHD are initiated by the recognition of HLA-bound polymorphic peptides, or minor histocompatibility (H) antigens, by donor T cells. Minor H antigens are typically encoded by dichotomous single nucleotide polymorphism (SNP) alleles, and may potentially be targeted by allo-immune reactions if the donor and recipient are mismatched at the minor H loci. Identification and characterization of minor H antigens that are specifically expressed in hematopoietic tissues, but not in other normal tissues, could contribute to the development of selective antileukemic therapies while minimizing unfavorable GVHD reactions, one of the most serious complications of allo-HSCT.[3,4] Unfortunately, the total number of such useful minor H antigens that are currently molecularly characterized is still disappointingly small, including HA-1,[5] HA-2,[6] ACC-1[Y] and ACC-2,[7] DRN-7,[8] ACC-6,[9] LB-ADIR-1F,[10] HB-1,[11] LRH-1,[12] and 7A7-PANE1,[13] limiting the number of patients eligible for such GVT-oriented immunotherapy.

Several techniques have been developed to identify novel minor H antigens targeted by CTLs generated from patients who have undergone transplantation. Among these, linkage analysis based on the cytotoxicity of the CTL clones against panels of lymphoblastoid cell lines (B-LCLs) from large pedigrees was proposed as a novel genetic approach,[14] and has been successfully applied to identify novel minor H epitopes encoded by the *BCL2A1* and *P2RX5* genes.[7,12] Nevertheless, the technology is still largely limited by its resolution, especially when large segregating families are not available. Linkage analysis using B-LCL panels from the Centre d'Etude du Polymorphisme Humain (CEPH) could only localize minor H loci within a range of 1.64 Mb to 5.5 Mb, which still contained 11 to 46 genes,[7,12,14] thus requiring additional selection procedures to identify the actual minor H genes.

On the other hand, clinically relevant minor H antigens might be associated with common polymorphisms within the human

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T.K. and Y.N. contributed equally to this work.

population, and therefore could be ideal targets of genetic association studies, considering recent advances of large-scale genotyping technologies and the assets of the International HapMap Project.[15,16] In this alternative genetic approach using the extensive linkage disequilibrium (LD) found within the human genome, target loci can be more efficiently localized within relatively small haplotype blocks without depending on limited numbers of recombination events, given the large number of genotyped genetic markers.[17] Moreover, since the presence of a target minor H allele in individual target cells can be determined by ordinary immunologic assays using minor H antigen-specific CTLs, the characterization of minor H antigens should be significantly more straightforward than identifying alleles associated with typical common complex diseases, for which typically weak-to-moderate genetic effects have been assumed.[18]

In this report, we describe a high-performance, cost-effective method for the identification of minor H antigens, in which whole genome association scans (WGASs) are performed based on SNP array analysis of pooled DNA samples constructed from cytotoxicity-positive (CTX^+) and cytotoxicity-negative (CTX^-) B-LCLs as determined by their susceptibility to CTL clones. Based on this method, termed WGA/CTL, we were able to map the previously characterized ACC-6 minor H locus to a 115-kb block containing only 4 genes, including *HMSD*.[9] Moreover, using the same approach, a novel minor H antigen encoded by the *BCL2A1* gene was identified within a 26-kb block containing only *BCL2A1* on chromosome 15q25. Surprisingly, the pool size required to identify these regions was no more than 100 individuals. Thus, this WGA/CTL method has significant potential to accelerate the discovery of minor H antigens that could be used in more selective, and thus more effective, allo-immune therapies in the near future.

Methods

Cell isolation and cell cultures

This study was approved by the institutional review board of the Aichi Cancer Center and the University of Tokyo. All blood or tissue samples were collected after written informed consent was obtained in accordance with the Declaration of Helsinki. B-LCLs were derived from allo-HSCT donors, recipients, and healthy volunteers. B-LCLs were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate.

Generation of CTL lines and clones

CTL lines were generated from peripheral blood mononuclear cells (PBMCs) obtained after transplantation by stimulation with irradiated (33 Gy) recipient PBMCs harvested before HSCT, thereafter stimulated weekly in RPMI 1640 supplemented with 10% pooled human serum and 2 mM L-glutamine. IL-2 was added on days 1 and 5 after the second and third stimulations. CTL clones were isolated by standard limiting dilution and expanded as previously described.[7] CTL-1B9 was isolated from PBMCs harvested on day 30 after transplantation from a patient receiving a marrow graft from his HLA-identical sibling (HLA A11, A24, B39, B51, Cw7, Cw14), and CTL-2A12 has been described recently.[9]

Chromium release assay

Target cells were labeled with 0.1 mCi (3.7 MBq) of ^{51}Cr for 2 hours, and 10^3 target cells/well were mixed with CTL at the effector-to-target (E/T) ratio indicated in a standard 4-hour cytotoxicity. All assays were performed at least in duplicate. Percent specific lysis was calculated as follows: ((Experimental cpm − Spontaneous cpm) / (Maximum cpm − Spontaneous cpm)) × 100.

Immunophenotyping by enzyme-linked immunosorbent assay

B-LCL cells (20 000 per well, which had been retrovirally transduced with restriction HLA cDNA for individual CTLs, if necessary) were plated in each well of 96-well round-bottomed plates, and corresponding CTL clones (10 000 per well) were added to each well. After overnight incubation at 37°C, 50 μL supernatant was collected and released IFN-γ was measured by standard enzyme-linked immunosorbent assay (ELISA).

Construction of pooled DNA and microarray experiments

Genomic DNA was individually extracted from immunophenotyped B-LCLs. After DNA concentrations were measured and adjusted to 50 μg/mL using the PicoGreen dsDNA Quantitation Reagent (Molecular Probes, Eugene, OR), the DNA specimens from CTX^+ and CTX^- B-LCLs were separately combined to generate individual pools. DNA pools were analyzed in pairs using Affymetrix GeneChip SNP-genotyping microarrays (Affymetrix, Tokyo, Japan) according to the manufacturer's protocol,[19,20] where 2 independent experiments were performed for each array type (for more detailed statistical analysis for generated microarray data, see Document S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article).

Estimation of LD blocks

LD structures of the candidate loci were evaluated based on empirical data from the International Hap Map Project (http://www.hapmap.org/).[15] LD data for the relevant HapMap panels were downloaded from the HapMap web site and further analyzed using Haploview software (http://www.broad.mit.edu/mpg/haploview/).[21]

Transfection of 293T cells and ELISA

Twenty thousand 293T cells retrovirally transduced with HLA-A*2402 were plated in each well of 96-well flat-bottomed plates, cultured overnight at 37°C, then transfected with 0.12 μg of plasmid containing full-length *BCL2A1* cDNA generated from either the patient or his donor using Trans IT-293 (Mirus, Madison, WI). B-LCLs of the recipient and his donor were used as positive and negative controls, respectively. Ten thousand CTL-1B9 cells were added to each well 20 hours after transfection. After overnight incubation at 37°C, 50 μL of supernatant was collected and IFN-γ was measured by ELISA.

SNP identification by direct sequencing

Complementary DNA prepared from B-LCLs was polymerase chain reaction (PCR) amplified for the coding region of *BCL2A1* using the following primers: sense: 5′-AGAAGATGACAGACTGTGAATTTGG-3′; antisense: 5′-TCAACAGTATTGCTTCAGGAGAG-3′.

PCR products were purified and directly sequenced with the same primer and BigDye Terminator kit (version 3.1) by using ABI PRISM 3100 (Applied Biosystems, Foster City, CA).

Confirmatory SNP genotyping

Genotyping was carried out using fluorogenic 3′-minor groove binding (MGB) probes in a PCR assay. PCR was conducted in 10-μL reactions containing both allelic probes, 500 nM each of the primers, 1 × TaqMan Universal PCR Master Mix (Applied Biosystems), and 1 μL (100 ng) DNA. PCR cycling conditions were as follows: predenature, 50°C for 2 minutes, 95°C for 10 minutes, followed by 35 cycles of 92°C for 15 seconds and 60°C for 1 minute in a GeneAmp PCR System 9700 (Applied Biosystems). The PCR products were analyzed on an ABI 7900HT with the aid of SDS 2.2 software (Applied Biosystems).

Epitope reconstitution assay

The candidate *BCL2A1*-encoded minor H epitope and its allelic counterpart (DYLQYVLQI) peptides were synthesized by standard Fmoc chemistry. ^{51}Cr-labeled CTX^- donor B-LCLs were incubated with graded concentrations of the peptides and then used as targets in standard cytotoxicity assays.

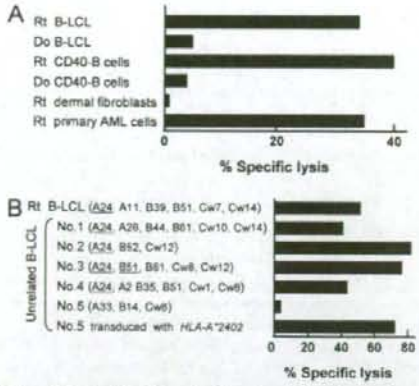

Figure 1. Specificity of CTL-1B9 against hematopoietic cells and its restriction HLA. (A) The cytolytic activity of CTL-1B9 was evaluated in a standard 4-hour ^{51}Cr release assay (E/T ratio, 20:1). Targets used were B-LCL, CD40-activated (CD40-B) B cells, dermal fibroblasts, and primary acute myeloid leukemia cells from the recipient (Rt), and B-LCL and CD40-B cells from his donor (Do). Rt dermal fibroblasts were pretreated with 500 U/mL IFN-γ and 10 ng/mL TNF-α for 48 hours before ^{51}Cr labeling. (B) Cytolytic activity of CTL-1B9 against a panel of B-LCLs derived from unrelated individuals, each of whom shared 1 or 2 class I MHC allele(s) with the recipient from whom the CTL-1B9 was generated. The shared HLA allele(s) with the recipient are underlined. B-LCLs (no. 5) which did not share any HLA alleles with the recipient, were retrovirally transduced with HLA-A*2402 cDNA and included to confirm HLA-A*2402 restriction by CTL-1B9. Results are typical of 2 experiments and data are the mean plus or minus the standard deviation (SD) of triplicates.

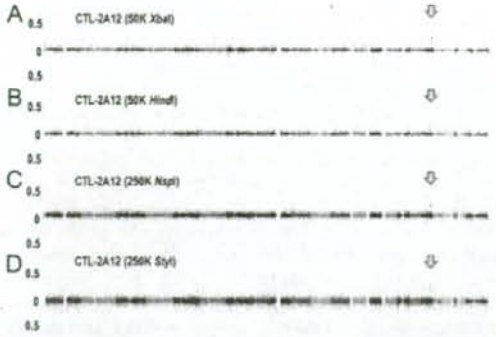

Figure 2. Whole genome association scans performed with pooled DNA generated based on immunophenotyping with CTL-2A12. Pooled DNAs generated from 44 CTX$^+$ and 44 CTX$^-$ B-LCLs were analyzed with 50 K *Xba*I (A), 50 K *Hind*III (B), 250 K *Nsp*I (C), and 250 K *Sty*I (D) arrays. Test statistics were calculated for all SNPs and plotted in the chromosomal order. In all SNP array types, a common association peak is observed at 18q21, to which the minor H antigen for CTL-2A12, encoded by the *HMSD* gene, had been mapped based on expression cloning[9] (arrows).

Results

CTL-based typing and SNP array analysis of pooled DNA

CTL-2A12 and CTL-1B9 are CTL clones established from the peripheral blood of 2 patients with leukemia who had received HLA-identical sibling HSCTs. Each clone demonstrated specific lysis against the B-LCLs of the recipient but not against donor B-LCLs, indicating recognition of minor H antigen (Figure 1A and Kawase et al[9]). The minor H antigen for CTL-2A12 had been previously identified by expression cloning[9]; on the other hand, the target minor H antigen for the HLA-A24–restricted CTL-1B9 clone, which was apparently hematopoietic lineage–specific (Figure 1A) and present in approximately 80% of the Japanese population (data not shown), had not yet been determined. Using these CTL clones, a panel of B-LCLs expressing the restriction HLA (HLA-B44 for CTL-2A12 and HLA-A24 for CTL-1B9) endogenously or retrovirally transduced, were subjected to "immunophenotyping" for the presence or absence of the minor H antigen by ELISA and, if necessary, by standard chromium release assay (CRA). Based on the assay results, for CTL-2A12 we initially collected 44 cytotoxicity-positive (CTX$^+$) and 44 cytotoxicity-negative (CTX$^-$) B-LCLs after screening 132 B-LCLs, while 57 CTX$^+$ and 38 CTX$^-$ B-LCLs were obtained from 121 B-LCLs for CTL-1B9. From these sets of B-LCL panels, pools of DNA were generated and subjected to analysis on Affymetrix GeneChip 100 K and 500 K microarrays in duplicate.[19,20]

Detection of association between minor H phenotypes and marker SNPs

Genetic mapping of the minor H locus was performed by identifying marker SNPs that showed statistically significant deviations in allele-frequencies between CTX$^+$ and CTX$^-$ pools based on the observed allele-specific signals in the microarray experiments. For this purpose, we evaluated the deviations of observed allele ratios between CTX$^+$ and CTX$^-$ pools for each SNP on a given array (Document S1). An SNP was considered as positive for association if its test statistic exceeded an empirically determined threshold that provided a "genome-wide" P value of .05 in duplicate experiments (Document S1, Figures S1,S2, and Table S1). Threshold values for different pool sizes are also provided in Table S2 for further experiments. The positive SNPs eventually obtained for both CTLs are summarized in Table 1, where the 10 SNPs showing the highest test statistics are listed for individual experiments.

Mapping of the minor H loci by WGASs

All the SNPs significantly associated with susceptibility to CTL-2A12 were correctly mapped within a single 115 kb LD block at chromosome 18q21 containing the *HMSD* gene (Figures 2 and 3A), which had been previously shown to encode the ACC-6 minor H antigen recognized by CTL-2A12.[9] According to the above criteria, no false-positive SNPs were reported in any array types (Table 1). Confirmation genotyping of individual B-LCLs from both panels revealed none of the 44 that had been immunophenotyped as CTX$^-$ were misjudged, while 8 of the 44 CTX$^+$ B-LCLs were found to actually carry no minor H-positive allele for ACC-6, which was likely due to the inclusion of individual B-LCLs showing borderline cytotoxicity (data not shown).

On the other hand, positive association of the target minor H antigen with CTL-1B9 was detected in 2 independent loci: SNP rs1879894 at 15q25.1 in 250 K *Nsp*I (Table 1, Figure 4A-B, and Figure S5) and SNP rs1842353 at 8q12.3 in 50 K *Hind*III (Table 1 and Figure S3A). We eventually focused on rs1879894, as it showed a much more significant genome-wide P value than SNP rs1842353 (Table 1). In contrast to the CTL-2A12 case, where many mutually correlated SNPs around the most significant one created a broad peak in the statistic plots (Figure 2 arrows and Figure S3), the adjacent SNPs (rs6495463 and rs2562756; Figure 3B solid arrows) around rs187894 (Figure 3B open arrow) did not show large test statistic values, reflecting the fact that no marker SNPs on 100 K and 500 K arrays exist in high LD (Figure 3B dashed red lines encompassing 26 kb) with this SNP according to the HapMap data. To further confirm the association, we generated additional B-LCL pools consisting of 75 CTX$^+$ and 34 CTX$^-$

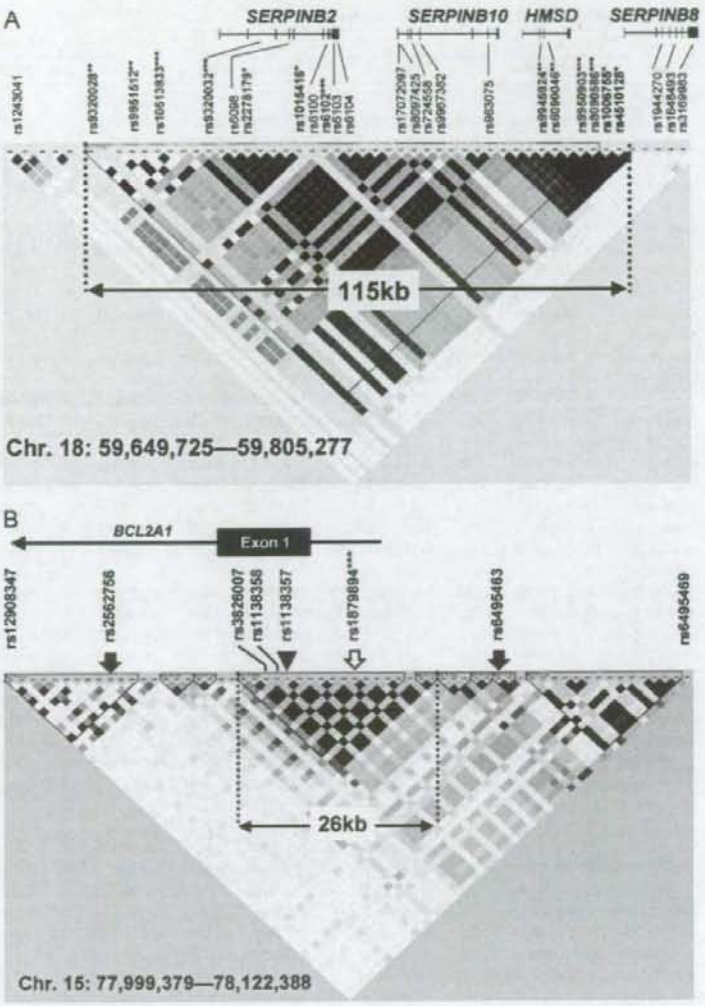

Figure 3. Linkage disequilibrium (LD) block mapped by CTL-2A12 and CTL-1B9. (A) An LD block map identified by pairwise r^2 plot from HapMap CEU data are overlayed with SNPs available from Affymetrix GeneChip SNP-genotyping microarrays (arrows) and 4 genes in the 115 kb block. SNPs that emerged repeatedly in the 2 independent experiments are indicated in blue. The genomewide *P* values for positive SNPs are shown as follows: $*P < .05$; $**P < .01$; $***P < .001$. The intronic SNP (rs9945924) controlling the alternative splicing of *HMSD* transcripts and expression of encoded ACC-6 minor H antigen is indicated in red. (B) LD blocks identified by pairwise r^2 plot from HapMap JPT data are overlayed with SNPs available from Affymetrix GeneChip SNP-genotyping microarrays (arrows) and exon 1 of the *BCL2A1* gene. The only SNP showing a high association with CTL-1B9 immunophenotypes (rs1879894) is shown as an open arrow. The nonsynonymous SNP (rs1138357) controlling the expression of the minor H antigen recognized by CTL-1B9 is indicated by a red arrowhead. ***SNP with genomewide $P < .001$. The 2 SNPs adjacent to the 26 kb LD block (rs2562756 and rs6495463) never gave a significant genomewide *P* value.

B-LCLs from another set of 128 B-LCLs, and performed a WGAS. As expected, the WGAS of the second pools also identified the identical SNP with the highest test statistic value in duplicate experiments, unequivocally indicating that this SNP is truly associated with the minor H locus of interest (Figure 4C,D and Table S3). The association was also detected when the references in the first and second pools were swapped (data not shown).

Identification of the minor H epitope recognized by CTL-1B9

The LD block containing SNP rs1879894 that was singled out from more than 500 000 SNP markers with 2 sets of DNA pools only encodes exon 1 of *BCL2A1* (Figure 3B). To our surprise, this was the region to which we had previously mapped an HLA-A24–restricted minor H antigen, ACC-1[Y].[7] We first confirmed that full-length *BCL2A1* cDNA cloned only from the recipient but not his donor could stimulate interferon-γ secretion from CTL-1B9 when transduced into donor B-LCL (Figure 5A), indicating that *BCL2A1* is a bona fide gene encoding minor H antigen recognized by CTL-1B9. We next genotyped 3 nonsynonymous SNPs in the *BCL2A1* exon 1 sequence (Figure 3B) and comparison was made between the genotypes and the susceptibility to CTL-1B9 of 9 HLA-A*2402$^+$ B-LCLs, including ones generated from the recipient (from whom CTL-1B9 was established) and his donor. Susceptibility to CTL-1B9 correlated completely with the presence of guanine at SNP rs1138357 (nucleotide position 238, according to the mRNA sequence for NM_004049.2) and thymine at SNP rs1138358 (nucleotide position 299) (Table 2), suggesting that the expression of the minor H epitope recognized by CTL-1B9 is controlled by either of these SNPs. We searched for nonameric amino acid sequences spanning the 2 SNPs using BIMAS software,[22] since most reported HLA-A*2402 binding peptides contain 9 amino acid residues.[23] Among these, a nonameric peptide, DYLQCVLQI (the polymorphic residue being underlined), has a predicted binding score of 75 and was considered as a candidate minor H epitope. As shown in Figure 5B, the DYLQCVLQI was strongly recognized by CTL-1B9, whereas its allelic counterpart,

Table 1. Positive SNPs from pooled DNA analysis

CTL-2A12, Exp 1				CTL-2A12, Exp 2				CTL-1B9, Exp 1				CTL-1B9, Exp 2			
rsID	Chr	Position	$\Delta R_{A\Delta R_B}$	rsID	Chr	Position	$\Delta R_{A\Delta R_B}$	rsID	Chr	Position	$\Delta R_{A\Delta R_B}$	rsID	Chr	Position	$\Delta R_{A\Delta R_B}$
50K X bal															
rs10513933	18	59699669	0.366*	rs10513933	18	59699669	0.511†	rs1363258	5	103297593	0.239	rs10499174	6	131209689	0.352*
rs9320028	18	59668150	0.255‡	rs9320028	18	59668150	0.360*	rs726083	3	67093729	0.203	rs30058	5	122325602	0.240
rs6102	18	59721450	0.221	rs10485873	7	3503743	0.157	rs639243	5	31392931	0.198	rs150724	16	61960443	0.213
rs724533	23	116440574	0.137	rs219323	14	59510440	0.150	rs1936461	10	56519024	0.186	rs1993129	8	63618836	0.208
rs1341112	6	104919391	0.136	rs10506892	12	82478539	0.147	rs763876	12	94922502	0.186	rs356946	13	69066751	0.201
rs470490	18	61182216	0.136	rs10492269	12	97786333	0.144	rs958404	7	133054441	0.179	rs2869268	4	86421898	0.184
rs2826718	21	21471423	0.134	rs10483466	14	35986827	0.139	rs10486727	7	41672315	0.178	rs287002	12	40312537	0.183
rs10506697	12	73241741	0.128	rs5910124	23	116408616	0.137	rs2833488	21	32010112	0.176	rs1146808	13	67688608	0.182
rs10506891	12	82393029	0.127	rs10512545	17	66337079	0.134	rs379212	5	60977687	0.172	rs10501287	11	42446011	0.180
rs308995	14	59657919	0.125	rs295678	5	58186928	0.131	rs1954004	14	58627872	0.170	rs564993	5	31393476	0.177
50K *Hind*III															
rs9320032	18	59712191	0.486†	rs9320032	18	59712191	0.506†	rs1842353	8	63617543	0.244*	rs9300692	13	101216476	0.225‡
rs8090046	18	59773066	0.207‡	rs8090046	18	59773066	0.245*	rs10521202	17	12755289	0.201‡	rs1842353	8	63617543	0.210‡
rs1474220	2	108525317	0.193‡	rs10498752	6	41876488	0.210‡	rs7899961	10	59696431	0.198‡	rs10520983	5	31314700	0.195‡
rs10498752	6	41876488	0.178	rs1941538	18	37994337	0.176	rs9320974	6	124421441	0.197‡	rs1334375	13	80897038	0.173
rs2298578	21	21632551	0.167	rs7682770	4	152748018	0.174	rs10520983	5	31314700	0.179	rs10519164	15	75412758	0.163
rs7516032	1	91618962	0.165	rs1445862	5	3675257	0.169	rs1862446	5	147460749	0.170	rs9322063	6	146852196	0.152
rs5030938	10	70645922	0.164	rs4696976	4	21058616	0.167	rs1358778	20	13266796	0.169	rs8067384	17	37926265	0.150
rs1883041	21	44921845	0.158	rs5030938	10	70645922	0.165	rs1873790	4	83422480	0.166	rs10521202	17	12755289	0.147
rs3902916	4	189045176	0.155	rs3902916	4	189045176	0.165	rs1220724	4	70888705	0.162	rs7914904	10	62749969	0.141
rs1000551	20	58709208	0.154	rs1883041	21	44921845	0.164	rs9300692	13	101216476	0.157	rs1220724	4	70888705	0.141
250K *Nsp*I															
rs9950903	18	59781783	0.534†	rs9950903	18	59781783	1.036†	rs1879894	15	78055874	0.846†	rs1879894	15	78055874	1.072†
rs1463835	3	23539615	0.532†	rs8090586	18	59781864	0.518†	rs9646294	16	6110019	0.484†	rs6771859	3	190642054	0.387†
rs16975459	18	37802275	0.383*	rs6473170	8	80664840	0.338*	rs17734332	5	134945240	0.365†	rs10512261	9	98804394	0.299*
rs8090586	18	59781864	0.367*	rs4510128	18	59782312	0.310‡	rs566619	7	41381538	0.345*	rs12122772	1	60384564	0.287*
rs16872621	4	22081055	0.312‡	rs1006755	18	59782026	0.300‡	rs17737566	6	50345280	0.310*	rs2153155	4	26034162	0.248‡
rs870582	6	125097114	0.301‡	rs7039378	9	118735938	0.258	rs3849955	9	28350374	0.285*	rs17126896	14	53320494	0.246‡
rs1015416	18	59720363	0.270‡	rs1860563	16	6418899	0.258	rs4616156	13	86561518	0.273*	rs1328652	13	35607527	0.240
rs2155907	11	97599883	0.227	rs4699126	4	105709109	0.212	rs2484698	1	217474460	0.263*	rs7021551	9	27446645	0.237
rs2112948	5	50994294	0.222	rs10275055	7	156212079	0.204	rs17139603	11	79638632	0.262*	rs252817	5	106752487	0.237
rs2919747	2	129681506	0.217	rs1526411	7	124658309	0.201	rs2156737	4	100642529	0.246‡	rs10772587	12	12681356	0.235
250K *Sty*I															
rs6102	18	59721450	0.597†	rs6102	18	59721450	0.495†	rs9383925	6	151975774	0.819†	rs201204	6	104842863	0.688†
rs9951512	18	59690885	0.374*	rs9945924	18	59771746	0.407*	rs6497397	16	19646258	0.311‡	rs12556155	23	108836419	0.442†
rs6496897	15	90493249	0.320‡	rs9951512	18	59690885	0.317‡	rs917252	7	22219990	0.289‡	rs4791422	17	10605304	0.435†
rs9945924	18	59771746	0.315‡	rs1983205	3	157782892	0.314‡	rs1019403	3	7823997	0.260‡	rs7749012	6	106459559	0.336*
rs12707805	8	107404746	0.303‡	rs950865	5	2720684	0.307‡	rs17053134	5	155373544	0.259‡	rs509951	5	31385483	0.308‡
rs10971778	9	33893184	0.296‡	rs2278179	18	59715512	0.292‡	rs11710880	3	72214965	0.246	rs16879024	8	32225711	0.256‡
rs6565076	16	81487818	0.294‡	rs10427722	22	36417752	0.289‡	rs17167866	7	13919264	0.237	rs2100054	15	75293482	0.252
rs2278179	18	59715512	0.291‡	rs17156659	7	82046820	0.271	rs10867062	9	137935241	0.237	rs11811023	1	143805934	0.240
rs7806238	7	29906442	0.290‡	rs4502324	18	4811261	0.262	rs5925800	23	23278707	0.235	rs17382798	15	75256074	0.231
rs965888	18	38062658	0.283‡	rs1348428	2	225927288	0.260	rs2255831	4	146614313	0.234	rs2030302	17	12526591	0.231

Significant SNPs that appeared on both experiments are underlined.
*Genomewide $P < .01$.
†Genomewide $P < .001$.
‡Genomewide $P < .05$.

DYLQ*Y*VLQI, was not. Decameric peptide, QDYLQ*C*VLQI, on the other hand, appeared to be weakly recognized; however, it is likely that the nonameric form was actually being presented after N-terminal glutamine cleavage by aminopeptidase in the culture medium. Because it was possible that the cystine might be cysteinylated, recognition of synthetic peptides DYLQCVLQI and cysteinylated DYLQC*VLQI were assayed using CTL-1B9. Half-maximal lysis for the former was obtained at a concentration of 200 pM, whereas recognition of the latter was several-fold weaker (Figure 5C). Thus, we concluded that DYLQCVLQI defines the cognate HLA-A*2402–restricted CTL-1B9 epitope, now designated ACC-1^C. This incidentally provides a second example of products from both dichotomous SNP alleles being recognized as HLA-A*2402–restricted minor H antigens, the first example being the HB-1 minor H antigen.[24] Finally, real-time quantitative PCR revealed that T cells carrying the complementarity-determining region 3 sequence identical to CTL-1B9 became detectable in the patient's blood at the frequencies of 0.22%, 0.91%, 1.07% and 0.01% among TCR$\alpha\beta^+$ T cells at days 30, 102, 196, and 395 after transplantation, respectively, suggesting that ACC-1^C minor H antigen is indeed immunogenic (Figure 5D).

Discussion

Recent reports have unequivocally demonstrated that WGASs can be successfully used to identify common variants involved in a wide variety of human diseases.[25-27] Our report represents a novel

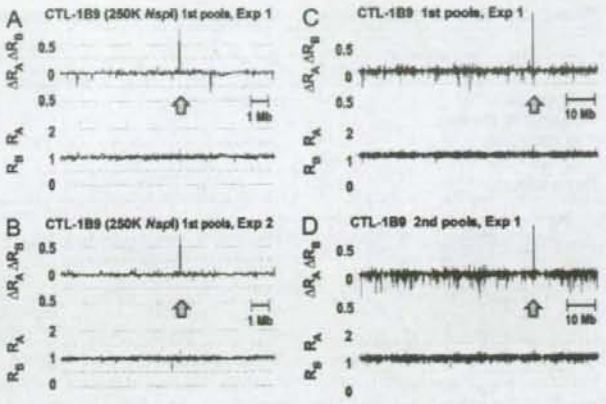

Figure 4. Reproducible detection of association with the immunophenotypes determined by CTL-1B9 at the *BCL2A1* locus. The maximum test statistic value was observed at a single SNP (rs1879894) within 15q25.1 in duplicate experiments for the first pools consisting of 57 CTX$^+$ and 38 CTX$^-$ B-LCLs (A-C). The peak association at the same SNP was reproduced in the experiments with the second pools consisting of 75 CTX$^+$ and 34 CTX$^-$ LCLs (D). Test statistic values ($\Delta R_A \Delta R_B$) are plotted by blue lines together with their R_A (red) and R_B (green) values. The expected $\Delta R_A \Delta R_B$ values multiplied by r^2 correlation coefficients for the adjacent SNPs within 500 kb from the SNP rs1879894 are overlaid by red lines (A,B).

application of WGASs to transplantation immunology, which provides a simple but robust method to fine-map the genetic loci of minor H antigens whose expression is readily determined by standard immunophenotyping with CTL clones established from patients who have undergone transplantation.

The current WGA/CTL method has several desirable features that should contribute to the acceleration of minor H locus mapping. In comparing the method to those of linkage analysis and other nongenetic approaches, including direct peptide sequencing of chemically purified minor H antigens[5,6,10,13] and conventional

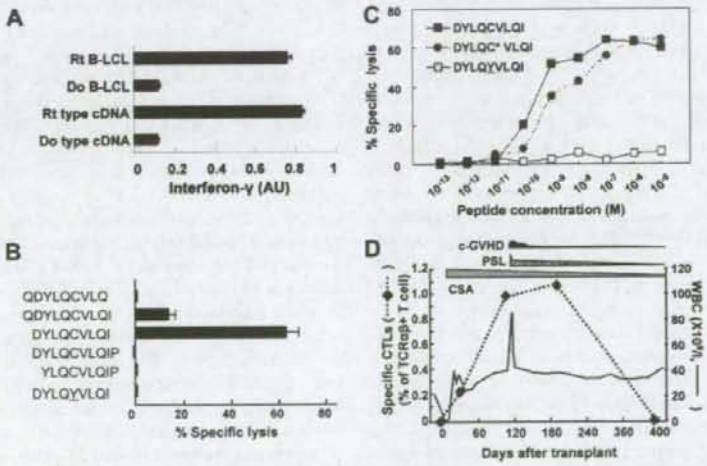

Figure 5. Identification of the CTL-1B9 minimal minor H epitope. (A) Interferon-γ production from CTL-1B9 against HLA-A*2402–transduced 293T cells transfected with plasmid encoding full-length *BCL2A1* cDNA cloned from either the recipient (Rt) from whom CTL-1B9 was isolated or his donor (Do). Rt B-LCL and Do B-LCL were used as positive and negative controls, respectively. Secreted interferon-γ was measured by ELISA and is expressed in arbitrary units (AUs) corresponding to optical density at 630 nm. Results are typical of 2 experiments and data are the mean plus or minus SD of triplicates. (B) A peptide reconstitution assay was conducted to determine the minimal epitope for CTL-1B9. Nonameric peptide (DYLQCVLQI), 2 nonameric peptides shifted by one amino acid to N- or C-terminus, N- and C-terminal extended decameric peptides, and its allelic counterpart (DYLQYVLQI) were synthesized and tested by adding to antigen-negative donor B-LCL at 10 nM in a standard ^{51}Cr release assay. Results are typical of 2 experiments and data are the mean plus or minus SD of triplicates. (C) Titration of the candidate minor H peptide by epitope reconstitution assay. Chromium-labeled donor B-LCLs were distributed to wells of 96-well round-bottomed plates, pulsed with serial dilutions of the indicated peptides for 30 minutes at room temperature, and then used as targets for CTL-1B9 in a standard ^{51}Cr release assay. A cysteinylated peptide (indicated by an asterisk) was included as an alternative form of the potential epitope. Results are typical of 2 experiments. (D) Tracking of ACC-1^{IC}–specific T cells in the recipient's peripheral blood. In order to longitudinally analyze the kinetics of the ACC-1^{IC}–specific CTLs in peripheral blood from the patient from whom CTL-1B9 was established, a real-time quantitative PCR was conducted. Complementary DNAs of peripheral blood mononuclear cells from the donor and patient before and after HSCT were prepared from the patient. Real-time PCR analysis was performed using a TaqMan assay as described previously.[9] The primers and fluorogenic probe sequences spanning the CTL-1B9 complementarity-determining region 3 (CDR3) were used to detect T cells carrying the CDR3 sequences identical to that of CTL-1B9. The primers and fluorogenic probe sequences spanning constant region of TCR beta chain (TCRBC) mRNA were used as internal control. Samples were quantified with the comparative CT method. The delta CT value was determined by subtracting the average CT value for TCRBC from the average CTL-1B9 CDR3 CT value. The standard curve for the proportion of CTL-1B9 among TCRαβ$^+$ T cells was composed by plotting mean delta CT values for each ratio, and the percentages of T cells carrying the CDR3 sequence identical to CTL-1B9 were calculated by using this standard curve. During this period, quiescent chronic GVHD, which required steroid treatment, developed; however, involvement of immune reaction to ACC-1^{IC} minor H antigen was unlikely since its frequency increased even after resolution of most chronic GVHD symptoms. c-GVHD, chronic GVHD; CSA, cyclosporine A; PSL, prednisolone; WBC, white blood cell count.

Table 2. Correlation of *BCL2A1* sequence polymorphisms with susceptibility to CTL-1B9

	HLA-A*2402–positive B-LCLs								
	Rt	Do	UR1	UR2	UR3	UR4	UR5	UR6	UR7
Cytolysis by CTL-1B9	+	−	+	+	+	+	+	−	−
Detected SNP, position*									
rs1138357, 238	G/A	A	G	G	G/A	G/A	G/A	A	A
rs1138358, 299	T/G	G	T	T	T/G	T/G	T/G	G	G
rs3826007, 427	G	G/A	G	G	G	G	G/A	G/A	G

Rt indicates recipient; Do, donor; UR, unrelated; +, yes; and −, no.
*Nucleotide positions are shown according to the NM_004092.2 mRNA sequence, available at http://www.ncbi.nlm.nih.gov/ as GEO accession GSE10044.

expression cloning,[8,9,11] there are differences in terms of power, sensitivity, and specificity. Direct sequencing of minor H antigen peptide guarantees that the purified peptide is surely present on the cell surface as antigen, but it requires highly specialized equipment and personnel. Expression screening of cDNA libraries is also widely used and has become feasible with commercially available systems. However, it depends highly on the quality of the cDNA library and expression levels of the target genes. In addition, it often suffers from false-positive results due to the forced expression of cDNA clones under a strong promoter. The current method of WGA/CTL genetically determines the relevant minor H antigen locus, not relying on highly technical protein chemistry using specialized equipment, or repetitive cell cloning procedures. It is also not affected by the expression levels of the target antigens.

As a genetic approach, the current method based on genetic association has several advantages over conventional linkage analysis: the mapping resolution has been greatly improved from several Mb in the conventional linkage analysis to the average haplotype block size of less than 100 kb,[17,25-27] usually containing a handful of candidate genes, compared with the dozens as typically found in linkage analysis. This means that the effort needed for the subsequent epitope mapping will be substantially reduced. In fact, the 115 kb region identified for CTL-2A12 contains 4 genes compared with 38 genes as revealed by the previous linkage study (data not shown), and the candidate gene was uniquely identified within the 26 kb region for CTL-1B9, for which linkage analysis had failed due to very rare segregating pedigrees among the CEPH panels with this trait (now ACC-1[C]; data not shown).[15,16] In addition, before moving on to epitope mapping, it would be possible to evaluate the clinical relevance of the minor H antigens by examining the tissue distribution of their expression, based on widely available gene expression databases such as Genomic Institute of the Novartis Research Foundation (GNF, http://symatlas.gnf.org/SymAtlas/).[28]

Second, the required sample size is generally small, and should be typically no more than 100 B-LCLs for common minor H alleles. This is in marked contrast to the association studies for common diseases, in which frequently thousands of samples are required.[17,25-27] In the current approach, sufficiently high test statistic values could be obtained for the relevant loci with a relatively small sample size, since the minor H allele is correctly segregated between the CTX^+ and CTX^- pools by the highly specific immunologic assay. Combined with high accuracy in allelic measurements, this feature allows for the use of pooled DNAs in WGAS, which substantially saves cost and time, compared with the genotyping of individual samples. Unexpectedly, our method allows for a considerable degree of error in the immunophenotyping, indicating the robustness of the current method; in fact, the minor H locus for CTL-2A12 was successfully identified in spite of the presence of 8 (~10%) immunophenotyping errors. When the minor H allele has an extreme allele frequency (eg, $< 5\%$ or $> 95\%$), which could be predicted by preliminary immunophenotyping, WGAS/CTL may not be an efficient method of mapping, due to the impractically large numbers of B-LCLs that would need to be screened to obtain enough CTX^+ or CTX^- B-LCLs. However, such minor H antigens would likely have limited clinical impact or applicability.

Sensitivity of the microarray analysis seems to be very high when the target SNP has good proxy SNPs on the array, because we were able to correctly identify the single SNP correlated with the target of CTL-1B9 from more than 500 000 SNP markers. On the other hand, genome coverage of the microarray is definitely important. In our experiments on CTL-2A12, the association was successfully identified by the marker SNPs showing r^2 values of approximately 0.74 with the target locus of ACC-6. Since the GeneChip 500 K array set captures approximately 65% of all the HapMap phase II SNPs with more than 0.74 of r^2,[29] and higher coverage will be obtained with the SNP 6.0 arrays having more than 1 000 K SNP markers, these arrays can be satisfactorily used as platforms for the WGA/CTL method.

As shown in the current study, the intrinsic sensitivity and specificity of the WGA/CTL method in detecting associated SNPs were excellent. In other words, as long as target SNPs are captured in high r^2 values with one or more marker SNPs within the Affymetrix 500 K SNP set, there is a high likelihood of capturing the SNP with the current approach. To evaluate the probability of a given minor H antigen being captured in high r^2 with marker SNPs, we checked the maximum r^2 values of known minor H antigen SNPs with the Affymetrix 500 K SNPs, according to empirical data from the HapMap project (www.hapmap.org). Among 13 known minor H antigens, 7 have their entries (designated minor H SNP) in the HapMap phase II SNP set (HA-3,[30] HA-8,[31] HB-1,[11] ACC-1 and ACC-2,[7] LB-ADIR-1F,[10] and 7A7-PANE1[13]), and were used for this purpose (note that absence of their entries in the HapMap data set does not necessarily mean that they could not be captured by a particular marker SNP set). As shown in Table S4, all 7 minor H SNPs are captured by at least one flanking SNP that is included in the Affymetrix 500 K SNP set with r^2 values of more than 0.74 in at least one HapMap panel. The situation should be more favorable in the recently available SNP 6.0 array set with 1 000 K SNPs, indicating the genome coverage with currently available SNP arrays would be sufficient to capture typical minor H antigens with our approach.

Most patients who have received allo-HSCT could be a source of minor H antigen-specific CTL clones to be used for this assay, since the donor T cells are in vivo primed and many CTL clones could be established using currently available methods. In fact, substantial numbers of CTL clones have been established worldwide and could serve as the probes to identify novel minor H antigens.[32,33] Once constructed, a panel of B-LCLs, including those transduced with HLA cDNAs, could be commonly applied to immunophenotyping with different CTL clones, especially when

CTLs are obtained from the same ethnic group. In addition, by adopting other immunophenotyping readouts such as production of IL-2 from $CD4^+$ T cells, this method could be applied to identification of MHC class II–restricted minor H antigens which have crucial roles in controlling CTL functions upstream. This may open a new field in the study of allo-HSCT since MHC class II–restricted mHags have been technically difficult to identify by conventional methods.

Finally, the discovery of ACC-1^C as a novel minor H antigen indicates that all the mismatched transplants at this locus could be eligible for allo-immune therapies, since we have previously demonstrated that the counter allele also encodes a minor H antigen, ACC-1^Y, which is preferentially expressed and presented on blood components including leukemic cells and may serve as a target of allo-immunity.[7,34] Indeed, CTLs specific for ACC-2, an HLA-B44–restricted minor H antigen restricted by the third exonic SNP on *BCL2A1*,[7] was independently isolated from the peripheral blood of a patient with recurrent leukemia re-entering complete remission after donor lymphocyte infusion.[32] The number of eligible allo-HSCT recipients has now been effectively doubled, accounting for 50% of transplants with HLA-A24 or 20% of all transplantations performed in the Asian population. In conclusion, we have described a simple but powerful method for minor H mapping to efficiently accelerate the discovery of novel minor H antigens that will be needed to contribute to our understanding of the molecular mechanism of human allo-immunity.

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Authorship

Contribution: T.K. performed most immunologic experiments and preparation of pooled DNA and quantative PCR, analyzed data, and wrote the manuscript; Y.N. performed the majority of genetic analyses and analyzed the data; H.T. performed T-cell receptor analysis and designed q-PCR primers and probes; G.Y. contributed to the organization of software for linkage analysis and simulation; S.M. prepared the pooled DNA; M.O., K.M., Y.K, and Y.M. collected clinical data and specimens; T.T. and K.K. contributed to data analysis and interpretation, and to the writing of the article; S.O. and Y.A. supervised the entire project, designed and coordinated most of the experiments in this study, contributed to manuscript preparation, and are senior coauthors.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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Ⅵ. 平成 20 年度研究成果作成原案

■新規認定施設調査チェックリスト①

新規認定施設調査チェックリスト

～非血縁者間末梢血細胞採取施設調査報告書～

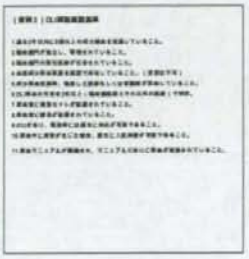
(案)

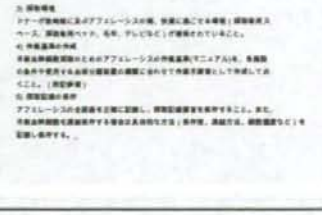
非血縁者間末梢血幹細胞採取施設認定基準

1. JMDP の非血縁者間骨髄採取施設認定基準(資料1)と DLI 採血施設基準(資料2)を満たすこと。
2. 日本造血細胞移植学会血縁者間 PBSCT 実施施設基準(資料3)を満たすこと。
3. CD34 測定可能であること。

非血縁者間末梢血幹細胞移植施設認定基準

1. JMDP の非血縁者間骨髄移植施設認定基準(資料4)を満たすこと。
2. 日本造血細胞移植学会血縁者間 PBSCT 実施施設基準(資料3)を満たすこと。
3. 末梢血幹細胞凍結可能であること。





施 設 情 報

※施設情報を下記へご記入ください。

申請施設名：

申請施設長名：

郵便番号 ：

住所 ：

Phone ： Fax：

責 任 体 制

※診療科情報を下記へご記入ください。

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申請診療科責任医師名：

Email：

事務取扱担当者名： (役職名：)

採取責任医師名 ： (役職名：)

■新規認定施設調査チェックリスト②

非血縁者間末梢血細胞採取

症例数　※症例数を下記にご記入下さい。

症例数

	ALLOGENEIC				AUTOLOGOUS			
	BM	PBSC	BM+ PBSC	CORD	BM	PBSC	BM + PBSC	CORD
20__								
20__								
Total								

末梢血細胞採取症例詳細

Unique Donor ID　　採取実施日

1. .....　.....
2. .....　.....
3. .....　.....
4. .....　.....
5. .....　.....
6. .....　.....
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14. .....　.....
15. .....　.....

Comments: .....

非血縁者間末梢血細胞採取

(Peripheral Blood Progenitor Cell Collection Facilities)

DONOR EVALUATION AND SELECTION
評価と選定

	申請者			調査指示者		
	Y	N	N/A	Y	N	N/A
C1.100 ドナーと患者を評価するためのマニュアルはあるか？						
これらの手順はドナーの既往歴、及び検査機関の結果から患者に感染する可能性に対応しているか？						
これらの手順は病歴と健康診断による PBSCH ドナーのリスクに対応しているか？						
ドナー評価の結果と検査結果はカルテの中で文書化されドナー選択のために利用されているか？						
C1.110 健康上の問題が発見された場合には見込みのある PBSCH ドナーに報告されるか？						

非血縁者間末梢血細胞採取

(Peripheral Blood Progenitor Cell Collection Facilities)

ALL PERIPHERAL BLOOD PROGENITOR CELL DONORS
PBSCT 提供者

	申請者			調査指示者		
	Y	N	N/A	Y	N	N/A
C1.311 医師は PBSCH のリスクのための病歴、身体検査、および臨床検査によって PBSCH ドナーを評価しているか？						
評価は中心静脈のアクセスが可能かを含むか？						
評価は CD34 動員の可能性を含むか？						
C1.312 術前健康診断の評価は各手続前に医師によって確認されているか？						
C1.313 最初の PBSCH の 72 時間以内とその後の 24 時間以内の血小板数を含む血球算定を実施しているか？						

非血縁者間末梢血細胞採取

(Peripheral Blood Progenitor Cell Collection Facilities)

C1.320 ALLOGENEIC PERIPHERAL BLOOD PROGENITOR CELL DONORS

	申請者			調査指示者		
	Y	N	N/A	Y	N	N/A
C1.211 ドナーは健康診断結果、既往歴と G-CSF 投与の適性で評価しているか？						
C1.212/ C1.213 既往歴はドナーの予防接種と輸血歴を含むか？						
C1.217 患者のカルテには、ドナーと患者、ウイルスマーカー、ABO 血液型、および他の HLA 適合試験でマッチしているドナーの適合性を記載しているか？						

■新規認定施設調査チェックリスト③

非血縁者間末梢血細胞採取
(Peripheral Blood Progenitor Cell Collection Facilities)

AUTOLOGOUS PERIPHERAL BLOOD PROGENITOR CELL DONORS:

	申請者 Y	申請者 N	申請者 N/A	調査担当者 Y	調査担当者 N	調査担当者 N/A
C1.221 女性ドナーに対して、同意の上、院内で妊娠検査が実施可能か？						
以下の感染症検査を施設内で実施している？						
ABO group and Rh type?						
Infectious disease tests:						
Anti-HIV-1						
Anti-HIV-2						
HIV-1-Antigen						
Anti-HTLV						
HbsAg						
Anti-HBc						
Anti-HCV						
Anti-CMV						
Serologic test for syphilis						
緊急時に、上記に示した検査を迅速に対応することは可能か？						

	申請者	調査担当者

非血縁者間末梢血細胞採取
(Peripheral Blood Progenitor Cell Collection Facilities)

PBSCH インフォームドコンセント

	申請者 Y	申請者 N	申請者 N/A	調査担当者 Y	調査担当者 N	調査担当者 N/A
C1.511 PBSH に精通している医療者がドナーのインフォームド・コンセントを得て、文書化しているか？						
同意は患者の疾患歴(多量投与療法・TBI)の開始に先だって得られて、文書化されているか？						
C1.512 PBSCH に関する説明をドナーの方は理解ではいるか？						
PBSC に関する説明には、リスクと利益についても説明しているか？						
ドナーと患者の安全と健康を保護するために措置を実行しているか						
ドナーの権利(自由意思)は保護されているか？						
C1.513 ドナーに質問の機会を与えているか？						
ドナーに提供の拒否を認めているか？						
C1.514 マイナーなドナーの場合に、ドナーの親または法律上の保護者からのインフォームド・コンセントは得られて、文書化されているか？						
C1.515 ドナーの名前が、ドナーレジストリーに追加されることになっているならば、具体的なインフォームド・コンセントは事前に得られて、文書化されるか？						

非血縁者間末梢血細胞採取
(Peripheral Blood Progenitor Cell Collection Facilities)

PERIPHERAL BLOOD PROGENITOR CELL COLLECTION FACILITIES

	申請者 Y	申請者 N	申請者 N/A	調査担当者 Y	調査担当者 N	調査担当者 N/A
PBSH を実施する際の責任医師は定めていますか？						
担当医師は、過去 1 年以内に 10 例以上 PBSCH を実施した経験がありますか。以下の対応は可能ですか？						
採取前評価						
採取許可						
採取行程管理						
採取後のドナーフォーロー						

何人の人員が PBSH を実施可能であるか？それらを記載したさい。

	申請者 Y	申請者 N	申請者 N/A	調査担当者 Y	調査担当者 N	調査担当者 N/A
C2.120 PBSCH のため適正なサポート人員がいるか？						
C2.130 ドナーの評価のために必要とされているすべてのテストを行う施設・機関はあるか？						

	申請者 Y	申請者 N	申請者 N/A	調査担当者 Y	調査担当者 N	調査担当者 N/A
C2.140 PBSCH のために必要でる試薬と機器の適切な準備とストレージのための専用のエリアがあるか？						
PBSCH のための専用のエリアがあるか？						
C2.150 PBSH はどこで実施しているか？						
C2.160 PBSH 中に健康被害が発生した場合の対応は可能か？						
C2.210 自家血小板の準備は可能か？						

輸血責任医師の氏名？

	申請者 Y	申請者 N	申請者 N/A	調査担当者 Y	調査担当者 N	調査担当者 N/A
C2.220 緊急時に対応できる ICU などの救急医療室を備えているか？						
C2.320 G-CSF 投与中に、何らかの健康被害が発生した場合に対応できるか？						

救急医療室の責任者の判断の氏名？

G-CSF の投与に伴う緊急対応の経験はあるか？

	申請者 Y	申請者 N	申請者 N/A	調査担当者 Y	調査担当者 N	調査担当者 N/A

■新規認定施設調査チェックリスト④

非血縁者間末梢血細胞採取
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PERIPHERAL BLOOD PROGENITOR CELL COLLECTION FACILITIES

		申請者			調査担当者		
		Y	N	N/A	Y	N	N/A
C3.100	ドナー評価と選択のために書かれたガイドラインはあるか？						
	これらのガイドラインは C1.000 にリストされた少なくともアイテムを含むか？						
C3.200	施設は PBSCH のために記載された手続を含んでいる標準操作手順(SOP)マニュアルがあるか？						
A2.100	PBSCH ドナーの基準(下記)はあるか？						
	ドナー適格性？						
	ドナーに対する説明文書？						
	品質管理と改善計画？						
	熱緩テスト？						
	ストレージ？						
	ラベル？						
	運搬？						
	日記録？						
	医療器具の廃棄方法？						
	許容限度と是正措置？						
	災害時の計画？						
A2.200	PBSCH の詳細な標準の操作手順マニュアルがあるか？						
	A2.210 以下は含まれるか：						
	A2.212 すべての手続のための規格化したフォーマット？						
	A2.213 手続きに関するシステム？						
	A2.211 すべての手続を準備し、実施し、レビューするための手続？						
A2.220	手続は、有資格のスタッフが首尾よく実施が可能にするためにクリアーであるか？						
	：各個別事項は含んでいる：						
	A2.221 A written description of the purpose?						
	A2.222 A clear description of equipment and supplies?						
	A2.223 The objectives of the procedure?						
	The range of expected results?						
	A2.224 A reference list?						
A2.270	新しく、改訂された手続はインプリメンテーションに先がけてスタッフによって読まれているか？						
	このレビューと関連したトレーニングは文書化されている？						
A2.280	これまでの臨床経験と今後の臨床は、使用の当初的な日付を含めて、無期限に保管されているか？						

非血縁者間末梢血細胞採取
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		申請者			調査担当者		
		Y	N	N/A	Y	N	N/A
C3.300	PBSCH を引き受けられる前に、患者の医師から移植のタイミングについてのコレクションチームとコレクションの結果の組織までの書かれた文書はあるか？						
C3.400	PBSCH のための方法は無菌のテクニックを採用するか？						
	PBSCH の手続は、容認できる生存能力と回復を結果として生じるために有効か？						
C3.500	すべての試薬は登録しているおり PBSCH のために使われるか？						
C3.600	試薬と使い捨て用品のロット番号と満了日付は記録された PBSCH に用いられるか？						
C3.700	PBSCH は D5.000 に従ってコレクションでラベルを貼られているか？						
	PBSCH のバッグはコレクション終了後に少なくとも以下の情報を記載してラベルを貼る：						
	D5.411 コンポーネントのユニークな文または英数字の識別子？						
	D5.412 固有名詞：PBSCH						
	D5.413 患者の名前と識別子(または無関係なドナーコンポーネントのためのドナーレジストリーとドナー識別子のアイデンティティ)？						
	D5.414 gDate とコレクションの時間？						
	D5.415 コレクションのボリューム？						
	D5.416 抗凝固剤の名前とボリューム？						