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# *NKG2D* gene polymorphism has a significant impact on transplant outcomes after HLA-fully-matched unrelated bone marrow transplantation for standard risk hematologic malignancies

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## ABSTRACT

### Background

NKG2D, an activating and co-stimulatory receptor expressed on natural killer cells and T cells, plays pivotal roles in immunity to microbial infections as well as in cancer immunosurveillance. This study examined the impact of donor and recipient polymorphisms in the *NKG2D* gene on the clinical outcomes of patients undergoing allogeneic T-cell-replete myeloablative bone marrow transplantation using an HLA-matched unrelated donor.

### Design and Methods

The *NKG2D* polymorphism was retrospectively analyzed in a total 145 recipients with hematologic malignancies and their unrelated donors. The patients underwent transplantation following myeloablative conditioning; the recipients and donors were matched through the Japan Marrow Donor Program.

### Results

In patients with standard-risk disease, the donor *NKG2D-HNK1* haplotype, a haplotype expected to induce greater natural killer cell activity, was associated with significantly improved overall survival (adjusted hazard ratio, 0.44; 95% confidence interval, 0.23 to 0.85; $p$=0.01) as well as transplant related mortality (adjusted hazard ratio, 0.42; 95% confidence interval, 0.21 to 0.86; $p$=0.02), but had no impact on disease relapse or the development of grade II-IV acute graft-versus-host disease or chronic graft-versus-host disease. The *NKG2D* polymorphism did not significantly influence the transplant outcomes in patients with high-risk disease.

### Conclusions

These data suggest an association between the donor *HNK1* haplotype and better clinical outcome among recipients, with standard-risk disease, of bone marrow transplants from HLA-matched unrelated donors.

Key words: *NKG2D*, *HNK1*, *LNK1*, unrelated donor; bone marrow transplantation, single nucleotide polymorphism.

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## Introduction

Hematopoietic stem cell transplantation (SCT) is a potentially curative treatment for a range of hematologic malignancies. Although the use of an HLA-matched unrelated donor is well accepted when an HLA-identical sibling donor is unavailable, the risk of transplantation- related complications may be increased.[1] Despite improvements in clinical and supportive care, transplant- related life-threatening complications, including graft-versus-host disease (GVHD), infections and disease relapse, remain an enormous obstacle to overcome.[2] Although HLA matching is the major genetic determinant of clinical outcome after allogeneic SCT, recent evidence suggests that non-HLA immune-associated genes are also implicated.[3] Previous investigations have revealed that several single nucleotide polymorphisms (SNP) which affect individual immune response to infections and inflammatory reactions are associated with the risk of GVHD and transplant outcomes.[4-15]

NKG2D is an activating and co-stimulatory receptor belonging to the C-type lectin-like family of transmembrane proteins and is expressed as a homodimer on natural killer (NK) cells, $CD8^+$ $\alpha\beta^+$ T cells, $\gamma\delta^+$ T cells and activated macrophages.[16-18] The ligands for NKG2D, such as MHC class I-chain related proteins (MICA and MICB), UL16 binding proteins are usually absent or expressed at very low levels in normal cells but are up-regulated by cellular stress including heat shock and microbial infections and are frequently expressed in epithelial tumor cells.[19] Ligand engagement of NKG2D triggers cell-mediated cytotoxicity and co-stimulates cytokine production through a DAP10-phosphoinositol 3-kinase dependent pathway and plays an important role in the elimination of tumors and infected cells.[16-18,20]

Recently, SNP were identified between *LNK1* and *HNK1* haplotypes of the *NKG2D* gene.[21] In Japanese individuals, the *HNK1* haplotype is associated with greater activity of NK cells in the peripheral blood[21,22] and a lower prevalence of cancers originating from epithelial cells.[21,23,24] The present study investigates the impact of donor and recipient polymorphisms in the *NKG2D* gene on the clinical outcomes of patients undergoing allogeneic myeloablative bone marrow transplantation using an HLA allele-matched unrelated donor.

## Design and Methods

### Patients

*NKG2D* genotyping was performed on a total 145 recipients with hematologic malignancies and their unrelated donors who were part of the Japan Marrow Donor Program (JMDP). The recipients underwent transplantation, following myeloablative conditioning, with T-cell-replete marrow from an HLA-A, -B, -C, -DRB1 allele-matched donor between November 1995 and March 2000. HLA genotypes of the HLA-A, -B, -C, and -DRB1 alleles of the patients and donors were determined by the Luminex microbead method described previously. (Luminex 100 System; Luminex, Austin, TX, USA).[25,26] No patient had a history of prior transplantation. The final clinical survey of these patients was completed by November 1, 2007. Diagnoses were acute myeloid leukemia (n=49; 34%), acute lymphoblastic leukemia (n=37; 26%), chronic myeloid leukemia (n=41; 28%), myelodysplastic syndrome (n=11; 8%) and malignant lymphoma (n=7; 5%), (Table 1). The recipients were defined as having standard risk disease if they had acute myeloid or lymphoblastic leukemia in first complete remission, malignant lymphoma in complete remission, chronic myeloid leukemia in any chronic phase or myelodysplastic syndrome. All other patients were designated as having high-risk disease. Myeloid malignancies included acute myeloid leukemia, chronic myeloid leukemia and myelodysplastic syndrome, whereas lymphoid malignancies included acute lymphoblastic leukemia and malignant lymphomas. Cyclosporine or tacrolimus- based regimens were used in all patients for GVHD prophylaxis whereas anti-T-cell therapy, such as anti-thymocyte globulin and *ex vivo* T-cell depletion, was not. All patients and donors gave their written informed consent to molecular studies, according to the declaration of Helsinki, at the time of transplantation. The project was approved by the Institutional Review Board of Kanazawa University Graduate School of Medicine and the JMDP.

### NKG2D genotyping

*NKG2D* was genotyped using the TaqMan-Allelic discrimination method[27] with a 9700-HT real time polymerase chain reaction (PCR) system (Applied Biosystems, Foster City, CA, USA) and results were analyzed using allelic discrimination software (Applied Biosystems). The genotyping assay was conducted in 96-well PCR plates. The amplification reaction contained template DNA, TaqMan universal master mix and a specific probe (product No. C_9345347_10; Applied Biosystems) for rs1049174, a single locus featuring a G-C substitution to distinguish between the *HNK1* (G) and *LNK1* (C) haplotypes of the *NKG2D* gene.[21,23,24]

### Data management and statistical analysis

Data were collected by the JMDP using a standardized report form. Follow-up reports were submitted at 100 days, 1 year and annually after transplantation. Pre-transplant cytomegalovirus serostatus was routinely tested only in patients but not in their donors. Engraftment was confirmed by an absolute neutrophil count of more than $0.5\times10^9$/L for at least 3 consecutive days. Acute and chronic GVHD were diagnosed and graded using established criteria.[28,29] Overall survival was defined as the number of days from transplantation to death from any cause. Disease relapse was defined as the number of days from transplantation to disease relapse. Transplant-related mortality was defined as death without relapse. Any patients who were alive at the last-follow-up date were censored. When collecting data, only the main cause of death was recorded if two or more causes were combined. Data on etiological agents of infections, post-mortem changes and supportive care (including prophylaxis of infections and therapy of GVHD, which were given on an institutional basis), were not available for this

cohort of patients. The analysis was performed using Excel 2007 (Microsoft Corp, Redmond, WA, USA), OriginPro version 8.0J (Lightstone Inc, Tokyo, Japan), and R (The R Foundation for Statistical Computing, Perugia, Italy).[30] The probability of overall survival was calculated using the Kaplan-Meier method and compared using the log-rank test. The probabilities of transplant-related mortality, disease relapse, acute GVHD, chronic GVHD, and each cause of death were compared using the Grey test[31] and analyzed using cumulative incidence analysis,[30] considering relapse, death without disease relapse, death without acute GVHD, death without chronic GVHD, and death without each cause as respective competing risks. The analysis was stratified for patients with standard-risk disease and high-risk disease to take into account the already recognized prognostic differences. The variables considered were recipient age at time of transplantation, sex, recipient cytomegalovirus serosta-

**Table 1. Characteristics of the donors and recipients.**

| | Standard-risk disease (n=93, 64%) Donor NKG2D haplotype | | | | | High-risk disease (n=52, 36%) Donor NKG2D haplotype | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *HNK1* negative n=55, 59% | | *HNK1* negative n=38, 41% | | | *HNK1* positive n=28, 54% | | *HNK1* negative n=24, 46% | | |
| Variable | N. | Ratio | N. | Ratio | p | N. | Ratio | N. | Ratio | p |
| Age, years | | | | | | | | | | |
| Recipient | | | | | 0.08 | | | | | 0.39 |
| Median | 31 | | 23 | | | 23 | | 22 | | |
| Range | 1-50 | | 1-50 | | | 7-46 | | 2-48 | | |
| Donor | | | | | 0.54 | | | | | |
| Median | 33 | | 28 | | | 34 | | 29 | | 0.02 |
| Range | 22-49 | | 21-50 | | | 21-47 | | 21-50 | | |
| Recipient *NKG2D* haplotype | | | | | 0.17 | | | | | 0.48 |
| *HNK1* positive | 33 | 60% | 28 | 74% | | 19 | 68% | 14 | 58% | |
| *HNK1* negative | 22 | 40% | 10 | 26% | | 9 | 32% | 10 | 42% | |
| Sex, male | | | | | 0.37 | | | | | 0.77 |
| Recipient | 30 | 55% | 23 | 61% | | 19 | 68% | 15 | 63% | |
| Donor | 42 | 76% | 23 | 61% | | 19 | 68% | 13 | 54% | |
| Recipient/donor sex | | | | | 0.23 | | | | | 0.86 |
| Sex matched | 31 | 56% | 20 | 53% | | 18 | 64% | 16 | 67% | |
| Male/female | 6 | 11% | 9 | 24% | | 5 | 18% | 5 | 21% | |
| Female/male | 18 | 33% | 9 | 24% | | 5 | 18% | 3 | 13% | |
| Disease | | | | | 0.86 | | | | | 0.99 |
| Acute myeloid leukemia | 14 | 25% | 9 | 24% | | 14 | 50% | 12 | 50% | |
| Acute lymphoblastic leukemia | 10 | 18% | 8 | 21% | | 10 | 36% | 9 | 38% | |
| Myelodysplastic syndrome | 6 | 11% | 5 | 13% | | 0 | 0% | 0 | 0% | |
| Malignant lymphoma | 2 | 4% | 3 | 8% | | 1 | 4% | 1 | 4% | |
| Chronic myeloid leukemia | 23 | 42% | 13 | 34% | | 3 | 11% | 2 | 8% | |
| ABO matching | | | | | 0.37 | | | | | 0.18 |
| Matched | 35 | 64% | 19 | 50% | | 14 | 50% | 17 | 71% | |
| Major mismatch | 11 | 20% | 10 | 26% | | 6 | 21% | 5 | 21% | |
| Minor mismatch | 9 | 16% | 9 | 24% | | 8 | 29% | 2 | 8% | |
| Bi-directional | 0 | 0% | 1 | 3% | | 0 | 0% | 1 | 4% | |
| Conditioning regimen | | | | | 0.93 | | | | | 0.51 |
| With total body irradiation | 43 | 78% | 30 | 79% | | 26 | 93% | 21 | 88% | |
| Without total body irradiation | 12 | 22% | 8 | 21% | | 2 | 7% | 3 | 13% | |
| Pretransplant CMV serostatus | | | | | 0.30 | | | | | 0.99 |
| CMV-negative recipient | 14 | 25% | 5 | 13% | | 6 | 27% | 5 | 21% | |
| Missing data | 4 | 7% | 2 | 5% | | 5 | 18% | 4 | 17% | |
| GVHD prophylaxis | | | | | 0.58 | | | | | 0.11 |
| With cyclosporine | 51 | 93% | 34 | 89% | | 27 | 96% | 20 | 83% | |
| With tacrolimus | 4 | 7% | 4 | 11% | | 1 | 4% | 4 | 17% | |
| TNC, $\times 10^8$/kg | | | | | 0.40 | | | | | 0.04 |
| Median | 5.4 | | 5.8 | | | 5.8 | | 8.2 | | |
| Range | 2.3-14.6 | | 2.3-57.6 | | | 2.9-20.0 | | 2.4-42.8 | | |
| Engraftment | 53 | 96% | 38 | 100% | 0.23 | 28 | 100% | 23 | 96% | 0.28 |

*CMV: cytomegalovirus; TNC: total nucleated cell count harvested.*

tus before transplantation, disease characteristics (disease type and disease lineage), donor characteristics (age, sex, sex compatibility, and ABO compatibility), transplant characteristics (total body irradiation-containing regimen, tacrolimus versus cyclosporine, and total nucleated cell count harvested per recipient weight). The median was used as the cut-off point for continuous variables. The $\chi^2$ test and Mann-Whitney test were used to compare results of two groups. The Hardy-Weinberg equilibrium for the *NKG2D* gene polymorphism was tested using the Haploview program.[32] Multivariate Cox models were used to evaluate the hazard ratio associated with the NKG2D polymorphism. Co-variates found to be statistically significant in univariate analyses ($p \leq 0.10$) were included in the models. For both the univariate and multivariate analyses, $p$ values were two-sided and outcomes were considered to be statistically significant with $p \leq 0.05$.

## Results

### Frequencies of NKG2D haplotype

The *NKG2D* gene polymorphism was analyzed in 145 pairs of unrelated donors-recipients of bone marrow following myeloablative conditioning (Table 1). The haplotype frequencies of *LNK1/LNK1*, *HNK1/LNK1* and *HNK1/HNK1* were 43%, 42% and 15%, respectively in donors and 35%, 45% and 20%, respectively in recipients. These frequencies were similar to those reported in previous studies in Japanese populations[21,24] and were in accordance with the Hardy-Weinberg equilibrium ($p$=0.80).

### Transplant outcomes according to NKG2D haplotype

With a median follow-up of 115 months among survivors (range, 74 to 140 months), 30 recipients (21%) had relapsed or progressed and 62 (47%) had died. Three patients (2%) died before engraftment. The analysis of the influence of the *NKG2D* genotype on clinical outcomes after transplantation was stratified according to whether the recipients had standard-risk disease or high-risk disease to account for the already recognized prognostic difference. The overall survival at 5 years in patients with standard-risk disease was 63% while that of patients with high-risk disease was 44% ($p$=0.06). The 5-year cumulative incidences of trasplant-related mortality were 32% and 27%, respectively ($p$=0.33) and those of disease relapse were 10% and 31%, respectively ($p$=0.0006).

The transplant outcomes according to *NKG2D* genotype are summarized in Table 2. Patients with standard-risk disease receiving transplants from donors with the *HNK1* haplotype had a significantly better 5-year overall survival (73% *vs.* 49%, $p$=0.01; Figure 1A) and lower transplant-related mortality rate (22% *vs.* 45%, $p$=0.02; Figure 1B) than those receiving transplants from donors without the *HNK1* haplotype. No difference was noted in disease relapse in relation to the donors' polymorphism (9% *vs.* 11%, $p$=0.81; Figure 1C) or in the development of grades II to IV acute GVHD (28% *vs.* 41%, $p$=0.25) or chronic GVHD (37% *vs.* 41%, $p$=0.83). When patients with acute myeloid leukemia or myelodysplastic syndrome were separately analyzed, there was still no difference in disease relapse in relation to NKG2D polymorphisms (*data not shown*). In patients with high-risk disease, the donor *HNK1* haplotype had no significant effects on transplant outcomes (Table 2).

### Multivariate analysis

Any factors found to be significant in univariate analyses were included in the multivariate analysis. When patients with standard-risk disease were analyzed, the *HNK1* haplotype in donors remained statistically significant in multivariate analyses for both overall survival and transplant-related mortality (Table 3). The presence of the *HNK1* haplotype in the donor resulted in better overall survival (hazard ratio, 0.44; 95% confidence interval, 0.23 to 0.85; $p$=0.01) and transplant-related mortality (hazard ratio, 0.42; 95% confidence interval, 0.21 to 0.86; $p$=0.02).



**Table 2. Univariate analysis of the association of *NKG2D* polymorphisms with clinical outcomes after transplantation.**

| | N. | 5-year OS | p | 5-year TRM | p | 5-year relapse | p | Grade II-IV acute GVHD | p | Chronic GVHD | p |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Standard-risk disease** | | | | | | | | | | | |
| Donor *NKG2D* haplotype | | | 0.01 | | 0.02 | | 0.81 | | 0.25 | | 0.83 |
| *HNK1*-positive | 55 | 73% | | 22% | | 9% | | 28% | | 37% | |
| *HNK1*-negative | 38 | 49% | | 45% | | 11% | | 41% | | 41% | |
| Recipient *NKG2D* haplotype | | | 0.39 | | 0.31 | | 0.93 | | 0.48 | | 0.98 |
| *HNK1*-positive | 61 | 62% | | 33% | | 10% | | 37% | | 39% | |
| *HNK1*-negative | 32 | 66% | | 28% | | 9% | | 25% | | 38% | |
| **High-risk disease** | | | | | | | | | | | |
| Donor *NKG2D* haplotype | | | 0.91 | | 0.77 | | 0.93 | | 0.08 | | 0.47 |
| *HNK1*-positive | 28 | 43% | | 26% | | 33% | | 54% | | 44% | |
| *HNK1*-negative | 24 | 46% | | 29% | | 29% | | 30% | | 35% | |
| Recipient *NKG2D* haplotype | | | 0.41 | | 0.43 | | 0.10 | | 0.40 | | 0.68 |
| *HNK1*-positive | 33 | 42% | | 23% | | 39% | | 39% | | 37% | |
| *HNK1*-negative | 19 | 47% | | 35% | | 18% | | 50% | | 47% | |

*OS: overall survival; TRM: transplant-related mortality.*

The donor and recipient *HNK1* haplotype did not significantly influence the transplant outcomes in patients with high-risk disease.

### Main causes of death

The main causes of death according to the *HNK1* haplotype of the donors and recipients are illustrated in Figure 2A for patients with standard-risk disease, and in Figure 2B for those with high-risk disease. In patients with standard-risk disease receiving transplants from *HNK1*-negative donors, the most frequent cause of death was acute GVHD, followed by interstitial pneumonia. Transplants from *HNK1*-positive donors resulted in a statistically significantly reduced incidence of death attributed to acute GVHD (Figure 3A; $p$=0.006) as well as a trend toward a lower incidence of death attributed to interstitial pneumonia (Figure 3B; $p$=0.09). Other causes of death did not differ according to the *HNK1* haplotype.

## Discussion

The current study showed an association between the *NKG2D-HNK1* haplotype in unrelated donors of HLA-matched myeloablative bone marrow transplants (haplotype frequency, 61%) and a significantly reduced transplant-related mortality and better overall survival for their recipients with standard-risk disease. The polymorphism of the donor *NKG2D* gene did not influence disease relapse or the development of grades II to IV acute GVHD or chronic GVHD in the patients. One possible explanation for the absence of the beneficial effects of the *HNK1* haplotype in patients with high-risk disease may be that the number of cases in the study was insufficient for a meaningful assessment of the effect. Alternatively, disease progression may precede the emergence of the potential advantageous effects of the *HNK1* donor haplotype that could protect the recipient from severe transplant-related complications. There was a larger difference in disease relapse between patients with standard-risk disease and those with high-risk disease: 10% and 31% at 3 years after transplantation, respectively.

*NKG2D* plays important roles in immunity to microbial infections and is especially prominent in controlling viral and bacterial infections.[16] Therefore, the reduced transplant-related mortality in patients with standard-risk disease receiving grafts from donors with the *HNK1* haplotype in this study might be a consequence of increased resistance to infections in the recipients. However, the hypothesis is too speculative because of the unavailability of data on causes of infections in this cohort. Further studies will be needed to clarify whether the *HNK1* haplotype in donors can effectively protect patients against infections.

Several studies have shown that NK cell activity has an important role in the outcomes of patients undergoing allogeneic transplantation.[33,34] Alloreactive NK cells reduced the risk of relapse of acute myeloid leukemia without increasing the incidence of GVHD, resulting in a marked improvement of event-free survival in a series of haploidentical transplant recipients.[35,36] In HLA-identical sibling transplants, the absence of HLA-C and HLA-B ligand for donor-inhibitory killer immunoglobulin-like receptors (KIR) provided benefits in terms of survival and relapse of patients with acute myeloid leukemia and myelodysplastic syndrome in recipients of T-cell-depleted SCT.[37] On the other hand, the JMDP found that KIR ligand mismatch was unfavorably correlated with relapse of leukemia and survival in patients undergoing T-cell-replete unrelated bone marrow transplants.[38] All patients in the present study received grafts from an HLA-A, -B, and -C allele-matched donor, implying KIR ligand match between each patient and donor. It is an open question whether the *NKG2D* polymorphism could affect the outcomes of patients undergoing transplantation with KIR-mismatched grafts.

In this study, major and minor ABO incompatibilities between the donor and recipient tended to be associated with poorer transplant outcomes, regardless of the risk



**Table 3. Multivariate analysis of the association of *NKG2D* polymorphisms with clinical outcomes after transplantation.**

| | Overall survival | | | Transplant-related mortality | | | Relapse | | | Grades II-IV acute GVHD | | | Chronic GVHD | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Variable | HR | 95% CI | p | HR | 95% CI | p | HR | 95% CI | p | HR | 95% CI | p | HR | 95% CI | p |
| **Standard-risk disease** | | | | | | | | | | | | | | | |
| *HNK1*-positive donor | 0.44 | 0.23-0.85 | 0.01 | 0.42 | 0.21-0.86 | 0.02 | 0.71 | 0.19-2.67 | 0.61 | 0.83 | 0.39-1.75 | 0.63 | 0.83 | 0.39-1.75 | 0.62 |
| *HNK1*-positive recipient | 1.22 | 0.60-2.50 | 0.58 | 1.32 | 0.61-2.87 | 0.48 | 1.11 | 0.28-4.48 | 0.88 | 1.54 | 0.66-3.57 | 0.32 | 1.06 | 0.49-2.31 | 0.88 |
| Donor age, >31 years | - | - | - | - | - | - | - | - | - | 2.17 | 0.95-4.96 | 0.07 | - | - | - |
| Major ABO incompatibility | - | - | - | - | - | - | - | - | - | 3.12 | 1.49-6.56 | 0.003 | 0.50 | 0.17-1.45 | 0.20 |
| Minor ABO incompatibility | 2.42 | 1.17-5.03 | 0.02 | - | - | - | - | - | - | - | - | - | 0.29 | 0.07-1.24 | 0.10 |
| **High-risk disease** | | | | | | | | | | | | | | | |
| *HNK1*-positive donor | 0.68 | 0.30-1.51 | 0.34 | 0.62 | 0.20-1.91 | 0.40 | 1.25 | 0.41-3.80 | 0.69 | 1.87 | 0.69-5.07 | 0.22 | 1.55 | 0.60-4.01 | 0.37 |
| *HNK1*-positive recipient | 1.41 | 0.65-3.07 | 0.39 | 0.76 | 0.25-2.29 | 0.63 | 2.35 | 0.66-8.44 | 0.19 | 0.47 | 0.18-1.22 | 0.12 | 0.92 | 0.35-2.38 | 0.86 |
| Age, >26 years | 1.95 | 0.93-4.09 | 0.08 | 6.30 | 1.86-21.32 | 0.003 | - | - | - | - | - | - | - | - | - |
| Donor age, >31 years | - | - | - | - | - | - | 0.53 | 0.17-1.65 | 0.27 | - | - | - | - | - | - |
| Minor ABO incompatibility | 2.94 | 1.19-7.25 | 0.02 | - | - | - | - | - | - | 5.10 | 2.08-12.52 | 0.004 | - | - | - |

category of the disease. These findings are compatible with those of a previous study by the JMDP,[39] although the impact of ABO incompatibilities on SCT outcomes is controversial.

This study also identified age as a significant predictive factor for transplant-related mortality in the patients with standard-risk disease. This is consistent with the results of a previous study[40] showing that age over 35 years increased the risk of transplant-related mortality after allogeneic myeloablative SCT in high-risk patients.

A possible limitation of this study is the fact that no direct evidence is yet available regarding the ability of *NKG2D* polymorphisms to protect against microbial infections. The association observed between the *NKG2D* haplotype and transplant outcome might be due to another genetic polymorphism in linkage disequilibrium responsible for a better transplant outcome. One candidate gene is *NKG2F* (*KLRC4*), which is located in the NK complex region adjacent to the *NKG2D* gene, because an intrinsic SNP (rs2617171) in the gene has been reported to be in complete linkage with the *NKG2D* genotype.[24] Alternatively, polymorphisms may not be directly associated with controlling infection, but rather may be associated with other factors, such as sensitivity to treatment against GVHD or protection against organ toxicities related to transplants, which also influence the transplant outcome. These hypotheses have yet to be verified give the insufficient evidence.

Polymorphisms in genes encoding for nucleotide-binding oligomerization domain 2 (NOD2)/caspase recruitment domain 15 (CARD15),[9] heme oxygenase-1 (HO-1) promoter,[6] the Toll-like receptor 4,[4] CC chemokine ligand (CCL) 5 promoter,[32] transforming growth factor (TGF) β1,[11] interleukin (IL) 12, tumor necrosis factor (TNF) α,[15] IL-23,[5] mannose-binding lectin (MBL),[10] Fcγ receptor IIa (FcγRIIa), myeloperoxidase (MPO), FcγRIIIb, IL-1Ra, IL-10,[12] Fc receptor-like 3 (FCRL3), peptidylarginine deimi-

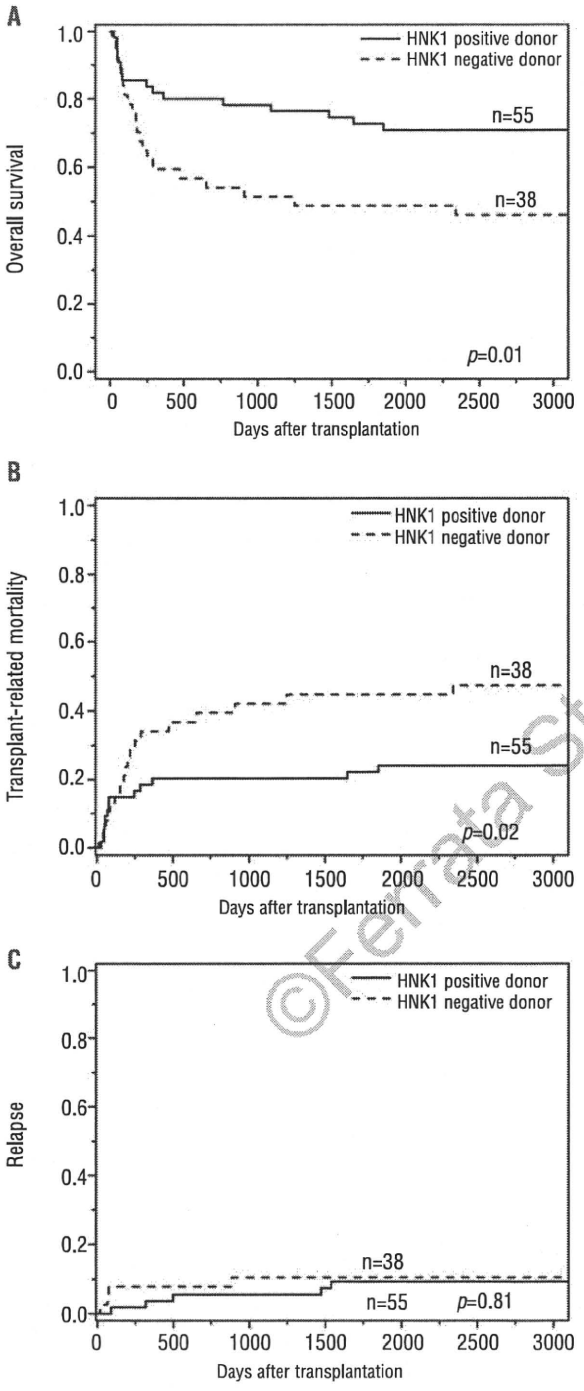


Figure 1. Kaplan-Meier analysis of (A) overall survival, (B) cumulative incidence of transplant-related mortality and (C) disease relapse after transplantation according to the donor *NKG2D* polymorphism in patients with standard-risk disease. Patients with donors with the *HNK1* haplotype had better overall survival and lower transplant-related mortality. Donor haplotype had no significant impact on disease relapse.

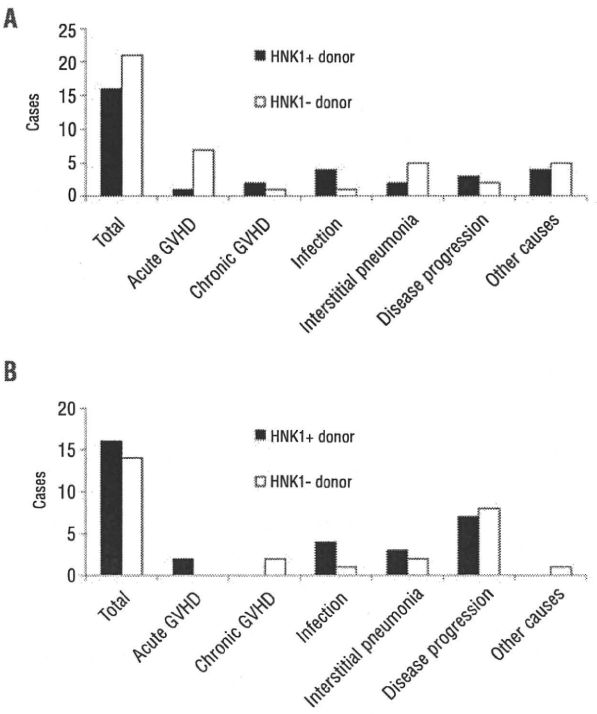


Figure 2. Main causes of death after transplantation according to the *NKG2D* polymorphism in patients with (A) standard-risk disease (B) high-risk disease.

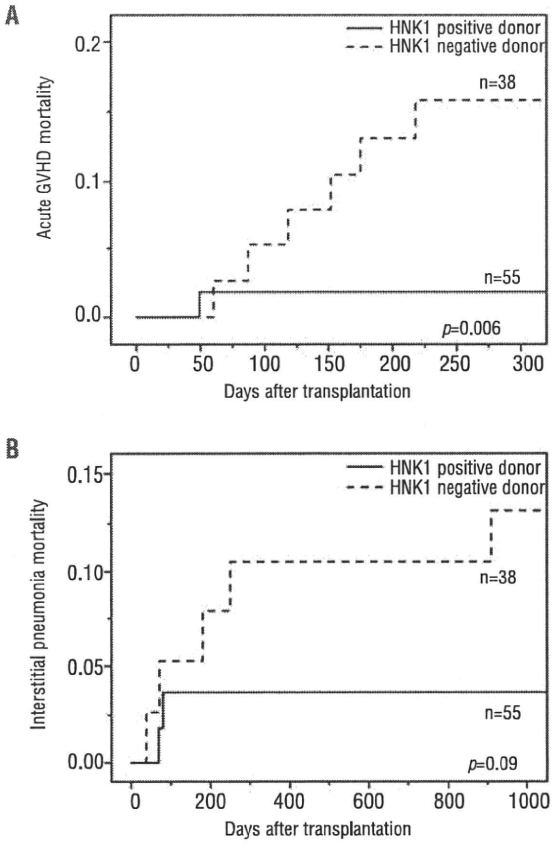


**Figure 3. Cumulative incidence of deaths due to (A) acute GVHD and (B) interstitial pneumonia after transplantation in patients with standard-risk disease. The *HNK1* haplotype in donors was associated with a significantly lower incidence of deaths due to acute GVHD ($p$=0.006) as well as a trend toward a lower incidence of deaths due to interstitial pneumonia ($p$=0.09).**

nase citullinating enzymes 4 (PADI4)[13] and methylenetetrahydrofolate reductase (MTHFR)[14] have been shown to influence the outcome after allogeneic SCT. Most of them are associated with the development of GVHD. Only the *NOD2/CARD15* and *HO-1* promoter polymorphisms have a significant impact on overall survival after SCT. Furthermore, the impact of the *HO-1* promoter polymorphisms depends on donor cells but not on recipient cells, as observed with the *NKG2D* polymorphism which, in the donor, was shown to be significantly associated with overall survival in the present study. This may prompt the determination of the donor *NKG2D* polymorphism prior to SCT in order to choose the best donor, expected to minimize transplant-related mortality after SCT, when multiple donors for a patient are available. Otherwise, prior information on the donor *NKG2D* polymorphism may be helpful in selecting risk-specific appropriate precautions following transplantation.

In conclusion, the present data suggest that the *NKG2D* polymorphism, in addition to HLA disparity between recipients and donors, affects prognosis after a bone marrow transplant from an unrelated donor. However, care should be made in drawing conclusions because the number of patients in the present study was small. The finding of a gene polymorphism may not be equivalent to differences in gene expression, which may be influenced by multiple factors because the NKG2D receptor is found on many tissues and cells.[41] Experimental evidence is required to substantiate the effect of the *NKG2D* polymorphism on immune function. We next plan to conduct a prospective study to confirm these results and to extend this investigation to other transplantation settings, such as related donor SCT, reduced-intensity SCT, HLA-mismatched SCT and SCT for patients with non-hematologic malignancies.



## Authorship and Disclosures

JLE and AT designed and performed the research, and contributed to the same aspects of the work; AT, JLE and SN wrote the paper; AT, YKa, and SOh performed the statistical analyses; MO, HS, HA, KM, SOk, MI, TF, YM, and YKo contributed to data collection.

The authors reported no potential conflicts of interest.

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# Exploration of the Genetic Basis of GVHD by Genetic Association Studies

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## INTRODUCTION

Graft-versus-host disease (GVHD), as well as graft-versus-leukemia effect (GVL), are essentially allo-immune reactions, which are induced by the engrafted donor T cells that recognize the host-derived allo-antigens presented on their targets (Figure 1). In HLA-matched transplantation, these antigens are called minor histocompatibility antigens (mHags), and are typically defined by the host single nucleotide polymorphisms (SNPs) that are not shared by the donor and therefore considered to be genetically mismatched between the donor and the recipient [1–3]. Thus, the development of both allo-reactions absolutely depends on the presence of 1 or more mismatched mHags, although these reactions could be further modified by other genetic as well as environmental factors, including, cytokine polymorphisms and GVHD prophylaxis. So, in view of better preventing GVHD and specifically targeting allo-immunity to the tumor component, central questions are what mHAgs are responsible for the development of GVHD or GVL and what genetic factors can influence the overall reactions, which are the plausible targets of genome-wide association studies (GWAS) [4-8].

To identify the genetic basis of GVHD, we conducted GWAS by genotyping more than 500,000 SNPs using Affymetrix GeneChip platforms [9,10] in donors and recipients from 1,598 unrelated transplants performed through the Japan Marrow Donor Program (JMDP). All transplants were matched for HLA-A, B, C, DRB1, and DQB1 by high-resolution DNA typing, while 1033 (63%) transplants were mismatched for HLA-DPB1. Six hundred fifty-six (41.7%) and 245 (14.9%) of transplants had developed grade II-IV and grade III-IV of acute GVHD (aGVHD), respectively. Overall SNP call rates exceeded 98% both in donors and in recipients. Unobserved HapMap PhaseII SNPs were rigorously imputed from the genotyped SNPs [11-13]. After excluding those disqualified SNPs showing <95% call rate, deviation from Hardy-Weinberg equilibrium, or <5% minor allele frequency, 1,276,699 SNPs were tested for association with development of aGVHD and chronic GVHD (cGVHD), relapse, and overall survival (OS), by calculating log-rank statistics for each SNP. Statistical thresholds for genome-wide $P$ value of .05 were determined empirically by doing 1,000 permutations for each analysis. Association tests were performed with regard to the simple genotype of donor and recipient SNPs. Alternatively, to identify possible mHag loci, GWAS were performed based on the allele-mismatch defined for each SNP locus, rather than simple SNP genotypes in donors and recipients. In the latter setting, associations were tested within the subgroups that shared particular HLA-types based on HLA-restriction. Generally speaking, the sample size of ~1,600 transplants in the current study was relatively small compared to the size of typical GWAS studies, and it was further reduced in the subgroup analysis [8]. Thus, it was likely that we could find only those mHag loci that were restricted to major HLA alleles and whose allele-mismatch conferred strong genetic effects on the development of GvHD [14,15]. However, this did not necessarily preclude conducting the current study, because it was such mHags that are thought to be clinically relevant.

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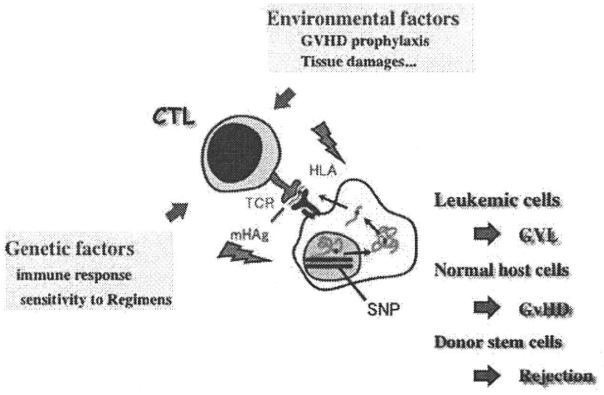


**Figure 1.** Allo-immunity plays central roles in HSCT.

In the analysis regarding genotype mismatch for aGVHD under the assumption of no HLA restriction, SNPs around the HLA-DPB1 locus showed strong association with the development of grade II-IV aGVHD with the maximum $P$ value of $1.81 \times 10^{-9}$ at rs6937034, and thus, the GWAS successfully captured the association of HLA-DPB1 allele mismatch as directly defined by high-resolution DNA typing (hazard ratio [HR] = 1.91, $P = 2.88 \times 10^{-13}$) (Figure 2) [16]. No other loci were identified that were significantly associated with aGVHD under the assumption of no HLA restrictions. To identify the target mHags for aGVHD, we further performed subgroup analyses, in which the analysis were confined to those transplants sharing major HLA types among the Japanese population [17]. Six loci were identified as candidate mHag loci. rs17473423 on chr12 was associated with the A*2402/B*5201/Cw*1201/DRB1*1501/DQB1*0601, which represents the most prevalent HLA haplotype among the Japanese population and shared in ~40% of unrelated transplants in Japanese (grade III-IV aGVHD, with maximum $P = 3.99 \times 10^{-13}$) (Figures 2b and 3b). rs9657655 on chr9 was associated with another common haplotype in Japanese, A*3303/B*4403/Cw*1403 (grade III-IV aGVHD with maximum $P = 8.56 \times 10^{-10}$) (Figures 2c and 3b). We found additional 4 loci that were associated with DQB1*0501, Cw*0102, B*5201, and Cw*1202. We also tested the association of GVHD with simple genotype in either recipients or donors, though which 2 recipient SNPs were found to be associated with aGVHD, rs5998746 on chr22 ($P = 3.41 \times 10^{-8}$) and rs11873016 on chr18 ($P = 1.26 \times 10^{-8}$), whereas no donor SNPs showed significant associations. Similarly, we identified 4 candidate SNPs associated with the development of severe cGVHD or relapse.

Our study provided a unique opportunity, in that a combination of 2 different genotypes, rather than mere genotypes in single individuals, is explored for association with particular disease phenotypes through whole genome association scanning. Although further replication studies and biologic confirmation are

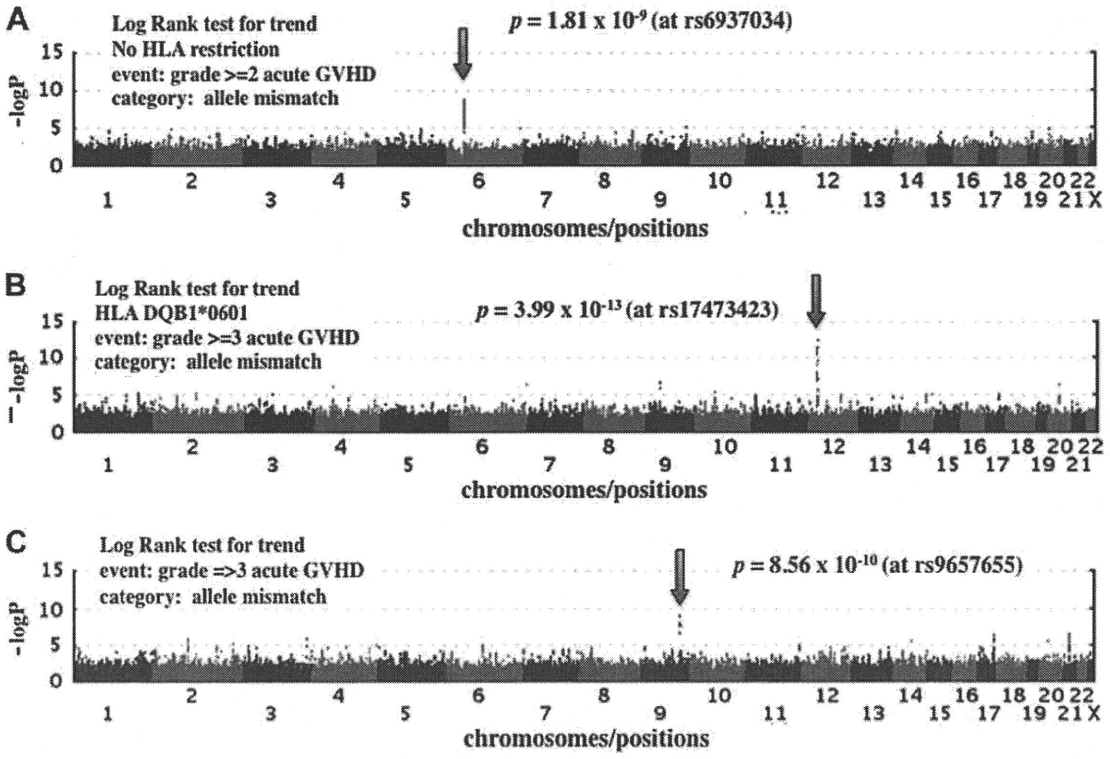


**Figure 2.** Representative results of GWAS based on genotype mismatch. $-\log_{10}$P values are plotted in genetic order. Results are presented for association tests for genotype mismatch under no HLA restriction (A), and under the restriction to HLA DQB1*0601 (B) and HLA B*4403 (C).

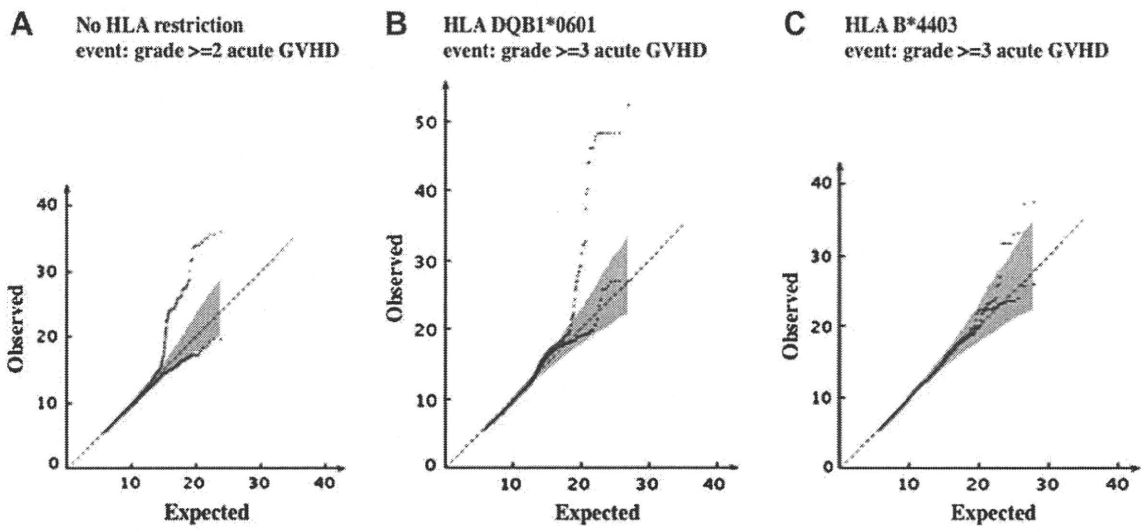


**Figure 3.** QQ-plots of the statistics. QQ-plots of the analysis of genotype mismatch under no restriction on HLA (A), and restriction to HLA DQB1*0601 (B) and HLA B*4403 (C) where observed test statistics values are plotted against expected values from 1000 random permutations (red); 95% confidence intervals are also provided by shadows. Only the plots for the top 20,000 results are presented. The QQ-plots excluding the SNPs that belong to the positive peak are also depicted in blue.

required, our results suggest that whole genome association studies of allo-SCT could provide a novel clue to our understanding of the genetic basis of 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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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.

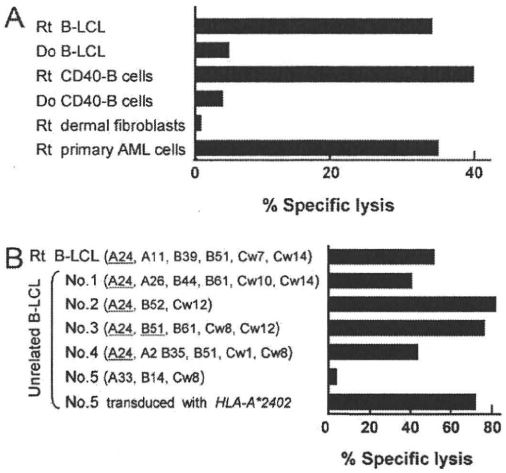


**Figure 1. Specificity of CTL-1B9 against hematopoietic cells and its restriction HLA.** (A) The cytolytic activity of CTL-1B9 was evaluated in a standard 4-hour $^{51}Cr$ release assay (E/T ratio, 20:1). Targets used were B-LCL, CD40-activated (CD40-B) B cells, dermal fibroblasts, and primary acute 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.

## 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.

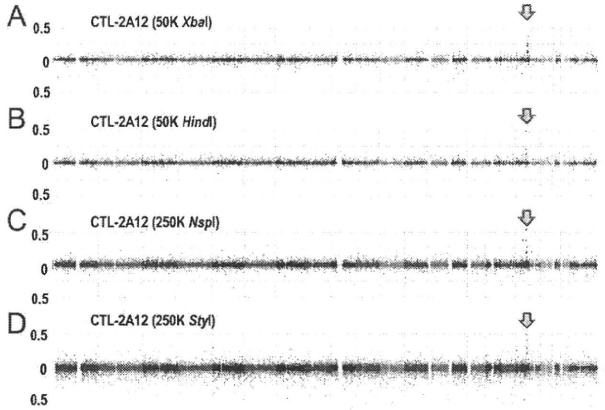


**Figure 2. Whole genome association scans performed with pooled DNA generated based on immunophenotyping with CTL-2A12.** Pooled DNAs generated from 44 $CTX^+$ and 44 $CTX^-$ B-LCLs were analyzed with 50 K *Xba*I (A), 50 K *Hind*III (B), 250 K *Nsp*I (C), and 250 K *Sty*I (D) arrays. Test statistics 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).

### 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 $CTX^-$

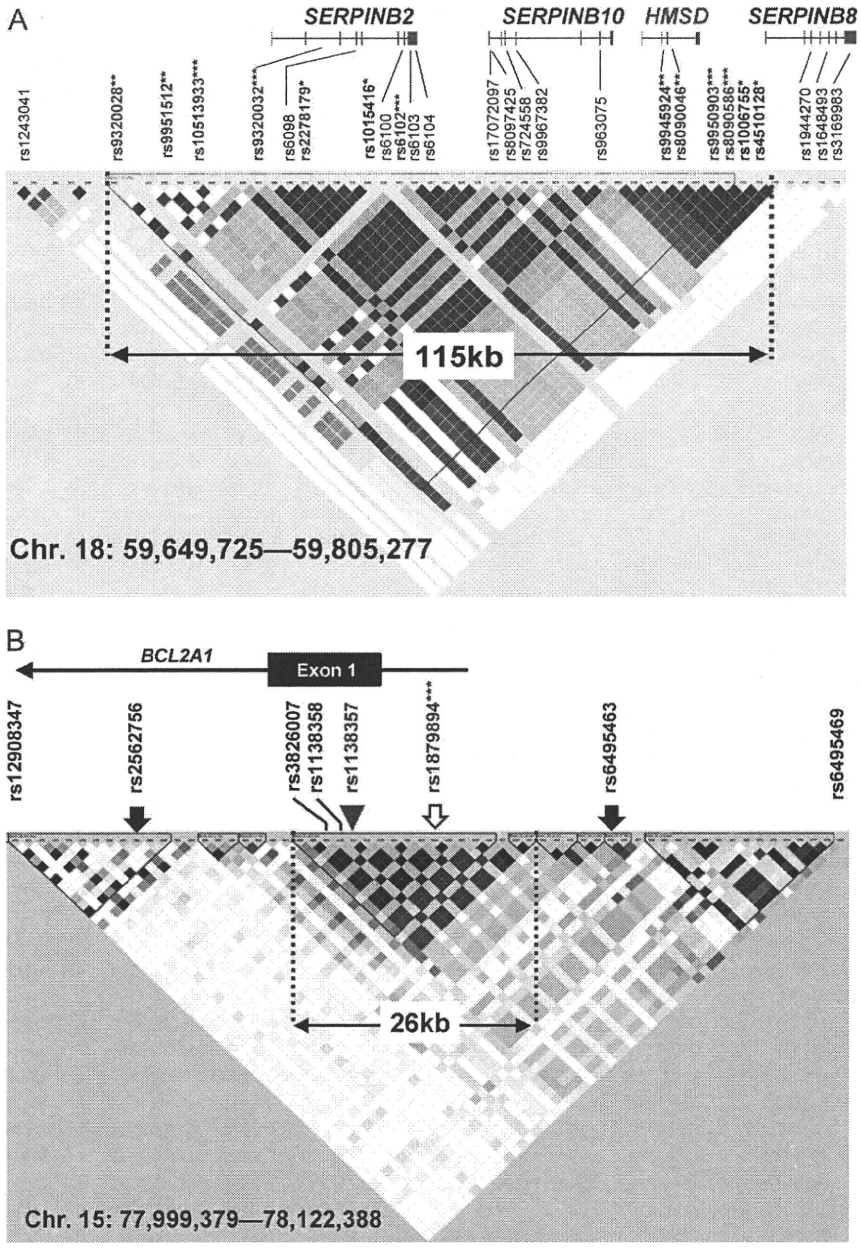


**Figure 3. Linkage disequilibrium (LD) block mapped by CTL-2A12 and CTL-1B9.** (A) An LD block map identified by pairwise $r^2$ plot from HapMap CEU data are overlayed with SNPs available from Affymetrix GeneChip SNP-genotyping microarrays (arrows) and 4 genes in the 115 kb block. SNPs that emerged 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, DYLQ*C*VLQI (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 DYLQ*C*VLQI 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 *HindIII*** | | | | | | | | | | | | | | | |
| 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 *NspI*** | | | | | | | | | | | | | | | |
| 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 | 86581518 | 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 *StyI*** | | | | | | | | | | | | | | | |
| 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

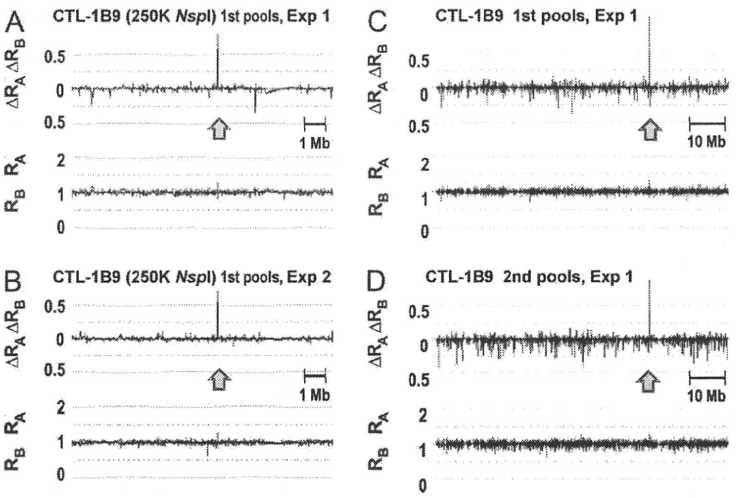


**Figure 4. Reproducible detection of association with the immunophenotypes determined by CTL-1B9 at the *BCL2A1* locus.** The maximum test statistic value was observed at a single SNP (rs1879894) within 15q25.1 in duplicate experiments for the first pools consisting of 57 $CTX^+$ and 38 $CTX^-$ B-LCLs (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

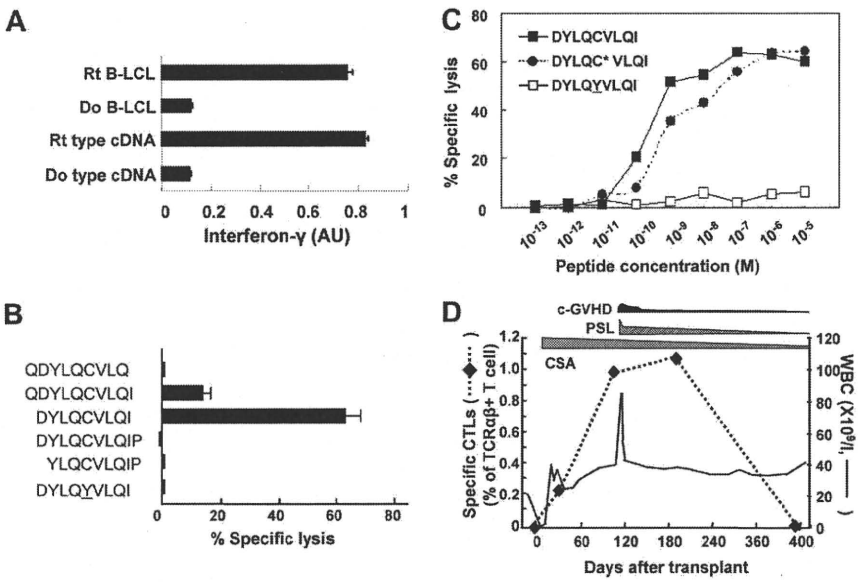


**Figure 5. Identification of the CTL-1B9 minimal minor H epitope.** (A) Interferon-γ production from CTL-1B9 against HLA-A*2402–transduced 293T cells transfected with plasmid encoding full-length *BCL2A1* cDNA cloned from either the recipient (Rt) from whom CTL-1B9 was isolated or his donor (Do). Rt 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-$1^C$–specific T cells in the recipient's peripheral blood. In order to longitudinally analyze the kinetics of the ACC-$1^C$–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 $TCR\alpha\beta^+$ 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-$1^C$ 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