A systematic scanning of the immunogenome with microsatellite markers in a Japanese HSCT population reveals multiple genetic risk loci for graft-versus-host disease

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Non-HLA gene polymorphisms contribute to the immune response leading to Graft-versus-host Disease (GVHD). We applied a systematic approach using microsattelite (ms) marker typing for a large number of immune response genes on pooled DNA of Japanese donors and recipients of haematopoietic stem cell transplants (HSCT) to identify recipient and donor risk loci for GVHD. Ms, due to their multiple alleles, are more informative than single nucleotide polymorphisms (SNP).

We selected 4,231 ms markers, tagging 3,093 target genes (representing the 'immunogenome') at close proximity (<100kb). We selected 922 unrelated HSCT donor/recipient pairs from the Japan Marrow Donor Programme (JMDP) registry, based on clinical homogeneity (acute leukaemia, age 4-40 years, myeloablative conditioning, bone marrow source). 35% of pairs had a 10/12 or 12/12 HLA match. The population was split into discovery and confirmation cohorts with 460/462 pairs each. Eight DNA pools, four for each of the two independent screening steps were created using a highly accurate DNA pooling method. While 4,321 ms were typed on the four pools of the 1st screening step, only markers positive here were typed on the 2nd screening pools. Fisher's exact test for 2x2 (each ms allele) and 2xm ChiSquare tests were performed, comparing allele frequencies of recipients with GVHD grade 0-1 with GVHD grade 2-4 (donors accordingly). Markers positive after both independent screening steps (p-value <0.05, same associated allele, consistent odd's ratio (OR)) were genotyped for confirmation on individual samples of all 922 pairs.

The independent, 2-step pooled DNA screening process has effectively reduced false-positive associations. In the final analysis, 39 (recipient) and 58 (donor) ms loci remain associated with risk or protection from GVHD. Of 14 microsatellite loci so far investigated by individual typing, four loci were confirmed while two showed a trend (donor: DXS0629i: p=0.001, OR 0.293; D6S0035i: p=0.005, OR 0.725; D17S0219i: p=0.001, OR 0.464; TNFc: p=0.052, OR 1.264; recipient: DXS0324i: p=0.008, OR 1.352; D16S3082: p=0.065, OR 1.372.

Our data show that genetic susceptibility to GVHD following HSCT is complex and depends on multiple recipient and donor risk loci. Large-scale genomic screening with microsatellites on pooled DNA, here described for the first time in a HSCT population, is a useful method for the systematic evaluation of multigenic traits.

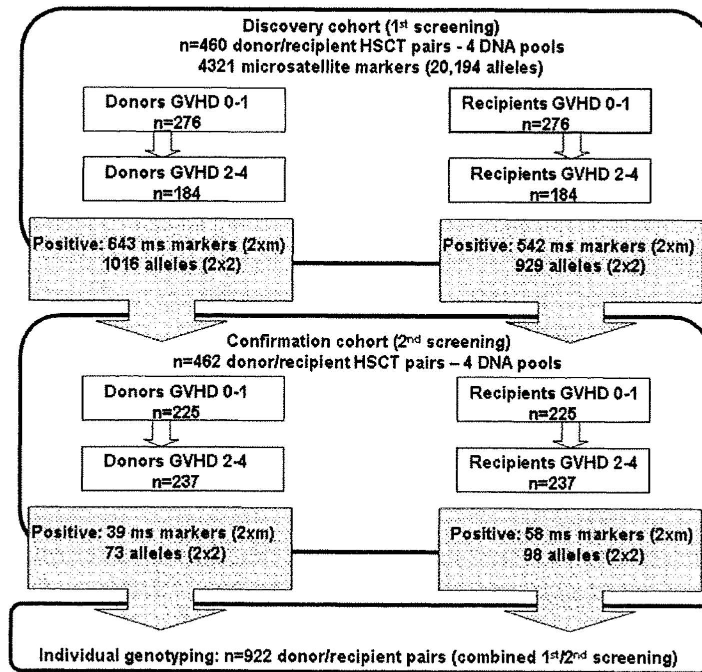

Fig.1: Study design and results, comparing microsatellite allele frequencies in pooled DNA.
Allele frequencies were derived from allele peak height in microsatellite typing.

厚生労働省がん研究助成金「成人難治性造血器腫瘍に対する非血縁者間の同種造血幹細胞移植法の確立に関する研究」班　班会議資料

ドナー由来のＴ細胞から見たHLA-Cの適合度とNK細胞受容体(KIR2DL ligand)適合度に基づいた治療成績の分析

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ドナー・患者間での HLA の適合度が非血血縁者間骨髄移植の臨床成績に関与している事がこれまでの解析で明らかにされてきた。HLA-C の適合度に関しては、ドナー由来のＴ細胞から見た適合度（適合、GVH 方向に適合、HVG 方向に不適合、両方向に不適合、以下 HLA-C 適合度と略す）とNK 細胞受容体(KIR2DL ligand)の適合度（以下 NK 細胞受容体適合度と略す）がある。JMDP を介した移植における各々の臨床的重要性はこれまでの解析で明らかとなっているが、相互の関係については十分な解析がなされていない。そこで今回、HLA-A、B、DR の血清型が適合した JMDP5210 症例を、ドナー由来の HLA-C 適合度と NK 細胞受容体適合度で下表のように群分けし解析を行った。方法は多変量解析の手法を用い、HLA-C 適合群を基準として、各群の重度急性 GVHD 発症リスク・死亡リスク(Hazard ratio (HR)) および 95%信頼区間(95%CI) を算出した。調整因子として臨床的因子と他座の適合度を用いた。

NK細胞受容体(KIR2DL ligand)適合度	ドナー由来のT細胞から見たHLA-Cの適合度				
	適合	HVG方向不適合 (Hetero to Homo)	GVH方向不適合 (Homo to Hetero)	両方向に1アリル不適合	2アリル不適合
適合	①	②	④	⑥	
HVG方向不適合			⑤	⑦	
GVH方向不適合		③			⑧
両方向不適合					

【結果】

HLA-C、NK 細胞受容体の HVG 方向の不適合はともに重症急性 GVHD 発症リスク、死亡リスクの上昇はわずかであり、一方、GVH 方向の不適合は重症急性 GVHD 発症リスク、死亡リスクを上昇させることが示唆された。さらに、HLA-C の GVH 方向の不適合、NK 細胞受容体の GVH 方向の不適合がともにある群（⑧）の重症急性 GVHD 発症リスクが最も高かった。
今後の更なる検討が必要であるが、今回の解析により、JMDP を介した移植におけるドナー由来のＴ細胞から見たHLA-C の適合度とNK 細胞受容体(KIR2DL ligand)適合度の相互関係がある程度明らかとなった。

「NK細胞受容体、サイトカイン遺伝子多型および検体保存事業協力」

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○屋部登志雄、平安恒幸、中本貴之、峯元睦子、柏瀬貢一

１、KIR遺伝子型、HLAリガンド型適合性

昨年度までに1993～2000年の非血縁者間骨髄移植症例を解析しHLA-C抗原のKIRリガンド型（C1,C2）GVH方向不適合の場合に急性GVHDの重症化および全生存率の低下が見られること、その効果はATG投与およびドナー活性化型KIR遺伝子の有無に影響されることを報告してきた。今年度は2001～2005年までの移植症例の解析を行い、これまでの結果の再現性確認およびHLA適合症例におけるKIR型と成績との関連について検討した。

２、LILR遺伝子型多型

マウスMHCクラスI抗原認識受容体PIR-Bが骨髄移植急性GVHD重症化と関連することが報告されている(Nat Immunol 2004)。ヒトの相同分子と考えられるLILRはNK,DC,顆粒球細胞などで発現する活性化型ならびに抑制型の11種類からなるHLAクラスⅠ抗原認識ペア型受容体ファミリーである。昨年度は機能的にPIR-Bと類似するLILRB2のSNPタイピングを行った。今年度はさらに他のLILR遺伝子SNPについてもHLA6座アリル一致症例ペアをタイピングして、移植成績との関連解析を行っている。

３、サイトカイン/サイトカイン受容体遺伝子多型

昨年度までに非血縁者間骨髄移植において、患者の抑制性のサイトカインであるIL-10遺伝子のプロモーター領域3か所のSNPハプロタイプが急性重症GVHD発症および無病生存率と関連することを報告した。本研究では、米国での血縁者間HLA一致骨髄移植解析（New Eng J Med 2003）と関連するハプロタイプが全く異なる結果となり、白人と東アジア人集団でのハプロタイプ多様性の違いによるものと考えられた。そこで今年度はさらに周辺領域のSNPと成績との関連を解析した。

４、検体保存事業協力

後方視野的研究のためにJMDPが収集、保存する患者ドナー検体の血液から抽出されたDNAを用いてHLA-AからDPの6座を蛍光ビーズ法でアリルタイピングしており、本年は2007年度に保存された1588検体について行った。またこれまでに保存されていたDNAの全ゲノム増幅（WGA）系の構築と検証作業および検体セットをロボットシステムで作成、配布する系の構築を終了し、解析希望研究者への検体配布作業（東海大学担当）に協力した。

別紙４

研究成果の刊行に関する一覧表

雑誌

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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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Acknowledgments: we are indebted to Drs. Hiroko Oshima, Masanobu Oshima and Atsushi Hirao, Mrs. Shinichi Ohmae and Katsuya Nakata, and Ms. Kaori Matsuura at Kanazawa University, and Drs. Keitaro Matsuo and Takakazu Kawase at the Aichi Cancer Center Research Institute for their technical assistance. We thank all of the JMDP transplant teams who contributed patients and donors to this study.

Funding: this study was supported by grants from the Ministry of Health, Labor and Welfare, and the Ministry of Education, Culture, Sports and Technology, and funds from the Mitani Research and Development Assistance Organization (Kanazawa, Japan) and by the Japan Leukemia Research Fund (Tokyo, Japan).

Manuscript received on March 5, 2009. Revised version arrived on April 13, 2009. Manuscript accepted on April 29, 2009.

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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.

Citation: Espinoza JL, Takami A, Onizuka M, Sao H, Akiyama H, Miyamura K, Okamoto S, Inoue M, Kanda Y, Ohtake S, Fukuda T, Morishima Y, Kodera Y, and Nakao S, for the Japan Marrow Donor Program. NKG2D *gene polymorphism has a significant impact on transplant outcomes after HLA-fully-matched unrelated bone marrow transplantation for standard risk hematologic malignancies. Haematologica 2009;94:1427-1434. doi:10.3324/haematol.2009.008318*

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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 0.5×10^9/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.

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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 χ^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; $p=0.02$).

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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.**

Variable	Overall survival			Transplant-related mortality			Relapse			Grades II-IV acute GVHD			Chronic GVHD		
	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-

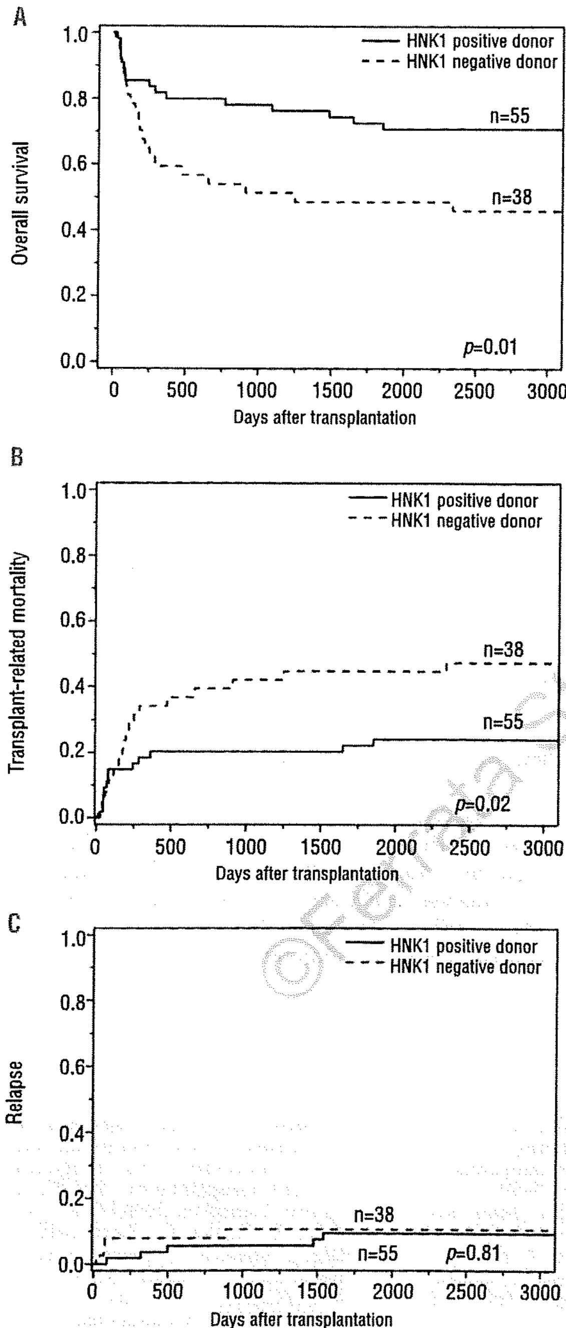

Figure 1. **Kaplan-Meier analysis of (A) overall survival, (B) cumulative incidence of transplant-related mortality and (C) disease relapse after transplantation according to the donor *NKG2D* polymorphism in patients with standard-risk disease. Patients with donors with the *HNK1* haplotype had better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

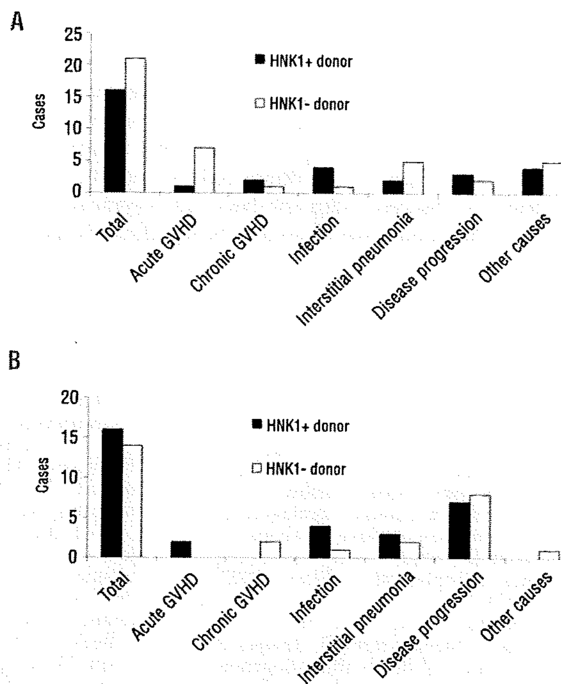

Figure 2. **Main causes of death after transplantation according to the *NKG2D* polymorphism in patients with (A) standard-risk disease (B) high-risk disease.**

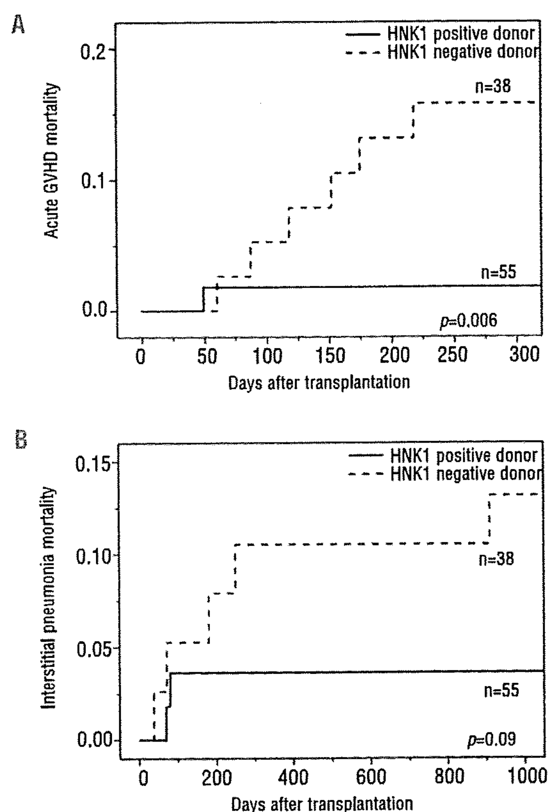

Figure 3. Cumulative incidence of deaths due to (A) acute GVHD and (B) interstitial pneumonia after transplantation in patients with standard-risk disease. The *HNK1* haplotype in donors was associated with a significantly lower incidence of deaths due to acute GVHD (p=0.006) as well as a trend toward 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/CARD*15 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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HapMap scanning of novel human minor histocompatibility antigens

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Minor histocompatibility antigens (mHags) are molecular targets of allo-immunity associated with hematopoietic stem cell transplantation (HSCT) and involved in graft-versus-host disease, but they also have beneficial antitumor activity. mHags are typically defined by host SNPs that are not shared by the donor and are immunologically recognized by cytotoxic T cells isolated from post-HSCT patients. However, the number of molecularly identified mHags is still too small to allow prospective studies of their clinical importance in transplantation medicine, mostly due to the lack of an efficient method for isolation. Here we show that when combined with conventional immunologic assays, the large data set from the International HapMap Project can be directly used for genetic mapping of novel mHags. Based on the immunologically determined mHag status in HapMap panels, a target mHag locus can be uniquely mapped through whole genome association scanning taking advantage of the unprecedented resolution and power obtained with more than 3 000 000 markers. The feasibility of our approach could be supported by extensive simulations and further confirmed by actually isolating 2 novel mHags as well as 1 previously identified example. The HapMap data set represents an invaluable resource for investigating human variation, with obvious applications in genetic mapping of clinically relevant human traits. (Blood. 2009;113:5041-5048)

Introduction

The antitumor activity of allogeneic hematopoietic stem cell transplantation (HSCT), which is a curative treatment for many patients with hematologic malignancies, is mediated in part by immune responses that are elicited as a consequence of incompatibility in genetic polymorphisms between the donor and the recipient.[1,2] Analysis of patients treated for posttransplantation relapse with donor lymphocytes has shown tumor regression to be correlated with expansion of cytotoxic T lymphocytes (CTLs) specific for hematopoiesis-restricted minor histocompatibility antigens (mHags).[3,4] mHags are peptides, presented by major histocompatibility complex (MHC) molecules, derived from intracellular proteins that differ between donor and recipient due mostly to single nucleotide polymorphisms (SNPs) or copy number variations (CNVs).[1,2,5] Identification and characterization of mHags that are specifically expressed in hematopoietic but not in other normal tissues could contribute to graft-versus-leukemia/lymphoma (GVL) effects, while minimizing unfavorable graft-versus-host disease, one of the most serious complications of allo-HSCT.[1,2] Unfortunately, however, efforts to prospectively target mHags to invoke T cell–mediated selective GVL effects have been hampered by the scarcity of eligible mHags, largely due to the lack of efficient methods for mapping the relevant genetic loci. Several methods have been developed to identify mHags, including peptide elution from MHC,[6,7] cDNA expression cloning,[8,9] and linkage analysis.[3,10] We have recently reported a novel genetic method that combines whole genome association scanning with conventional chromium release cytotoxicity assays (CRAs). With this approach the genetic loci of the mHag gene recognized by a given CTL clone can be precisely identified using SNP array analysis of pooled DNA generated from immortalized lymphoblastoid cell lines (LCLs) that are immunophenotyped into mHag$^+$ and mHag$^-$ groups by CRA.[11] The mapping resolution has now been improved from several Mb for conventional linkage analysis to an average haplotype block size of less than 100 kb,[12] usually containing a handful of candidate genes. Nevertheless, it still requires laborious DNA pooling and scanning of SNP arrays with professional expertise for individual

Submitted July 29, 2008; accepted August 27, 2008. Prepublished online as *Blood* First Editon paper, September 22, 2008; DOI 10.1182/blood-2008-07-171678.

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An Inside *Blood* analysis of this article appears at the front of this issue.

The online version of this article contains a data supplement.

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CTLs.[11] To circumvent these drawbacks, we have sought to take advantage of publicly available HapMap resources. Here, we describe a powerful approach for rapidly identifying mHAg loci using a large genotyping data set and LCLs from the International HapMap Project for genome-wide association analysis.[13-15]

Methods

Cell lines and CTL clones

The HapMap LCL samples were purchased from the Coriell Institute (Camden, NJ). All LCLs were maintained in RPMI1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 1 mM sodium pyruvate. Because the recognition of a mHag requires presentation on a particular type of HLA molecule, the LCLs were stably transduced with a retroviral vector encoding the restriction HLA cDNA for a given CTL clone when necessary.[16]

CTL lines were generated from recipient peripheral blood mononuclear cells obtained after transplantation by stimulation with those harvested before HSCT after irradiation (33 Gy), and thereafter stimulated weekly in RPMI 1640 supplemented with 10% pooled human serum and 2 mM L-glutamine. Recombinant human inerleukin-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.[10,17] HLA restriction was determined by conventional CRAs against a panel of LCLs sharing HLA alleles with the CTLs. All clinical samples were collected based on a protocol approved by the Institutional Review Board Committee at Aichi Cancer Center and the University of Tokyoand after written informed consent was obtained in accordance with the Declaration of Helsinki.

Immunophenotyping of HapMap LCLs and high-density genome-wide scanning of mHag loci

Case ($mHag^+$) - control ($mHag^-$) LCL panels were generated by screening corresponding restriction HLA-transduced CHB and JPT HapMap LCL panels with each CTL clone using CRAs. Briefly, target cells were labeled with 0.1 mCi of ^{51}Cr for 2 hours, and 10^3 target cells per well were mixed with CTL at a predetermined E/T ratio in a standard 4-hour CRA. All assays were performed at least in duplicate. The percent specific lysis was calculated by ((Experimental cpm - Spontaneous cpm) / (Maximum cpm - Spontaneous cpm)) × 100. After normalization by dividing their percent specific lysis values by that of positive control LCL (typically recipient-derived LCL corresponding to individual CTL clones), the mHag status of each HapMap LCL was defined as positive, negative, or undetermined.

To identify mHag loci, we performed association tests for all the Phase II HapMap SNPs, by calculating χ^2 test statistics based on 2 × 2 contingency tables with regard to the mHag status as measured by CRA and the HapMap genotypes (presence or absence of a particular allele) at each locus. χ^2 were calculated for the 2 possible mHag alleles at each locus and the larger value was adopted for each SNP. While different test statistics may be used showing different performance, the χ^2 statistic is most convenient for the purpose of power estimation as described below. The maximum value of the χ^2 statistics was evaluated against the thresholds empirically calculated from 100 000 random permutations within a given LCL set. The program was written in C++ and will run on a unix clone. It will be freely distributed on request. Computation of the statistics was performed within several seconds on a Macintosh equipped with 2 × quadcore 3.2 GHz Zeon processors (Apple, Cupertino, CA), although 100 000 permutations took several hours on average.

Evaluation of the power of association tests using HapMap samples

The genotyping data of the Phase II HapMap[14] were obtained from the International HapMap Project website (http://www.hapmap.org/genotypes/latest_ncbi_build35), among which we used the nonredundant data sets (excluding SNPs on the Y chromosome) from 60 CEU (Utah residents with ancestry from northern and western Europe) parents, 60 YRI (Yoruba in Ibadan, Nigeria) parents, and the combined set of 45 JPT (Japanese in Tokyo, Japan) and 45 CHB (Han Chinese in Beijing, China) unrelated people. They contained 3 901 416 (2 624 947 polymorphic), 3 843 537 (295 293 polymorphic), and 3 933 720 (2 516 310 polymorphic) SNPs for CEU, YRI, and JPT + CHB, respectively.

To evaluate the power, we first assumed that the Phase II HapMap SNP set contains the target SNP of the relevant mHag or its complete proxies, and that the immunologic assays can completely discriminate i $mHag^+$ and j $mHag^-$ HapMap LCLs. Under this ideal condition, the test statistic, or χ^2, for these SNPs takes a definite value, $f(i,j) = i+j$, which was compared with the maximum χ^2 value, or its distribution, under the null hypothesis, that is, no SNPs within the Phase II HapMap set should be associated with the mHag locus. Unfortunately, the latter distribution cannot be calculated in an explicit analytical form but needs to be empirically determined based on HapMap data, because Phase II HapMap SNPs are mutually interdependent due to extensive linkage disequilibrium within human populations. For this purpose, we simulated 10 000 case-control panels by randomly choosing i $mHag^+$ and j $mHag^-$ HapMap LCLs for various combinations of (i,j) and calculated the maximum χ^2 values (χ^2_{max}) for each panel to identify those (i,j) combinations, in which $f(i,j)$ exceeds the upper 1 percentile point of the simulated 10 000 maximum values, $g(i,j)^{P=.01}$.

When proxies are not complete (ie, $r^2 < 1$), the expected values will be decayed by the factor of r^2, and further reduced due to the probabilities of false positive (f_P) and negative (f_N) assays, and expressed as $\hat{f}(i,j) = (i + j) \times \hat{r}^2$ through an apparent r^2 ($\hat{r}^2$) as provided in formula 1.[1] Under given probabilities of assay errors and maximum LD strength between markers and the mHag allele, we can expect to identify target mHag loci for those (i,j) sets that satisfy $\hat{f}(i,j) > g(i,j)^{P=.01}$.

Empirical estimation of distributions of r^2

The maximum r^2 value (r^2_{max}) between a given mHag allele and one or more Phase II HapMap SNPs was estimated based on the observed HapMap data set. Each Phase II HapMap SNP was assumed to represent a target mHag allele, and the (r^2_{max}) was calculated, taking into account all the Phase II HapMap SNPs less than 500 kb apart from the target SNP.

Confirmatory genotyping

Genotyping was carried out either by TaqMan MGB technology (Applied Biosystems, Foster City, CA) with primers and probes for HA-1 mHag according to the manufacturer's protocol using an ABI 7900HT with the aid of SDS version 2.2 software (Applied Biosystems) or by direct sequencing of amplified cDNA for the *SLC1A5* gene. cDNA was reverse transcribed from total RNA extracted from LCLs, and polymerase chain reaction (PCR) was conducted with cDNA with the corresponding primers. Amplified DNA samples were sequenced using BigDye Terminator version 3.1 (Applied Biosystems). The presence or absence (deletion) of the *UGT2B17* gene was confirmed by genomic PCR with 2 primer sets for exons 1 and 6 as described previously[18] using DNA isolated from LCLs of interest.

Epitope mapping

A series of deletion mutant cDNAs were designed and cloned into pcDNA3.1/V5-His TOPO plasmid (Invitrogen, Carlsbad, CA). Thereafter, 293T cells that had been transduced with restricting HLA class I cDNA for individual CTL clones were transfected with each of the deletion mutants and cocultured with the CTL clone overnight to induce interferon (IFN)-γ release, which was then evaluated by enzyme-linked immunosorbent assay (ELISA) as previously described.[9]

For *SLC1A5*, expression plasmids encoding full-length cDNA and the exon 1 of recipient and donor origin were first constructed because only the SNP in the exon 1 was found to be concordant with susceptibility to CTL-3B6. Next, amino (N)- and (carboxyl) C-terminus–truncated minigenes encoding polypeptides around the polymorphic amino acid defined by the SNP were amplified by PCR from *SLC1A5* exon 1 cDNA as template and cloned into the above plasmid. The constructs all encoded a Kozak

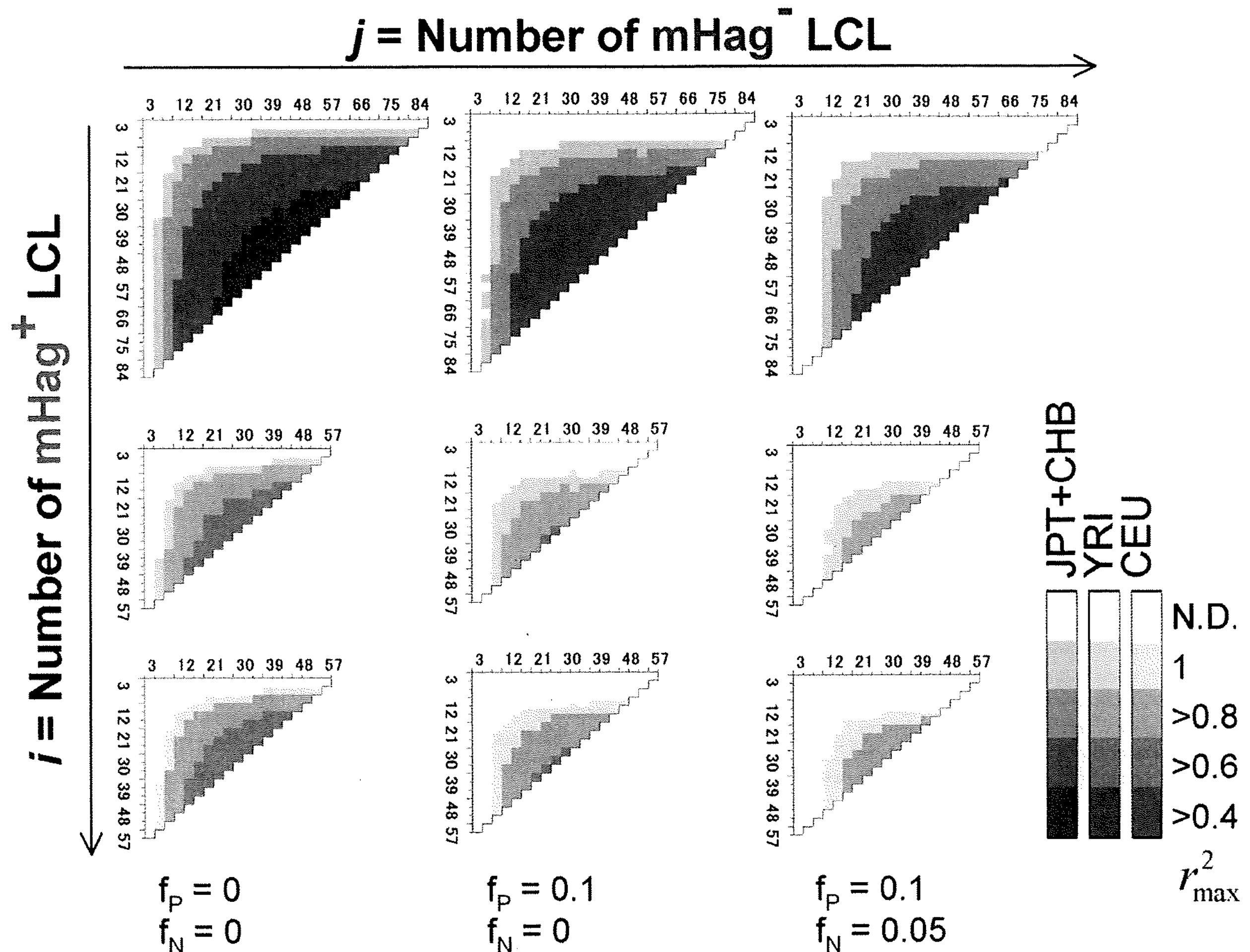

Figure 1. Numbers of positive and negative LCLs required for successful mHag mapping. The target locus was assumed to be uniquely identified, if the expected χ^2 value for the target SNP ($\hat{f}(i,j)$, see Document S1) exceeded the upper 1 percentile point of the maximum χ^2 values in 10 000 simulated case-control panels ($g(i,j)^{P=.01}$). Combinations of the numbers of mHag$^+$ (vertical coordinates) and mHag$^-$ (horizontal coordinates) samples satisfying the above condition are shown in color gradients corresponding to different max r^2 values between the target SNP and one or more nearby Phase II HapMap SNPs (r^2_{max}), ranging from 0.4 to 1.0. Calculations were made for 3 HapMap population panels, CHB + JPT (top), YRI (middle), and CEU (bottom) and for different false positive and negative rates, $f_P = f_N = 0$ (left), $f_P = 0.1, f_N = 0$ (middle), and $f_P = 0.1, f_N = 0.05$ (right), considering the very low false negative assays for CRAs.

sequence and initiator methionine (CCACC-ATG) and for C-terminus deletions a stop codon (TAG).

For *UGT2B17*, a series of C-terminus deletion mutants with approximately 200 bp spacing was first constructed as above. For further mapping, N-terminus deletion mutants were added to the region that was deduced to be potentially encoding the CTL-1B2 epitope. For prediction of a CTL epitope, the HLA Peptide Binding Predictions algorithm on the BioInformatics & Molecular Analysis Section (BIMAS) website (http://www.bimas.cit.nih.gov/molbio/hla_bind/)[19] was used because HLA-A*0206 has a similar binding motif to that of A*0201.

Epitope reconstitution assay

The candidate mHag epitopes and allelic counterpart peptides (in case of SLC1A5) were synthesized by standard Fmoc chemistry. ^{51}Cr-labeled mHag$^-$ donor LCL were incubated with graded concentrations of the peptides and then used as targets in standard CRAs.

Results and discussion

Statistical approach and estimation of potential overfitting

We reasoned that the mHag locus recognized by a given CTL clone could be defined by grouping LCLs from a HapMap panel into mHag$^+$ and mHag$^-$ subpanels according to their susceptibility to lysis by the CTL clone and then performing an association scan using the highly qualified HapMap data set containing more than 3 000 000 SNP markers. The relevant genetic trait here is expected to show near-complete penetrance, and the major concern with this approach arises from the risk of overfitting observed phenotypes to one or more incidental SNPs with this large number of HapMap SNPs under the relatively limited size of freedom due to small numbers of independent HapMap samples (90 for JPT + CHB and 60 for CEU and YRI, when not including their offspring).[13]

To address this problem, we first estimated the maximum sizes of the test statistics (here, χ^2 values) under the null hypothesis (ie, no associated SNPs within the HapMap set) by simulating 10 000 case-control HapMap panels under different experimental conditions, and compared them with the expected size of test statistic values from the marker SNPs associated with the target SNP, assuming different linkage disequilibrium (LD), or r^2 values in between. As shown in Figure 1, the possibility of overfitting became progressively reduced as the number of LCLs increased, which would allow for identification of the target locus in a broad range of r^2 values, except for those mHags having very low minor allele frequencies (MAF) below

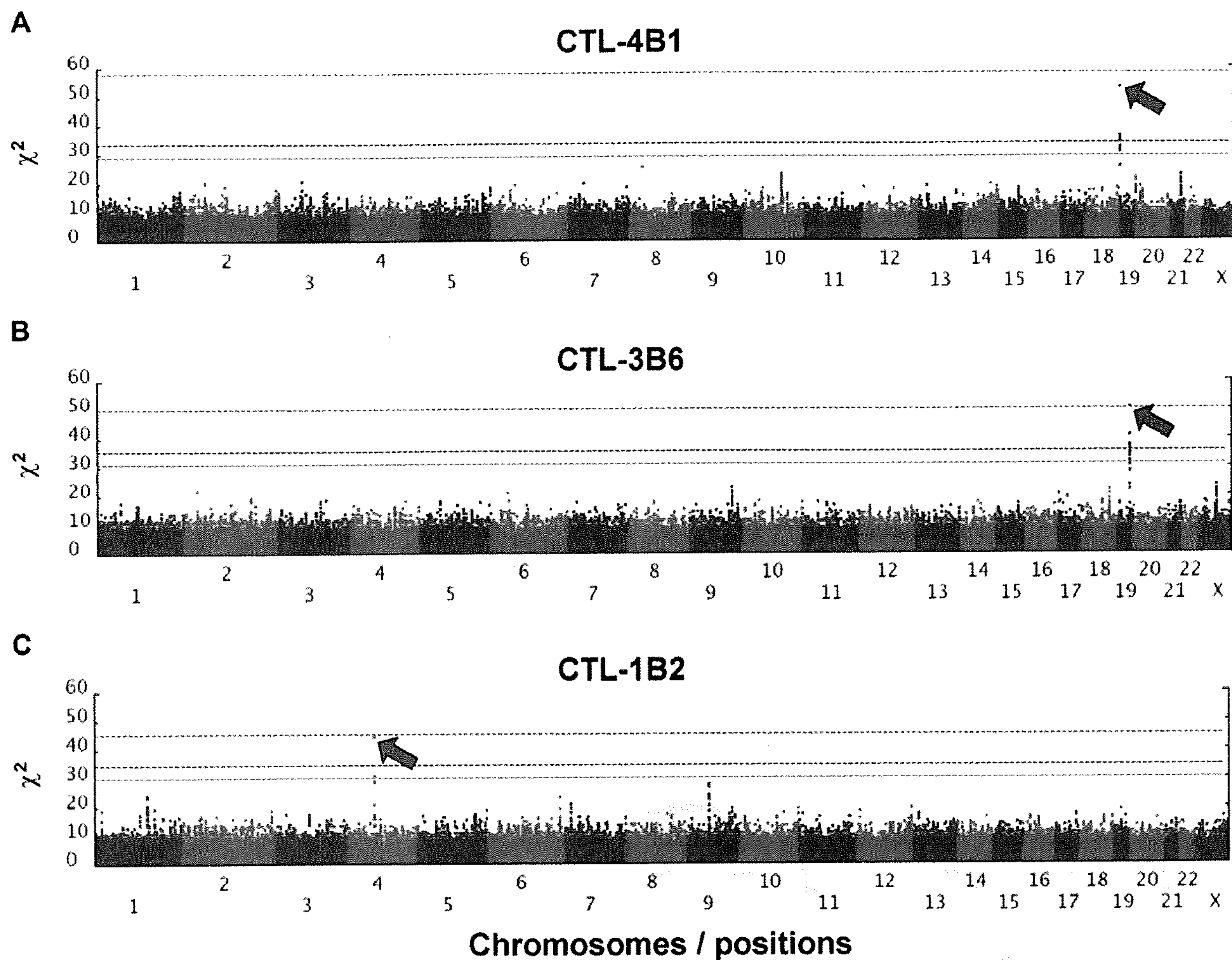

Figure 2. Genome-wide scanning to identify chromosome location of mHag. χ^2 values were plotted against positions on each chromosome for each of 3 mHags recognized by CTL-4B1 (A), CTL-3B6 (B), and CTL-1B2 (C). Chromosomes are displayed in alternating colors. Threshold χ^2 values corresponding to the genome-wide $P = 10^{-3}$ (dark blue) and 10^{-2} (light blue), as empirically determined from 100 000 random permutations, are indicated by broken lines, while the theoretically possible maximum values are shown with red broken lines. The highest χ^2 value in each experiment is indicated by a red arrow.

approximately 0.05. According to our estimation using the Phase II HapMap data (see "Methods"), the majority (> 90%) of common target SNPs (MAF > ~ 0.05) could be captured by one or more HapMap SNPs with more than 0.8 of r^2 (Figure S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article), ensuring a high probability of detecting an association (Figure 1 left panels). The simulation of pseudo-Phase II sets generated from the ENCODE regions provided a similar estimation.[13] False positive and negative immunophenotyping results could also complicate the detection, reducing the expected test statistics through the "apparent" r^2 values ($\hat{r}^2$), as defined by

$$\hat{r}^2 = r^2 \times \frac{(l - f_P - f_N)^2}{(l - f_P + f_N q)(l - f_N + f_P/q)} \quad (1)$$

where f_P, f_N, and q represent false typing probabilities with positive and negative LCL panels, and the ratio of the positive to the negative LCL number, respectively. However, the high precision of cytotoxicity assays ($f_P \sim < 0.1$, $f_N \sim = 0$) limits this drawback from the second term to within acceptable levels and allows for sensitive mHag locus mapping with practical sample sizes (Figure 1 middle and right panels), suggesting the robustness of our novel approach.

Evaluation of the detection power for known mHags

Based on these considerations, we then assessed whether this approach could be used to correctly pinpoint known mHag loci (Table S1). Because the relevant mHag alleles are common SNPs and directly genotyped in the Phase II HapMap set, or if not, located within a well-defined LD block recognized in this set (Figure S2), their loci would be expected to be uniquely determined with an acceptable number of samples, as predicted from Figure 1. To test this experimentally, we first mapped the locus for HA-1^H mHag[7] by evaluating recognition of the HLA-A*0206–transduced HapMap cell panel with HLA-A*0206–restricted CTL-4B1.[20] After screening 58 well-growing LCLs from the JPT + CHB panel with CRAs using CTL-4B1 (Figure S3A; Tables S2,S3), we obtained 37 mHag$^+$ and 21 mHag$^-$ LCLs, which were tested for association at 3 933 720 SNP loci. The SNP (rs1801284) encoding the mHag is located within a HapMap LD block on chromosome 19q13.3, but is not directly genotyped within this data set. The genome-wide scan clearly indicated a unique association with the HA-1^H locus within the *HMHA1* gene, showing a peak χ^2 statistic of 52.8 (not reached in 100 000 permutations) at rs10421359 (Figures 2A,3A; Tables S2,S3).

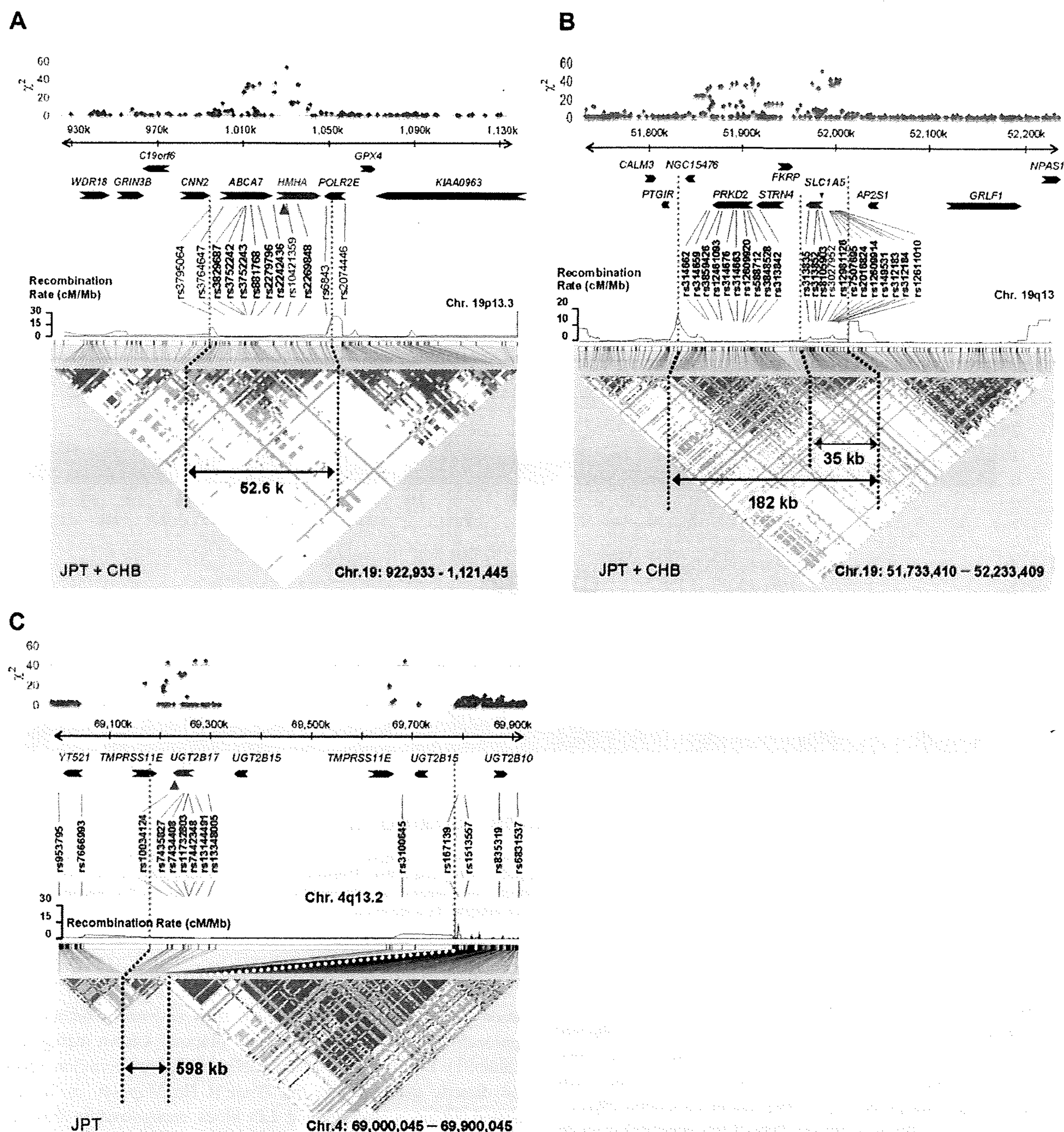

Figure 3. Regions of mHag loci identified by HapMap scanning. LD structures around the SNPs showing peak statistical values (in JPT + CHB) are presented for each mHag locus identified with (A) CTL-4B1, (B) CTL-3B6, and (C) CTL-1B2. Regional χ^2 plots are also provided on the top of each panel. LD plots in pairwise D's with recombination rates along the segment were drawn with HaploView software version 4.0 (http://www.broad.mit.edu/mpg/haploview/). The size and location of each LD block containing a mHag locus are indicated within the panels. Significant SNPs (blue letters), as well as other representative SNPs, are shown in relation to known genes. The positions of the SNPs showing the highest statistic values (red letters) are indicated by red arrowheads.

Identification of novel mHags

We next applied this method to mapping novel mHags recognized by CTL clone 3B6, which is HLA-B*4002–restricted; and CTL clone 1B2, which is HLA-A*0206–restricted. Both clones had been isolated from peripheral blood samples of post-HSCT different patients. In preliminary CRAs with the JPT + CHB panel, allele frequencies of target mHags for CTL-3B6 and CTL-1B2 in this panel were estimated as approximately 25% and approximately 45%, respectively (data not shown). After screening 72 JPT + CHB LCLs with CTL-3B6, 36 mHag$^+$ and 14 mHag$^-$ LCLs were obtained, leaving 22 LCLs undetermined based on empirically determined thresholds (> 51% for mHag + LCLs and < 11% for mHag-LCLs; Figure S3B, Tables S2,S4). As shown in Figure 2B, the χ^2 statistics calculated from the immunophenotyping data produced discrete peaks in the LCL sets. The peak in chromosome 19q13.3 for the CTL-3B6 set showed the theoretically maximum χ^2 value of 50 (not reached in 100 000 permutations) at rs3027952, which was mapped within a small LD block of

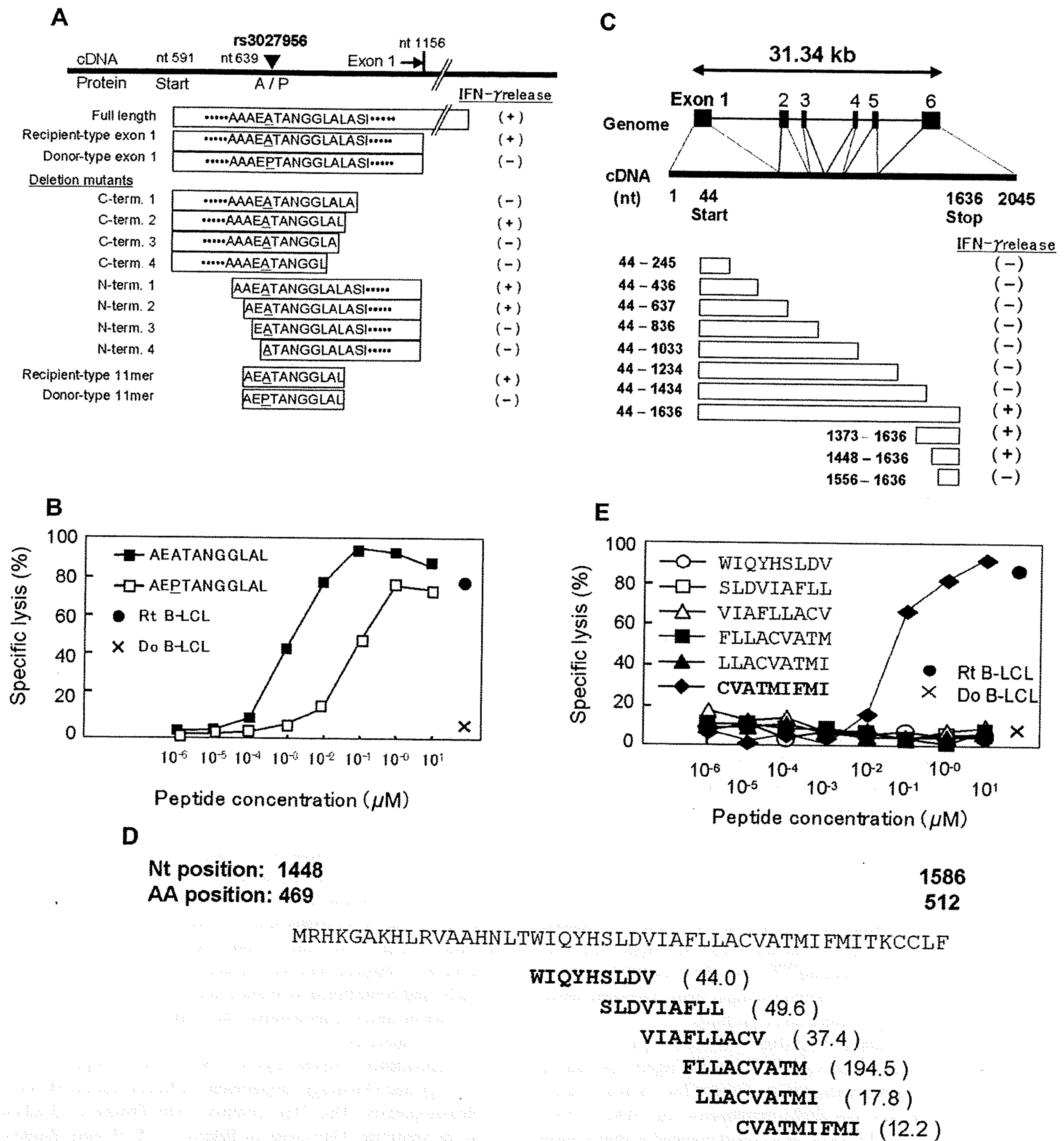

Figure 4. Epitope mapping. (A) Determination of the *SLC1A5* epitope by deletion mapping. Plasmids encoding recipient full-length *SLC1A5*, exon 1 of recipient and donor, exon 1 with various N- and C-terminus deletions around the amino acid encoded by SNP rs51983014, and minigenes encoding AEATANGGLAL and its allelic counterpart AEPTANGGLAL were constructed and transfected into HLA-B*4002–transduced 293T cells. Interferon (IFN)-γ was assessed by ELISA (right column) after coculture of CTL-3B1 with 293T transfectants. (B) Epitope reconstitution assay with synthetic undecameric peptides, AEATANGGLAL and AEPTANGGLAL. (C) Structure of the *UGT2B17* gene and screening of *UGT2B17* cDNA and deletion mutants. HLA-A*0206–transduced 293T cells were transfected with each plasmid and cocultured with CTL-2B1. IFN-γ production from CTL-1B2 (right column) indicated that the epitope was likely encoded by nucleotides 1448-1586, including 30 nucleotides from position 1566 that could potentially encode part of the epitope. (D) Epitope prediction using the HLA Peptide Binding Predictions algorithm.[19] Because HLA-A*0201 and -A*0206 have similar peptide binding motifs,[30] the algorithm for HLA-A*0201 was used to predict candidate epitopes recognized by CTL-1B2. Values in parentheses indicate the predicted half-time of dissociation. (E) Epitope reconstitution assays with graded concentrations of synthetic nonameric peptides shown in panel D.

approximately 182 kb, or more narrowly within its 35 kb sub-block containing a single gene, *SLC1A5*, as a candidate mHag gene (Figure 3B). In fact, when expressed in 293T cells with HLA-B*4002 transgene, recipient-derived, but not donor-derived, *SLC1A5* cDNA was able to stimulate IFN-γ secretion from CTL-3B6 (Figure 4A), indicating that *SLC1A5* actually encodes the target mHag recognized by CTL-3B6. Conventional epitope mapping using a series of deletion mutants of *SLC1A5* cDNA finally identified an undecameric peptide, AEATANGGLAL, as the minimal epitope (Figure 4A). The donor-type AEPTANGGLAL induced IFN-γ with a 2-log lower efficiency, suggesting that AEPTANGGLAL may not be transported efficiently into the ER

because endogenous expression of a minigene encoding AEPTANG-GLAL was not recognized by CTL-3B1 (Figure 4B). Unfortunately, although the peak statistic value showed the theoretically maximum value for this data set, it did not conform to the relevant SNP for this mHag (rs3027956) due to high genotyping errors of the HapMap data at this particular SNP. However, the result of our resequencing showed complete concordance with the presence of the rs3027956 SNP and recognition in the cytotoxicity assay (Table S4).

Similarly, 13 mHag$^+$ and 32 mHag$^-$ LCLs were identified from the screening of 45 JPT LCLs from the same panel using CTL-1B2 (Figure S3C; Tables S2,S5). The χ^2 statistics calculated from the immunophenotyping data produced bimodal discrete peaks with this LCL set. The target locus for the mHag recognized by CTL-1B2 was identified at a peak (max $\chi^2 = 44$, not reached in 100 000 permutations) within a 598-kb block on chromosome 4q13.1, coinciding with the locus for a previously reported mHag, *UGT2B17*[18] (Figures 2C, 3C). In fact, our epitope mapping using *UGT2B17* cDNA deletion mutants (Figure 4C), prediction of candidate epitopes by HLA-binding algorithms[19] (Figure 4D) and epitope reconstitution assays (Figure 4E), successfully identified a novel nonameric peptide, CVATMIFMI. Of particular note, this mHag was not defined by a SNP but by a CNV (ie, a null allele[18]) that is in complete LD with the SNPs showing the maximum χ^2 value (Table S5). Transplanted T cells from donors lacking both *UGT2B17* alleles are sensitized in recipients possessing at least 1 copy of this gene.[18] Although LD between SNPs and CNVs has been reported to be less prominent,[21] this is an example where a CNV trait could be captured by a SNP-based genome-wide association study.

The recent generation of the HapMap has had a profound impact on human genetics.[13,15] In the field of medical genetics, the HapMap is a central resource for the development of theories and methods that have made well-powered, genome-wide association studies of common human diseases a reality.[22-28] The HapMap samples provide not only an invaluable reference for genetic variations within human populations, but highly qualified genotypes that enable gene-wide scanning. Here, we have demonstrated how effectively HapMap resources can be used for genetic mapping of clinically relevant human traits. No imputations and tagging strategies are required[25,28] and the potential loss of statistical power due to very limited sample sizes is circumvented by accurate immunologic detection of the traits.

Using publicly available HapMap resources, high-throughput identification of mHag genes is possible without highly specialized equipment or expensive microarrays. Except for clinically irrelevant mHags with very low allele frequencies (eg, MAF $< 5\%$), the target of a given CTL can be sensitively mapped within a mean LD block size, typically containing just a few candidate genes. The methodology described here will facilitate construction of a large panel of human mHags including those presented by MHC class II molecules, and promote our understanding of human allo-immunity and development of targeted allo-immune therapies for hematologic malignancies.[1,2] The HapMap scan approach may be useful for exploring other genetic traits or molecular targets (eg, differential responses to some stress or drugs), if they can be discriminated accurately through appropriate biologic assays. In this context, the recent report that we may reprogram the fate of terminally differentiated human cells[29] is encouraging, indicating possible exploration of genotypes that are relevant to cell types other than immortalized B cells.

Acknowledgments

We thank Drs P. Martin and W. Ho for critically reading the manuscript; and Ms Keiko Nishida, Dr Ayako Demachi-Okamura, Dr Yukiko Watanabe, Ms Hiromi Tamaki, and the staff members of the transplant centers for their generous cooperation and technical expertise.

This study was supported in part by a grant for Scientific Research on Priority Areas (B01; no.17016089) from the Ministry of Education, Culture, Science, Sports, and Technology, Japan; grants for Research on the Human Genome, Tissue Engineering Food Biotechnology and the Second and Third Team Comprehensive 10-year Strategy for Cancer Control (no. 26) from the Ministry of Health, Labor, and Welfare, Japan; and a grant-in-aid from Core Research for Evolutional Science and Technology (CREST) of Japan.

Authorship

Contribution: M.K. performed most of immunologic experiments and analyzed data and wrote the manuscript; Y.N. performed the majority of genetic analyses and analyzed the data; H.T., T.K., M.Y., S.M. and K.Tsujimura performed research; K.Taura contributed to the computational simulation; Y.I., Taro T., K.M., Y.K. and Y.M. collected clinical data and specimens; T.I., H.T., S.R.R., Toshitada T. and K.K. contributed to data analysis and interpretation, and writing of the article; and Y.A. and S.O. supervised the entire project, designed and coordinated most of the experiments in this study, and contributed to manuscript preparation.

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

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