Table 3. Univariate analyses of acute GVHD and survival according to HLA disparity in the GVH direction

	Acute GVHD II-IV			2-year overall survival		
No. of HLA mismatches in the GVH direction	n	Cumulative incidence, %	*P*	n	Survival rate, %	*P*
HLA-A, -B, -DR (antigen)			.08			.5
0 + 1	50	33		59	36	
2	84	48		104	35	
HLA-A, -B (class I antigen)			.5			.2
0	22	36		24	54	
1	80	42		96	32	
2	32	46		43	32	
HLA-DR (class II antigen)			.5			.9
0	71	38		91	32	
1 + 2	63	47		72	38	
HLA-A, -B (antigen), -DR (allele)			.4			1.0
0 + 1	25	32		29	38	
2	48	51		60	38	
3 + 4	30	44		38	39	
HLA-A, -B, -DR (allele)			.3			.4
0 + 1	15	27		16	56	
2	35	49		41	37	
3 + 4 + 5	36	51		50	35	

GVHD in which activated donor T cells secreted various cytokines.[25] HLA disparity in the GVH direction may augment alloimmune reactions, which evoke hypercytokinemia and macrophage activation and occasionally result in establishment of hemophagocytic syndrome, one of the major complications directly related to graft failure in recipients.[26-28] Indeed, a considerable number of patients showed hemophagocytosis in the BM with donor dominancy, leading to graft failure, even though we cannot exclude the possibility of graft rejection caused by recipient lymphocytes in some cases. In addition, among those who achieved donor cell engraftment, delayed neutrophil recovery was prominent for those with more HLA mismatch in the GVH direction rather than in the HVG direction. Myelosuppression is commonly observed during acute or chronic GVHD, indicating that GVHD can negatively affect hematopoietic function of the graft, possibly because of an attack on the hematopoiesis-supporting recipient stromal cells[29] or production of cytokines from immune cells, such as transforming growth factor-β, known to regulate hematopoiesis negatively.[30] The delayed engraftment observed in our study may have been caused by similar mechanisms during the recovery of donor cells. Furthermore, our results demonstrated that HLA class I antigen mismatch in the GVH direction was associated with inferior engraftment. Higher impact of HLA class II disparity on the development of acute GVHD has been reported in National Marrow Donor Program data.[31] On the contrary, the Japan Marrow Donor Program registry data showed that mismatch in class I had higher impact than that in class II.[32] The discrepancy may be explained by unique ethnic background of the Japanese population. The observation shown here may further strengthen our hypothesis that GVH reactions play a crucial role in engraftment process. In the analysis using allele data, the statistical power of HLA disparity in the GVH direction on engraftment had decreased. This discrepancy probably results from the small sample size in each mismatched category but may be suggestive of more powerful immunogenicity of mismatch in antigen rather than allele level.

In the Eurocord registry data, which includes 550 CBTs, HLA disparity was shown to have a negative impact on engraftment, although the effect of direction of mismatch was not described.[14,33] More specifically, it was reported from the Düsseldorf Cord Blood Bank and Eurocord-Netcord Registry that HLA-A locus high-resolution typing in the HVG direction was associated with reduced cumulative incidence of engraftment in 122 patients receiving CBT.[34] Several reasons may explain this discrepancy from our observations. First, patients included in our study received relatively uniform pretransplantation conditioning regimens consisting mainly of fludarabine, melphalan, and TBI, whereas those in the Eurocord database had more variable pretransplantation conditioning regimens. Second, all of our patients had GVHD prophylaxis using single calcineurin inhibitors, whereas most of those in the Eurocord Registry received additional chemicals or anti-thymocyte globulin. Many institutes use methotrexate,[35,36] mycophenolate mofetil,[19,37] corticosteroids,[13] or anti-thymocyte globulin[38,39] in combination with a calcineurin inhibitor as GVHD prophylaxis in CBT. Narimatsu et al demonstrated that use of short-term methotrexate was associated with a lower rate of posttransplantation immune reactions without compromising engraftment.[36] Thus, more intensive immunosuppression may be beneficial for controlling early immune reactions and overcoming the issue of HLA mismatch. In addition, the unavoidable high incidence of gastrointestinal tract damage caused by TBI or melphalan in preparative regimens may have increased the chance of triggering GVH reactions.[40]

In the present study, HLA disparity had little association with the development of GVHD and survival, despite its obvious impact on engraftment. According to the Eurocord Registry data, better HLA match was not associated with better outcome in hematologic malignancies receiving CBT.[20] Further analyses are required to determine whether this is the result of the unique immunologic immaturity of CB or to the heterogeneous patient population with the majority being in the high-risk disease status.

In conclusion, HLA disparity in the GVH direction, especially class I disparity, was found to have a significant impact on engraftment. These results shed light on a novel mechanism responsible for graft failure in CBT and add a valuable clue for choosing a better CB unit to avoid graft failure.

Acknowledgments

The authors thank data coordinators Kaori Kobayashi and Naomi Yamada for their invaluable help in making this study possible; Dr Akinori Kimura, Department of Molecular Pathogenesis, Division of Pathophysiology, Medical Research Institute, Tokyo Medical and Dental University, for critical review of the manuscript; and the physicians, nurses, pharmacists, and support personnel for their care of patients in this study.

This work was supported in part by a Research Grant for Tissue Engineering (H17-014) from the Japanese Ministry of Health, Labor, and Welfare.

Authorship

Contribution: N.M. and A.W. performed research and extracted data; A.Y. reviewed histopathologic methods; N.M. and Y.K. performed statistical analysis; N.U. and S. Taniguchi reviewed study design and methods; and K.I., H.A., S. Takagi, M.T., H.Y., D.K., Y.M., S.S., K.M., S. Miyakoshi, and S. Makino contributed to the writing of the paper.

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

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HapMap scanning of novel human minor histocompatibility antigens

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Minor histocompatibility antigens (mHags) are molecular targets of alloimmunity 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 mini-genes 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

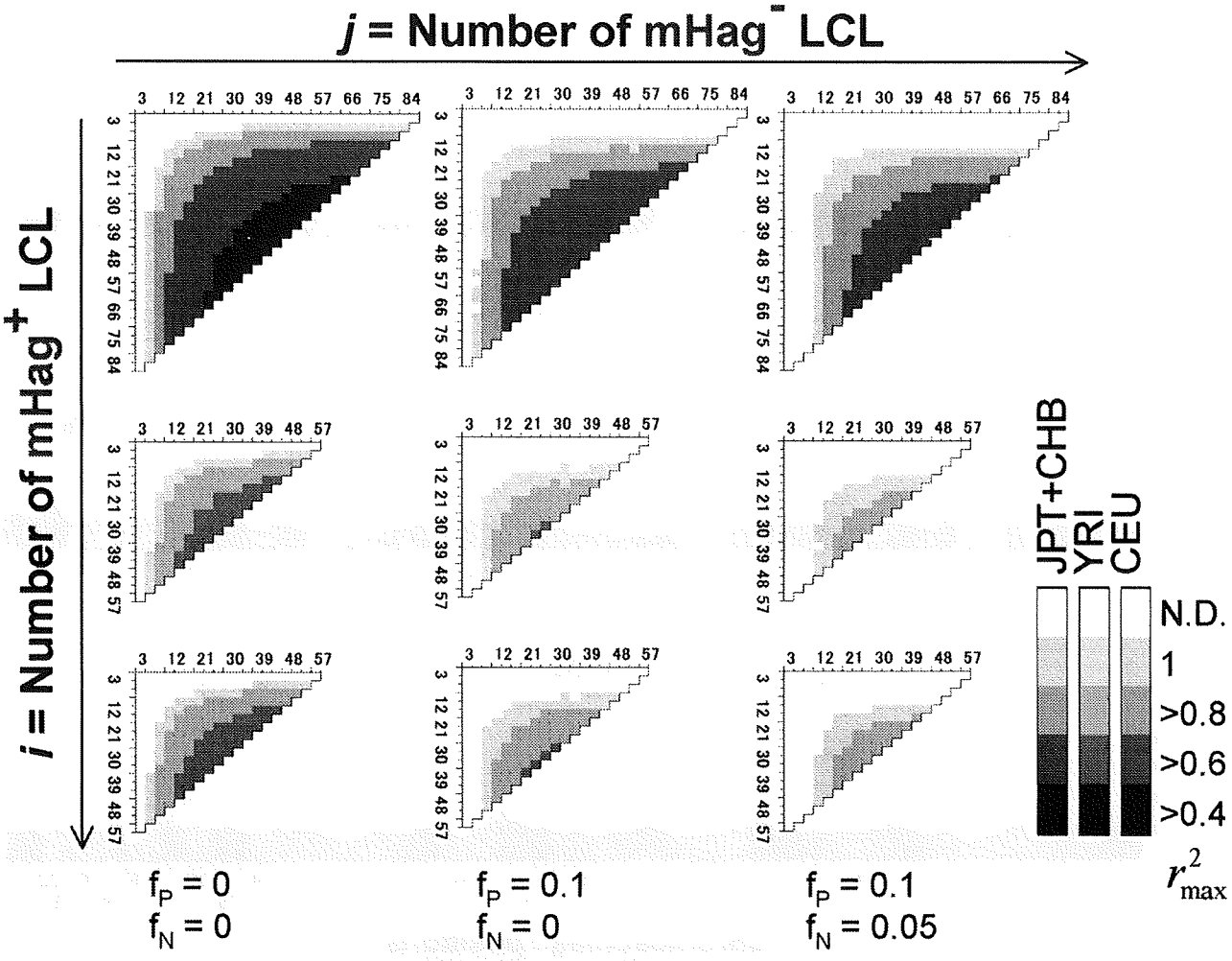

Figure 1. Numbers of positive and negative LCLs required for successful mHag mapping. The target locus was assumed to be uniquely identified, if the expected χ^2 value for the target SNP ($\hat{\chi}(i,j)$, see Document S1) exceeded the upper 1 percentile point of the maximum χ^2 values in 10 000 simulated 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), $f_P = 0.1, f_N \cong 0$ (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

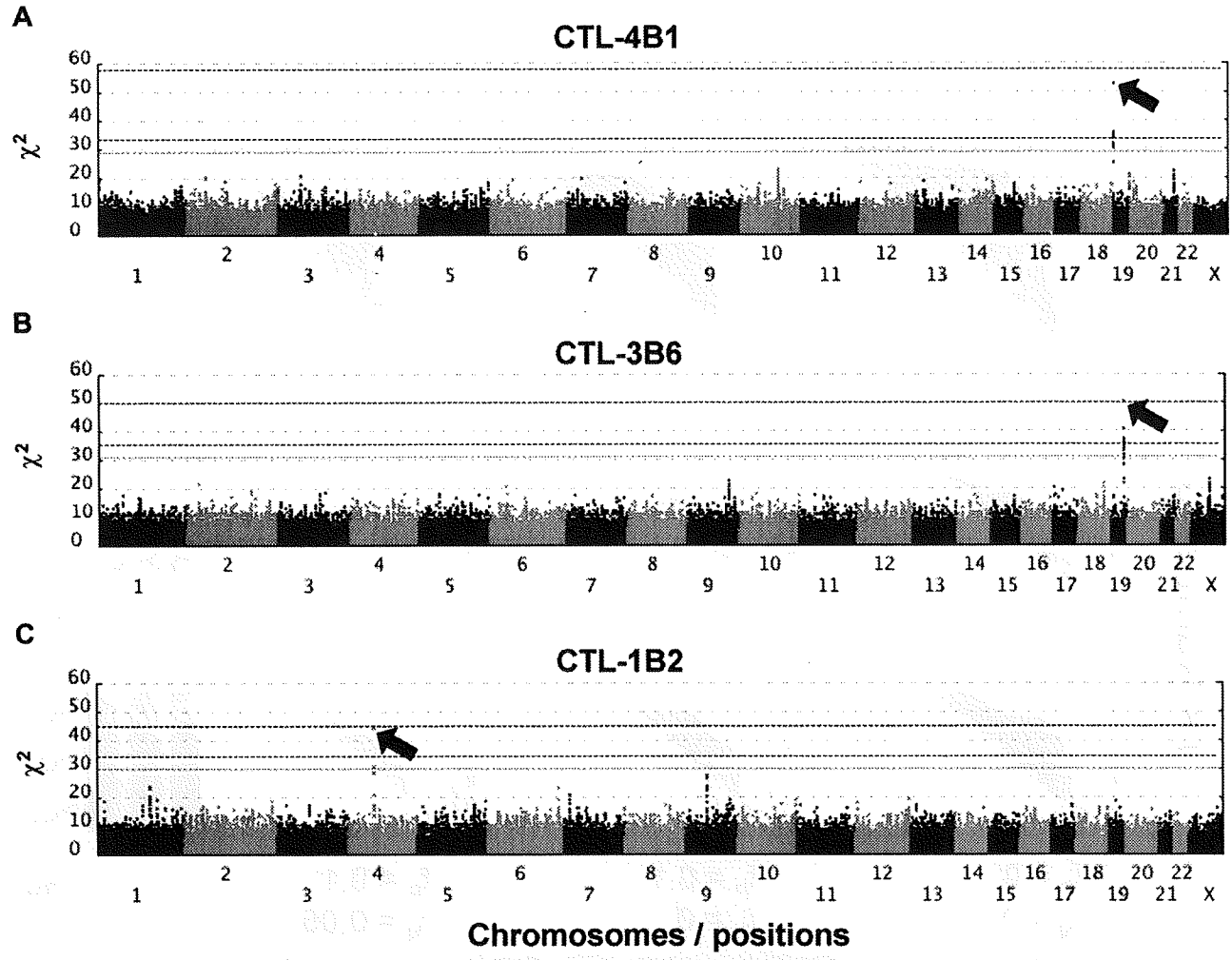

Figure 2. Genome-wide scanning to identify chromosome location of mHag. χ^2 values were plotted against positions on each chromosome for each of 3 mHags recognized by CTL-4B1 (A), CTL-3B6 (B), and CTL-1B2 (C). Chromosomes are displayed in alternating colors. Threshold χ^2 values corresponding to the 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

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

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 ~ < 0.1, f_N ~ = 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).

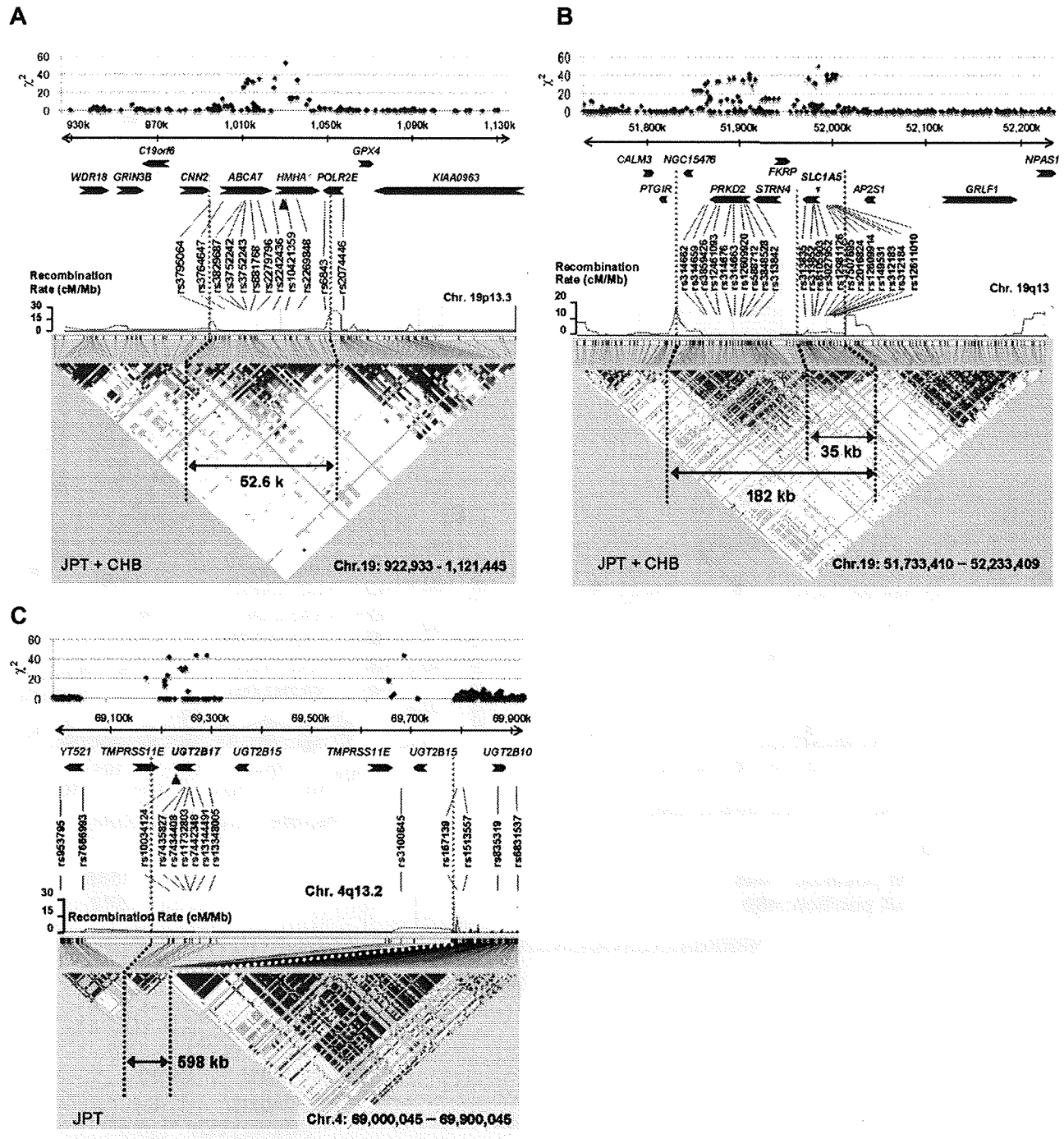

Figure 3. Regions of mHag loci identified by HapMap scanning. LD structures around the SNPs showing peak statistical values (in JPT + CHB) are presented for each mHag locus identified with (A) CTL-4B1, (B) CTL-3B6, and (C) CTL-1B2. Regional χ^2 plots are also provided on the top of each panel. LD plots 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

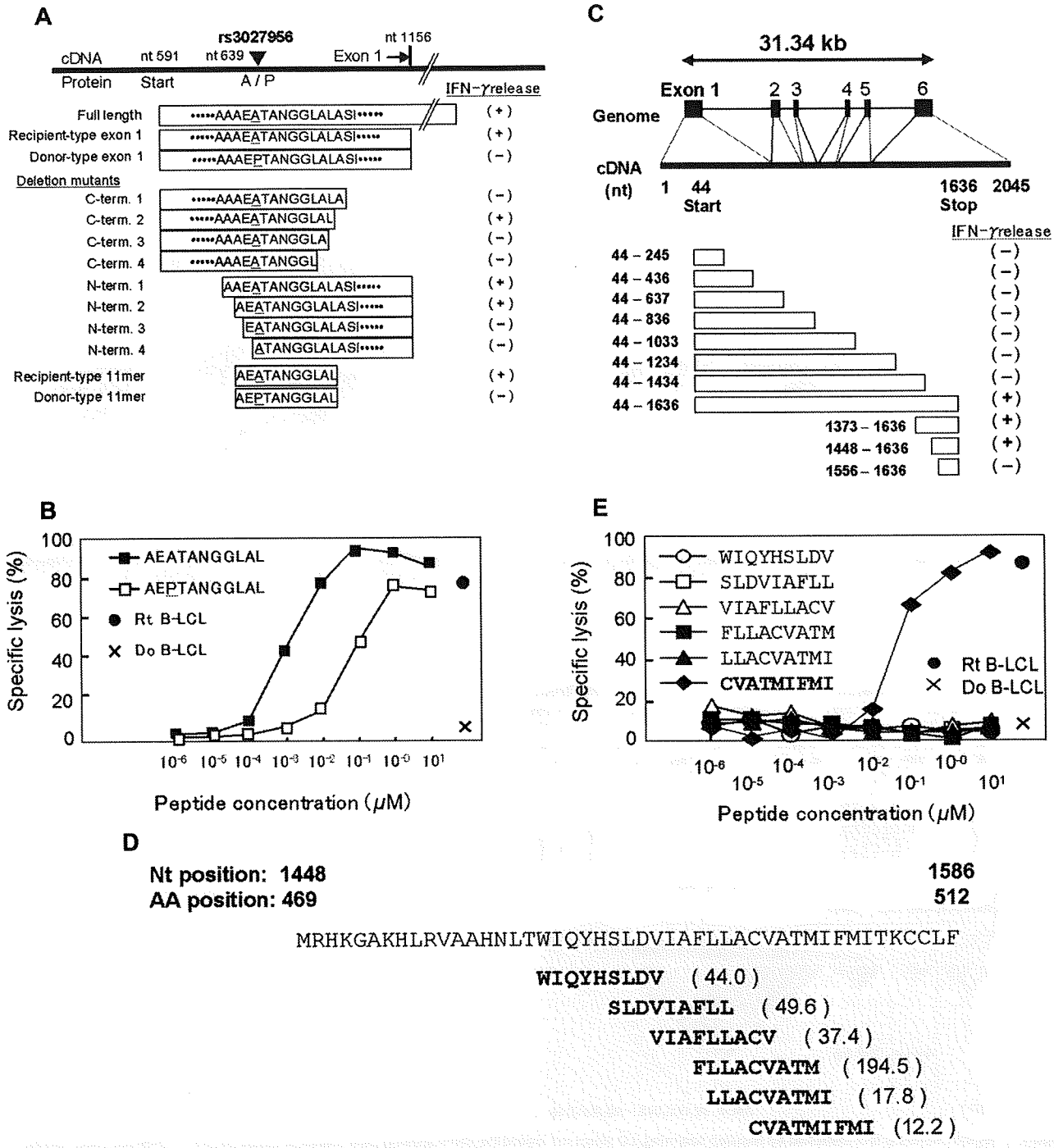

Figure 4. Epitope mapping. (A) Determination of the *SLC1A5* epitope by deletion mapping. Plasmids encoding recipient full-length *SLC1A5*, exon 1 of recipient and donor, exon 1 with various N- and C-terminus deletions around the amino acid encoded by SNP rs51983014, and minigenes encoding AEATANGGLAL 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 AEPTANGGLAL 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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Hematopoietic stem cell transplantation for core binding factor acute myeloid leukemia: t(8;21) and inv(16) represent different clinical outcomes

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We analyzed 338 adult patients with acute myeloid leukemia (AML) with t(8;21) and inv(16) undergoing stem cell transplantation (SCT) who were registered in the Japan Society for Hematopoietic Cell Transplantation database. At 3 years, overall survival (OS) of patients with t(8;21) and inv(16) was 50% and 72%, respectively ($P = .002$). Although no difference was observed when restricted to allogeneic SCT in first complete remission (CR; 84% and 74%), OS of patients with t(8;21) and inv(16) undergoing allogeneic SCT in second or third CR (45% and 86% at 3 years; $P = .008$) was different. OS was not different between patients in first CR who received allogeneic SCT and those who received autologous SCT for both t(8;21) AML (84% vs 77%; $P = .49$) and inv(16) AML (74% vs 59%; $P = .86$). Patients with inv(16) not in CR did better after allogeneic SCT than those with t(8;21) (70% and 18%; $P = .03$). Patients with t(8;21) and inv(16) should be managed differently as to the application of SCT. SCT in first CR is not necessarily recommended for inv(16). For t(8;21) patients in first CR, a prospective trial is needed to clarify the significance of autologous SCT and allogeneic SCT over chemotherapy. (Blood. 2009;113:2096-2103)

Introduction

Core binding factor (CBF) acute myeloid leukemia (AML) including t(8;21)(q22;q22) and inv(16)(p13q22)/t(16;16)(p13;q22) [t(8;21) and inv(16)] is considered to be a favorable cytogenetic subgroup in clinical studies.[1-4] Patients with t(8;21) and inv(16) have shown a markedly improved outcome with repetitive use of high-dose cytarabine.[5-13] However, the major treatment failure is disease recurrence.[14-16] These patients frequently become stem cell transplantation (SCT) candidates.

Both t(8;21) and inv(16) AMLs are associated with disruption of genes encoding subunits of the CBF, a heterodimeric transcriptional factor involved in the regulation of hematopoiesis.[17,18] Although these 2 different cytogenetics also share common clinical characteristics, they are associated with different clinical features such as morphologic presentation and immunophenotypic marker expression.[19]

Several reports demonstrated inferior outcome of t(8;21) compared with inv(16), but the number of patients who underwent transplantation was limited.[14,15,20] A recent study from the Dana-Farber Cancer Institute reported that both patients with t(8;21) and inv(16) de novo AML who underwent allogeneic transplantation performed favorably compared with other karyotypes.[21] To identify the survival data and prognostic factors among the CBF leukemia population who received SCT, we conducted a retrospective analysis using a Japanese multi-institution database with a large number of patients.

Methods

Study population

A total of 2802 adult patients who underwent autologous or allogeneic SCT from 1996 and 2004 for AML were registered in the Japan Society for Hematopoietic Cell Transplantation (JSHCT) database. Patients who underwent SCT from unrelated donors were registered in the different registry in the study period, but not all of the patients undergoing unrelated SCT were registered in the JSHCT database. Demographic, diagnostic, clinical, cytogenetics, induction, and outcome information were collected for each patient, and were sent to a central registration center. Cytogenetic studies were performed in each center, but a central review of cytogenetic analysis was not performed.

Patients with de novo AML aged 16 to 70 years who received hematopoietic SCT as the first transplant were included in the study. No patients with prior history of autologous or allogeneic SCT were included in the study. Of the remaining 2164 patients, 178 patients with t(15;17) or PML/RARα were excluded from the analysis below (Table 1). Finally, of the 1986 patients included in the analysis, 255 were reported to have t(8;21) abnormality, and 83 to have inv(16). A total of 194 patients had no available cytogenetic data. The remaining 1454 patients with normal karyotype and other cytogenetic abnormalities were further coded and analyzed according to published Southwest Oncology Group (SWOG) criteria.[3] The intermediate risk category included patients characterized by +8, −Y, +6, del(12p), or normal karyotype. The unfavorable risk category was defined by the presence of one or more of −5/del(5q), −7/del(7q), abn 3q, 11q, 20q, or

Submitted March 18, 2008; accepted December 17, 2008. Prepublished online as *Blood* First Edition paper, January 6, 2009; DOI 10.1182/blood-2008-03-145862.

The online version of this article contains a data supplement.

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Table 1. Cytogenetic risk groups of patients with AML who received autologous SCT and allogeneic SCT

Cytogenetic risk groups	No. patients		
	Auto-SCT	Allo-SCT	Total
t(8;21)	61	194	255
inv(16)	17	66	83
t(15;17)*	65	113	178
Intermediate	140	749	889
Unfavorable	35	325	360
Unknown			
Unknown cytogenetic risk	27	178	205
No available cytogenetic data	44	150	194
Total	389	1775	2164

Auto-SCT indicates autologous stem cell transplantation; Allo-SCT, allogeneic stem cell transplantation.

*Patients with t(15;17) were excluded from the analysis.

21q, del(9q), t(6;9), t(9;22), abn 17p, and complex karyotypes defined as 3 or more abnormalities. Patients with other cytogenetic aberrations were considered an unknown risk group, and were analyzed together with 194 patients with no cytogenetic data.

This study was approved by the Committee for Nationwide Survey Data Management of the JSHCT. Informed consent was obtained in accordance with the Declaration of Helsinki.

Transplantation

A total of 1662 patients underwent allogeneic SCT, and 324 underwent autologous SCT. Patients were treated with various conditioning regimens, but most of those who underwent autologous transplantation received non–total body irradiation (TBI) regimens (97%), including busulfan (BU), cytarabine (CA), and etoposide. The most frequently used conditioning regimens before allogeneic SCT were cyclophosphamide (Cy) plus TBI (n = 327 patients), and BU plus Cy (n = 267). Conditioning regimens before allogeneic SCT also included more intensified regimens such as CA plus Cy plus TBI (n = 262) and BU plus Cy plus TBI (n = 146), or reduced-intensity conditioning regimens with fludarabine (n = 241) or cladribine (n = 19).

Stem cell sources for allogeneic SCT were bone marrow in 871 patients, peripheral blood stem cell in 570 patients, bone marrow plus peripheral blood stem cell in 23 patients, and cord blood in 190 patients. A total of 1242 patients underwent allogeneic SCT from a related donor, and 404 patients underwent SCT from an unrelated donor.

Of the 1637 patients who had available data, 74% received transplants from human leukocyte antigen (HLA)–matched donors. Among patients who received unrelated bone marrow transplants, 156 patients were HLA genotypically matched and 51 were HLA mismatched. HLA data for 39 mismatched unrelated bone marrow transplantation patients were available. A total of 32 patients were one locus mismatched, and 7 patients were 2 loci mismatched. Among patients receiving unrelated cord blood transplants, 19 patients were serologically HLA matched and 170 patients were mismatched. HLA incompatibility was 5 of 6 HLA matched in 57 patients, 4 of 6 HLA matched in 99 patients, 3 of 6 HLA matched in 7 patients, and 1 of 6 HLA matched in 1 patient.

Graft-versus-host disease (GVHD) prophylaxis mostly consisted of methotrexate and a calcineurin inhibitor, either cyclosporin A or tacrolimus. Several other prophylaxes include mycophenolate mofetil, antithymocyte globulin, and $CD34^+$ selection. The incidence of acute GVHD was evaluated in 1488 patients who survived more than 28 days, and chronic GVHD was evaluated in 1302 patients who survived more than 100 days after allogeneic SCT. GVHD was evaluated in each center.

Statistical analysis

Correlation between the 2 groups was examined with the chi-square test, Fisher exact test, and the Mann-Whitney *U* test. Disease-free survival (DFS) was calculated from the date of transplantation until the date of relapse or the date of death in CR. Patient survival data were analyzed with the method of Kaplan and Meier and compared by the log-rank test.

Univariate and multivariate analyses for OS were performed with the aid of the Cox proportional hazard regression model, and variables were selected with the stepwise method. The following variables were evaluated: age, sex, and disease status at transplantation; CR versus not in CR; the number of induction courses to achieve CR; one course versus more than one course and failure; type of transplantation (allogeneic SCT vs autologous SCT); conditioning regimen (reduced intensity vs myeloablative); TBI regimen or not; and the existence of additional karyotype abnormalities or not. For those who received allogeneic SCT, in addition to these variables, the following were also evaluated: type of GVHD prophylaxis; short-course methotrexate plus cyclosporin A or short methotrexate plus FK506; acute GVHD, grade II to IV or grade III to IV; chronic GVHD; HLA mismatch; donor; and donor source. The doses of methotrexate were not surveyed. Each factor was considered to be prognostic if the *P* value was less than .05. Data were analyzed with the Stata 9.2 statistical software (College Station, TX).

Results

Initial characteristics of patients

The median age of all patients with AML in total was 41 years old (range, 16-70 years old). Median follow-up period of living patients was 37.3 months (range, 0.4-108 months). Patients were categorized into 5 cytogenetic subgroups: with t(8;21), with inv(16), intermediate risk cytogenetics, unfavorable cytogenetics, and an unknown risk group. Table 1 shows the number of patients in each cytogenetic subgroup and patients with t(15;17), who were excluded from the analysis.

Characteristics of the patients with CBF who underwent allogeneic SCT or autologous SCT are shown in Table 2. No significant difference was observed between characteristic of 2 groups of patients with CBF who received autologous SCT, except for the initial white blood cell count.

Of the 259 patients with CBF who received allogeneic SCT, significantly more patients with t(8;21) had failed to achieve CR with a single course of induction chemotherapy at diagnosis ($P = .002$), and were not in CR at the time of transplantation ($P < .001$). Among patients in CR at transplantation, the ratio of those in first, second, or third CR was not different between t(8;21) and inv(16) subgroups. Significantly more patients with inv(16) received transplants from an unrelated donor ($P = .004$). Table 3 and Table S1 (available on the *Blood* website; see the Supplemental Materials link at the top of the online article) summarize the transplantation data of those undergoing allogeneic SCT. More patients with inv(16) received unrelated transplants compared with t(8;21) patients ($P = .004$).

Overall survival

The OS of 1986 patients with AML at 3 years was 48%, and those with t(8;21), inv(16), intermediate, unfavorable, and unknown cytogenetic risks showed OS of 50%, 72%, 52%, 35%, and 45%, respectively ($P < .001$). Figure 1 shows survival curves of patients with AML patients who underwent allogeneic SCT in first CR (Figure 1A), in second or third CR (Figure 1B), or not in CR (Figure 1C), categorized by the cytogenetic abnormalities. Survival data are listed in Table 4. The OS of patients with t(8;21), inv(16), and intermediate, unfavorable, and unknown risk undergoing allogeneic SCT in first CR was 84%, 74%, 69%, 53%, and 52%, respectively ($P < .001$), and that of patients undergoing allogeneic-SCT

Table 2. Characteristics of patients with CBF AML

	Auto-SCT			Allo-SCT		
	t(8;21) (n = 61), no.	inv(16) (n = 17), no.	P	t(8;21) (n = 194), no.	inv(16) (n = 66), no.	P
Median age, y (range)	44 (17-68)	37 (19-61)	.59	39 (16-70)	34 (16-64)	.054
Median WBC, g/L (range)	8.8 (0.2-94)	33 (2.1-199)	.02	11 (.6-366)	53 (1.8-284)	< .001
Sex						
Male	41	12	.79	117	40	.93
Female	20	5		74	26	
No. of induction chemotherapy at diagnosis of AML						
1 course	48	15	.72	125	55	.002
> 1 or failure*	11	2		56	7	
Additional cytogenetic abnormalities						
None	53	15	> .999	153	54	.61
Positive	8	2		41	12	
Disease status at SCT						
CR	55	16	> .999	108	52	< .001
Not in CR	6	1		85	11	
CR1	43	13	.98	49	21	.29
CR2	7	1		45	26	
CR3	0	1		5	4	
Conditioning regimen						
TBI	0	1	.22	118	47	.078
Not TBI	61	16		71	16	

Correlation between the two groups was examined.
WBC indicates white blood cell count; g/L, 10^9/L; CR1, first complete remission; and CR2 or 3, second or third CR.
*More than 1 or failure includes patients who did not achieve complete remission after first course of induction chemotherapy, and those who were resistant to induction chemotherapy.

in second or third CR was 45%, 86%, 57%, 44%, and 64%, respectively ($P = .09$). OS of patients undergoing allogeneic SCT not in CR was 18%, 70%, 25%, 15%, and 18%, respectively ($P = .003$).

Table 3. Summary of allogeneic SCT

	t(8;21) (n = 194), no.	inv(16), (n = 66), no.	P
Conditioning regimen			
RIST	31	9	.66
Myeloablative	161	56	
GVHD prophylaxis*			
sMTX+CyA	136	48	.78
sMTX+FK	20	8	
HLA			
Match	146	47	.5
Mismatch	45	18	
Donor			
Related	161	44	.004
Unrelated	32	22	
Stem cell source			
BM	101	40	.27
PB	72	17	
CB	18	7	
aGVHD grade			
0-I	117	37	.54
II-IV	60	22	
cGVHD type			
None	64	28	.28
Lmt/Ext	67	20	

Correlation between the two groups was examined. Some of the missing data was not available, and total numbers do not add up to the number of the patients in each group.
RIST indicates reduced intensity stem cell transplantation; sMTX, short-course methotrexate; CyA, cyclosporin A; FK, tacrolimus; BM, bone marrow; PB, peripheral blood; CB, cord blood; aGVHD, acute graft-versus-host disease; cGVHD, chronic graft-versus-host disease; Lmt, limited; and Ext, extensive.
*Dose of methotrexate was not surveyed in the study. Detail of other GVHD prophylaxis regimens are in Table S1.

When patients undergoing allogeneic SCT in first CR were analyzed, 3-year OS was not significantly different between patients with t(8;21) and inv(16) (84% and 74%, respectively; $P = .28$), between inv(16) and intermediate risk groups (74% and 69%, respectively; $P = .84$), or between t(8;21) and intermediate risk groups (84% and 69%, respectively; $P = .06$). However, when patients undergoing allogeneic SCT in second or third CR were analyzed, the 3-year OS of patients with inv(16) was significantly better than patients with t(8;21) (86% and 45%, respectively; $P = .008$), and better than intermediate risk patients (86% and 57%, respectively; $P = .03$). Difference was not significant between patients in the intermediate risk group and t(8;21) undergoing allogeneic SCT in second or third CR ($P = .36$). The OS of inv(16) patients undergoing allogeneic SCT not in CR was 70% at 3 years, which was also significantly better than that of t(8;21) (18%; $P = .03$) and the intermediate risk group (25%; $P = .045$).

In addition, the OS of t(8;21) undergoing allogeneic SCT in first CR was significantly better than that of the unfavorable risk group (84% and 53%, respectively; $P < .001$), but the difference between the 2 groups was not significant among patients undergoing allogeneic SCT in second or third CR. In contrast, OS was not different between inv(16) and unfavorable groups undergoing allogeneic SCT in first CR, but it was significantly different when they underwent allogeneic SCT in second or third CR (86% and 44%, for inv(16) and unfavorable groups, respectively; $P = .01$) or allogeneic SCT in non-CR (70% and 15%, respectively; $P = .006$).

Survival curves of patients who underwent autologous SCT in first CR, second or third CR, and not in CR are shown in Figure 2A, 2B, and 2C, respectively. The overall survival of patients with t(8;21), inv(16), and intermediate, unfavorable, and unknown cytogenetic risks in first CR was 77%, 59%, 74%, 38%, and 71%, respectively ($P = .049$), while that of patients undergoing autologous SCT in second or third CR was 43%, 50%, 59%, 44%, and 42%, respectively ($P = .8$). The OS of patients undergoing autologous SCT not in CR with t(8;21), inv(16), intermediate, and

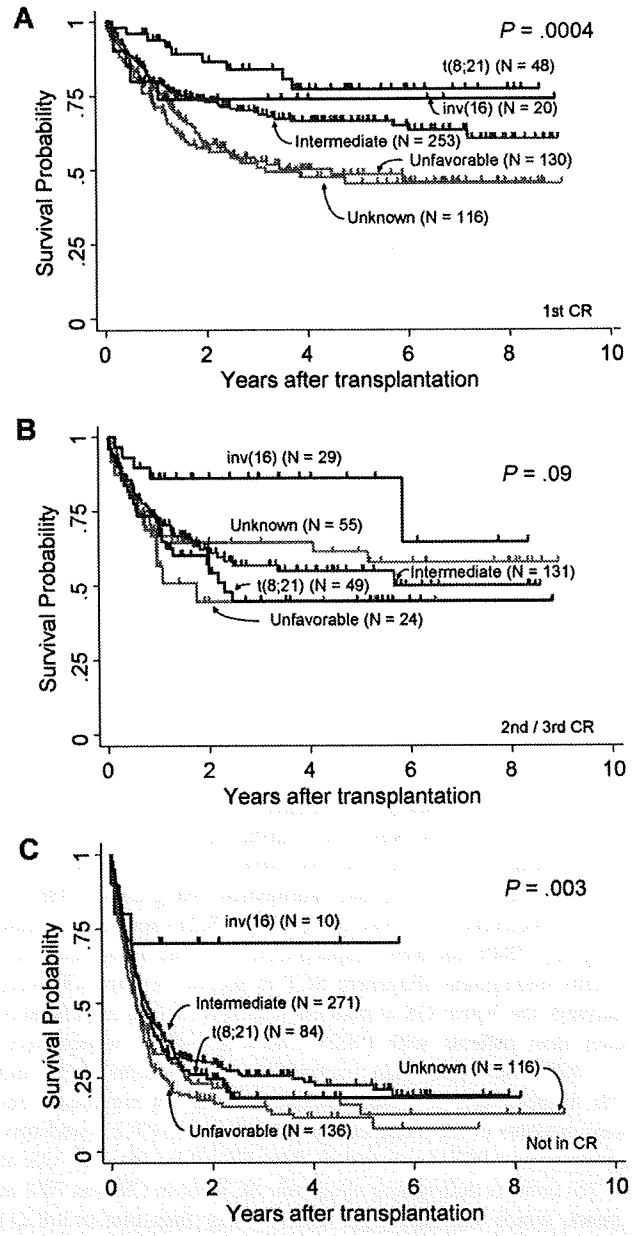

Figure 1. OS difference of patients undergoing allogeneic SCT between cytogenetic subgroups. (A) Survival curves of patients undergoing allogeneic SCT in first CR. (B) Survival curve of patients undergoing allogeneic SCT in second or third CR. (C) Survival curves of patients undergoing allogeneic SCT not in CR. Each are categorized by cytogenetic risk groups, respectively.

unknown risks was 17%, 100%, 25%, and 13%, respectively, and the survival curve of patients in the unfavorable risk group did not reach 3 years ($P = .35$).

Figure 3A and B focus on t(8;21) and inv(16) patients, stratified according to the type of (allogeneic or autologous) and disease status at the time of transplantation (first CR, second or third CR, and not in CR). The 3-year overall survival of t(8;21) patients in first CR was not different between allogeneic and autologous transplantation (84% and 77%, respectively), as well as that of patients in second or third CR (45% and 43%, respectively) and patients not in CR (18% and 17%, respectively). Similarly, the 3-year OS of inv(16) patients was not different between allogeneic and autologous transplantation when they underwent transplantation in first CR (74% and 59%). A significant difference was observed among the 3 disease status groups of t(8;21) patients ($P < .001$; Figure 3A), but not inv(16) patients ($P = .75$; Figure 3B).

The OS of allogeneic SCT, excluding cord blood transplantation, was not different from the analysis presented here, including bone marrow, peripheral blood, and cord blood transplantation (Table S2; Figures S1,S2).

DFS after SCT was also different among cytogenetic risk groups ($P < .001$). DFS of patients with inv(16) (69% at 3 years) was better compared with t(8;21) (49%), intermediate (46%), unfavorable (31%), and unknown (41%) risk groups. Among patients undergoing allogeneic SCT in first CR, DFS was also different among cytogenetic subgroups ($P < .001$). When t(8;21), inv(16), and intermediate cytogenetic subgroups undergoing allogeneic SCT in first CR were compared, the difference was not statistically significant between t(8;21) and inv(16) (78% and 73% at 3 years; $P = .58$), between t(8;21) and intermediate risk group (78% and 63%; $P = .1$), nor between inv(16) and intermediate risk group (73% and 63%; $P = .65$). DFS of patients with t(8;21) undergoing allogeneic SCT in first CR was better than that of the unfavorable risk group (78% and 47%, respectively; $P < .001$), but the difference was not significant between inv(16) and unfavorable risk groups (73% and 47%, respectively; $P = .16$).

DFS was not significantly different when 5 cytogenetic subgroups among patients undergoing allogeneic SCT in second or third CR were compared ($P = .32$). The DFS of patients undergoing allogeneic SCT in second or third CR was not significantly different between t(8;21) and inv(16) (43% and 71% at 3 years; $P = .053$), t(8;21) and the intermediate group (43% and 47%; $P = .76$), or inv(16) and the intermediate group (71% and 47%; $P = .06$). The difference was also not significant between t(8;21) and unfavorable risk groups (43% and 42%; $P = .7$), nor between inv(16) and unfavorable risk groups (71% and 42%; $P = .06$). The DFS of patients undergoing allogeneic SCT who were not in CR was significantly different among the 5 cytogenetic subgroups ($P = .005$), and that of inv(16) (75% at 3 years) was significantly better than t(8;21) (18%; $P = .02$), the intermediate risk group (22%; $P = .03$) and the unfavorable risk group (10%; $P = .003$).

Relapse and TRM

The relapse rate (RR) after SCT also differed among cytogenetic subgroups ($P < .001$). The RR of patients with inv(16) (18% at 3 years) was lower than t(8;21) (38%), intermediate (38%), and unfavorable (56%) risk groups. The RR of t(8;21) and inv(16) after allogeneic SCT was not statistically different in either first CR (16% and 6%; $P = .45$) or second or third CR (34% and 16%, respectively; $P = .09$).

Transplantation-related mortality (TRM) of all patients with AML was 22% at 3 years. The TRM of t(8;21) (18%), inv(16) (11%), and intermediate (21%), unfavorable (24%), and unknown risk groups (27%) was significantly different among cytogenetic risk groups ($P = .02$).

Evaluation of prognostic variables in CBF

Univariate analyses of t(8;21) showed that age ($P = .004$), not in CR at transplantation ($P < .001$), allogeneic SCT ($P = .01$), and TBI regimen ($P = .006$) were significant prognostic factors indicating poor OS (Table 5). Multivariate analysis for OS revealed older age ($P = .01$) and not in CR at transplantation ($P < .001$) as the independent prognostic variables. Univariate analyses of t(8;21) patients who received allogeneic SCT in CR showed that age ($P = .02$), TBI regimen ($P = .01$), and second and third CR at

Table 4. Outcome of the AML patient population by cytogenetic risk groups

	t(8;21)		inv(16)		Intermediate		Unfavorable		Unknown		
	%	N	%	N	%	N	%	N	%	N	*P*
OS											
Allogeneic SCT											
CR1	84	48	74	20	69	253	53	130	52	116	< .001
CR2/CR3	45	49	86	29	57	131	44	24	64	55	.09
Non-CR	18	84	70	10	25	271	15	136	18	116	.003
Autologous SCT											
CR1	77	42	59	13	74	89	38	15	71	39	.05
CR2/CR3	43	7	50	2	59	15	44	6	42	18	.8
Non-CR	17	6	100	1	25	16	0	10	13	8	.35
DFS											
Allogeneic SCT											
CR1	78	48	73	19	63	249	47	129	48	113	< .001
CR2/CR3	43	48	71	27	47	129	42	22	57	54	.32
Non-CR	18	81	75	8	22	255	10	128	16	107	.005
Autologous SCT											
CR1	73	41	62	13	64	81	33	15	61	36	.09
CR2/CR3	43	7	50	2	36	14	50	6	39	18	.89
Non-CR	17	6	100	1	25	16	0	10	17	6	.45

transplantation ($P < .001$) were also significantly prognostic for poor OS. These variables remained significant after multivariate analysis. Univariate analyses for inv(16) patients showed only age ($P = .009$) to be a significant prognostic factor (Table 5). The univariate analysis of inv(16) patients who underwent allogeneic SCT in CR showed only additional karyotype abnormalities to be an unfavorable prognostic variable ($P = .009$).

Additional cytogenetic abnormalities to CBF

A total of 49 patients with t(8;21) and 14 with inv(16) had additional cytogenetic abnormalities. Data for additional cytogenetic abnormalities were obtained in 42 patients with t(8;21) and 13 patients with inv(16) (Table 6). Additional abnormalities were selected that have been reported to be prognostic by others, including loss of sex chromosome (X or Y), trisomy 8, trisomy 4, del(7q), and del(9q) for the t(8;21) group, and trisomy 22, trisomy 8, trisomy 21, del(7q), and del(9q) for the inv(16) group.[14,15,20,22,23] There were no patients with trisomy 21 in the data of patients with CBF. Patients with t(8;21) and patients with inv(16) were analyzed separately. Among t(8;21) patients undergoing allogeneic SCT, survival was not different between patients with and without additional karyotype abnormalities. When patients with inv(16) were analyzed, the survival was not different between patients with (n = 13) and without (n = 67) additional abnormalities (61% and 74%, respectively; $P = .07$). The survival of patients undergoing allogeneic SCT without additional abnormality (n = 52) was significantly better than that with additional abnormality (n = 11), (85% and 53%, respectively; $P = .004$). When analysis was restricted to patients in CR with inv(16) undergoing allogeneic SCT, a similar difference was observed (86% without additional abnormality [n = 42], and 60% with additional abnormality [n = 8], respectively; $P = .03$). Difference in OS was observed among non-CR patients with (n = 9) and without (n = 1) additional abnormality, but this difference may not be relevant with too few patients in the analysis. We further analyzed subgroups of additional abnormalities of the patients with inv(16). Although the number of patients were limited, significant difference was found among 3 groups of patients; trisomy 8 or trisomy 22 as a sole abnormality (n = 4), without additional abnormality (n = 69), and other additional abnormality to inv(16) (n = 10). The OS at 3 years were 100%, 74%, and 42%, respectively ($P = .002$). The OS of patients undergoing allogeneic SCT was also different among these 3 groups (100%, n = 3; 85%, n = 52; and 33%, respectively; $P < .001$).

Discussion

We analyzed the outcome of a large group of patients with adult CBF AML in Japan who were treated with SCT. The current study focused on the different outcome of the 2 different cytogenetic subgroups of patients with CBF AML undergoing SCT. Our study demonstrated a comparable outcome between patients with t(8;21) and inv(16) undergoing SCT in first CR, but the prognosis between these 2 cytogenetic subgroups was different beyond first CR.

In the literature, there have been several reports showing inferior survival of patients with t(8;21) compared with inv(16) patients undergoing induction chemotherapy and SCT.[14,15,20] Other studies categorized both patients with t(8;21) and inv(16) undergoing allogeneic SCT together as good-risk CBF AML,[1,21] with a relatively comparable prognosis. In our study, OS of patients with t(8;21) undergoing allogeneic SCT in first CR was not statistically different from intermediate cytogenetic subgroup (84% and 79% at 3 years, respectively; $P = .058$). Moreover, the survival of inv(16) (74% at 3 years) and intermediate cytogenetic subgroups showed no statistically significant difference.

In contrast, we have here demonstrated that the prognosis of patients with t(8;21) undergoing allogeneic SCT with second or third CR disease was significantly poor compared with those with inv(16). This finding is consistent with those of other studies reporting differences between the 2 types of CBF AML.[14,15] In the present study, non-CR disease with t(8;21) was also significantly poor compared with patients with inv(16). The Acute Leukemia French Association reported that allogeneic donor availability among patients with CBF AML who were in second CR was a prognostic factor for better survival.[16] We believe that different treatment strategies should be applied for patients with t(8;21) and those with inv(16) other than first CR.

Patients with t(8;21) undergoing allogeneic SCT and autologous SCT had a similar survival rate when they underwent transplantation in first CR, and in further CR. No survival difference between allogeneic SCT and autologous SCT was also

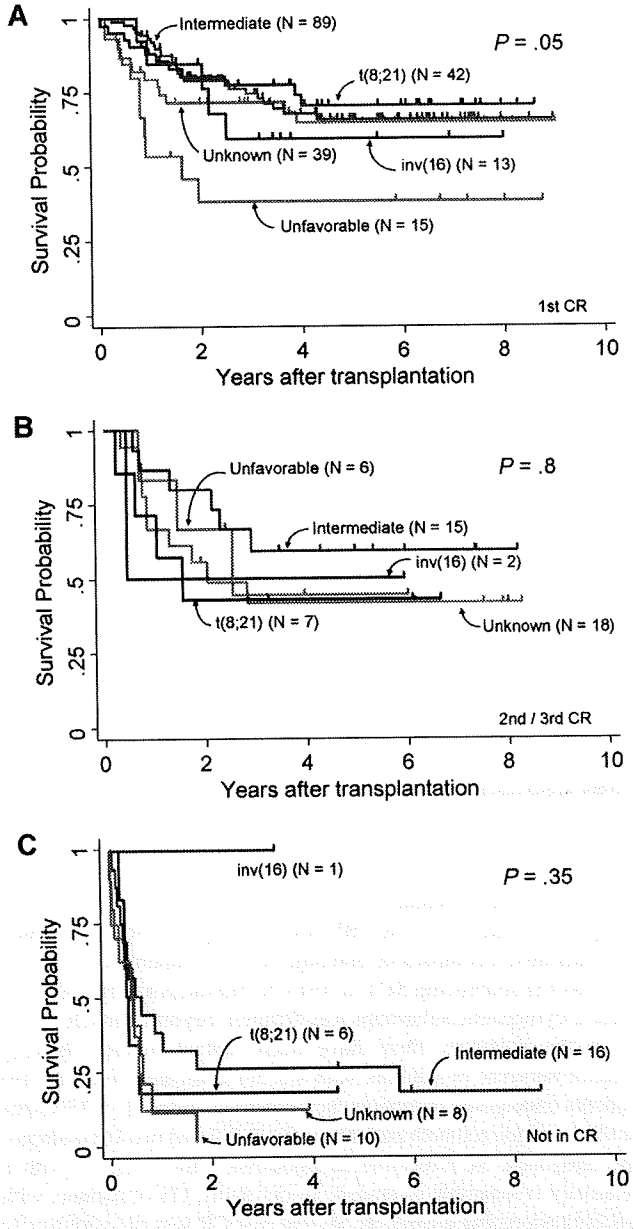

Figure 2. OS difference of patients undergoing autologous SCT between cytogenetic subgroups. (A) Survival curves of patients undergoing autologous SCT in first CR. (B) Survival curves of patients undergoing autologous SCT in second or third CR. (C) Survival curves of patients undergoing autologous SCT not in CR. Each are categorized by cytogenetic risk groups, respectively.

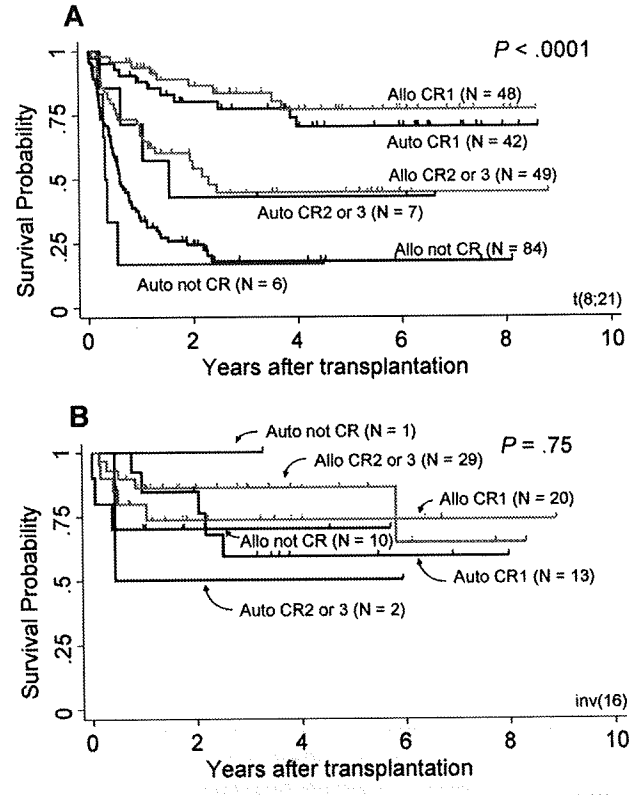

Figure 3. OS of patients with CBF. Survival curves of patients with t(8;21) (A) and with inv(16) (B). Both are stratified according to the type of transplantation (allogeneic or autologous) and disease status at the time of transplantation (first CR, second or third CR, and not in CR).

observed among inv(16) patients receiving SCT in first CR (74% and 59%, respectively). The University of California, San Francisco (UCSF) group described the good results of patients with advanced AML undergoing autologous SCT in second or third remission, including patients with CBF.[24] As in our study, the European Group for Blood and Marrow Transplantation (EBMT) reported that the survival rate of t(8;21) patients who received allogeneic bone marrow transplantation was not significantly different from that of patients who received autologous SCT.[1] Results by others showed that allogeneic SCT in first CR did not benefit good-risk cytogenetic subgroups.[3,25,26] Schlenk et al also demonstrated that t(8;21) patients receiving allogeneic SCT or chemotherapy showed no difference in outcome.[23] These results suggest that autologous SCT can be considered as postremission therapy for patients with CBF AML, but it remains unclear whether SCT is more beneficial for patients with CBF than high-dose cytarabine. Survival of patients with inv(16) was favorable beyond first CR. Patients with inv(16) in second or third CR, or even non-CR patients, are good candidates for allogeneic SCT. There are long-term survivors after allogeneic SCT in non-CR disease, so t(8;21) patients with no other choice of treatment, such as those in further CR or non-CR, can proceed to allogeneic SCT. In order to confirm the appropriate treatment for t(8;21) patients in first CR, a prospective trial is needed to compare the results of autologous SCT for t(8;21) in first CR with standard chemotherapy. t(8,21) patients with suitable related or well-matched donors should be recommended to participate in a risk-adopted prospective trial when they receive allogeneic SCT in first CR.

There were differences between the 2 types of CBF AML with respect to prognostic valuables. Age was a significant and independent prognostic variable in both t(8;21) and inv(16) patients, a finding in agreement with reports from some,[14,27] but not all,

Table 5. Prognostic factors affecting overall survival of patients with t(8;21)

Variables	Unfavorable factors	Hazard ratio	95% CI	P
t(8;21)				
Age		1.02	1.01-1.04	.004
Disease status at SCT	Not in CR	4.4	3.1-6.5	< .001
Transplantation	Allo-SCT	1.9	1.2-3.0	.01
Conditioning regimen	TBI	1.7	1.2-2.5	.005
inv(16)				
Age		1.1	1.0-1.1	.009

CI indicates confidence interval.

Table 6. Additional cytogenetic abnormalities among patients with CBF

Additional cytogenetic abnormalities	t(8;21), no.	inv(16), no.
None	206	69
With additional abnormalities	49	14*
−Y	10	0
−X	5	0
Trisomy 22	0	3†
Trisomy 8	0	2†
Trisomy 4	2*	0
Complex	7	4
del(7q)	1†	2
del(9q)	6	0
Other abnormalities	27	9‡
Unknown	7	1

*Patients with additional change to inv(16) and trisomy 4 with t(8;21) tended to show poor survival tendency, with $P < .1$.

†All patients with trisomy 22, trisomy 8 with inv(16), and del(7q) with t(8;21) were alive and censored at survival analysis.

‡Other abnormalities with inv(16) was poorly prognostic, with $P < .001$.

investigators.[28] Transplantation in CR was a significant and independent prognostic factor for patients with t(8;21), but not for those with inv(16). The Cancer and Leukemia Group B (CALGB) also reported differences between t(8;21) and inv(16) in prognostic factors, in terms of race, sex, and secondary cytogenetic abnormalities.[14] Among patients with CBF AML, t(8;21) and inv(16) patients undergoing SCT should be considered 2 separate clinical entities in future clinical studies.

Several specific additional karyotype abnormalities have been reported to be prognostic in patients with CBF AML. Among t(8;21) patients, no specific additional karyotype abnormality was prognostic for overall survival. The poor prognosis of t(8;21) patients with trisomy 4 has been reported by others,[22] but the survival difference was not statistically significant ($P = .085$) in our case series. Since there were limited numbers of patients with additional abnormalities, the real significance of each additional abnormality should be investigated in large numbers of patients.

The reason for the different survival results between patients with t(8;21) and inv(16) undergoing allogeneic SCT in our study remains unclear. The impact of additional mutational events such as c-Kit, FLT3, RAS, and gene-expression profiles was reported to be associated with the clinical outcome of patients with CBF AML.[29-34] The effects of these additional mutational events and gene-expression profiles on the clinical outcome of autologous and allogeneic SCT have not yet been studied. Which proportion of the patients with CBF AML benefited from earlier SCT remains to be identified in future clinical studies. Recent studies by others also suggested that prognosis of CBF AML could differ among different ethnic groups or races.[14,35-37] The background molecular basis among the Japanese population must also be taken into account in future studies.

In conclusion, the survival outcome of patients with CBF AML was similar when they received allogeneic or autologous SCT in first CR. However, the outcomes were significantly different between t(8;21) and inv(16) when they received allogeneic SCT beyond first CR. Therefore, these 2 kinds of CBF AML should be managed differently when applying SCT.

Acknowledgments

We thank all of the staff of the participating institutions of the Japan Society for Hematopoietic Cell Transplantation Registry. We thank Dr Y. Inamoto for thoughtful discussion.

Authorship

Contribution: Y. Kuwatsuka, K.M., and R.S. contributed to data collection, designed and performed the study, analyzed the data, and wrote the manuscript; M.K., A.M., H.O., R.T., S.T., K.K., K.Y., Y.A., T.Y., and H.S. contributed to data collection and analysis and writing of the paper; and Y. Kodera contributed to data collection and writing of the paper, conceived the study, and provided intellectual input.

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

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Brief report

Impact of macrophage infiltration of skin lesions on survival after allogeneic stem cell transplantation: a clue to refractory graft-versus-host disease

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We retrospectively reviewed 104 biopsy specimens of previously untreated skin acute graft-versus-host disease (GVHD) within 100 days after allogeneic stem cell transplantation, and analyzed the relationship between types of infiltrating cells and clinical outcomes. Counting the total number of $CD8^+$ T cells, $CD163^+$ macrophages, and $CD1a^+$ dendritic cells in 4 fields under original magnification ×200, the infiltration of more than 200 cells of $CD163^+$ macrophages (many macrophages [MM]) was the only significant predictor for refractory GHVD (odds ratio, 3.79; 95% confidence interval, 1.22-11.8; $P = .02$). In 46 patients given steroid treatments, MM was the only significant predictor for refractory acute GVHD (odds ratio, 5.05; 95% confidence interval, 1.19-21.3; $P = .03$). Overall survival of patients with MM was significantly lower than that of those with an infiltration of less than 200 cells of $CD163^+$ macrophages. Macrophage infiltration of skin lesions could be a significant predictive factor for refractory GVHD and a poor prognosis. (Blood. 2009;114: 3113-3116)

Introduction

Macrophages are phagocytic cells with various abilities, such as phagocytosis, antigen-presenting, and secretion of cytokines.[1,2] Recently, it was revealed in human sequential biopsy data that recipient macrophages contributed to acute graft-versus-host disease (GVHD) by antigen-presenting and secreting cytokines, causing the activation and proliferation of $CD8^+$ T cells.[3] We focused on macrophage involvement in acute GVHD, especially on the relationship between the macrophage infiltration of skin lesions and refractory GVHD.

Methods

Between January 1997 and October 2007 at the Japanese Red Cross Nagoya First Hospital, we used skin biopsy specimens within 100 days after allogeneic stem cell transplantation (allo-SCT) of skin lesions clinically considered acute GVHD without any GVHD treatment from 104 patients who underwent allo-SCTs. We analyzed the relationship between types of infiltrating cells and clinical outcomes by counting the total number of $CD8^+$ T cells, $CD163^+$ macrophages, and $CD1a^+$ dendritic cells in 4 fields of a skin biopsy specimen under original magnification ×200. Immunohistochemical analysis using paraffin sections was performed using monoclonal antibodies against CD8, CD163, and CD1a (Novocastra). CD163 is a member of the scavenger receptor cystein-rich superfamily and is an exclusive marker for macrophages, playing a major role in the scavenging components of damaged cells.[4-7]

The endpoints of this study were the outcomes of acute GVHD and overall survival (OS). Acute GVHD was diagnosed and graded according to the consensus criteria.[8] We defined refractory GVHD as that exhibited by patients who had persistent lesions after primary steroid treatments. To establish parameters, we analyzed the numbers of infiltrating $CD8^+$ T cells (≤ 100/4 fields [few T cells; FT] vs > 100/4 fields [many T cells; MT]), numbers of infiltrating $CD163^+$ macrophages (≤ 200/4 fields [few macrophages; FM] vs > 200/4 fields [many macrophages; MM]), disease risk (low vs high), human leukocyte antigen (HLA) disparity (match vs mismatch), donor source (related vs unrelated), graft source (bone marrow vs peripheral blood), age at allo-SCT (≤ 50 years vs > 50 years), conditioning regimen (conventional regimens vs reduced intensity regimens), and skin GVHD stage at biopsy (stages 1-2 vs stages 3-4). A significance level of $P < .05$ was used for all analyses, which were based on all data available as of August 31, 2008. Protocols were approved by the Japanese Red Cross Nagoya First Hospital's Institutional Review Board, and all patients provided informed consent in accordance with the Declaration of Helsinki.

Results and discussion

Table 1 summarizes the characteristics of patients and information gathered about GVHD. We divided patients into 4 groups according to the amount of infiltrating cells (FM and FT, 60.6%; MT and FM, 18.2%; MT and MM, 10.6%; and FT and MM, 10.6%). We noted a striking difference among patients in the types of infiltrating cells in skin GVHD lesions (Figure 1A). The distributions of numbers of infiltrating cells also exhibited a

Submitted March 9, 2009; accepted June 28, 2009. Prepublished online as *Blood* First Edition paper, July 30, 2009; DOI 10.1182/blood-2009-03-209635.

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Table 1. Information on patient characteristics, acute GVHD, and skin biopsy

Characteristic	Value
Patient characteristics	
Total no. of patients	104
Median age at allo-SCT, y (range)	40.5 (19-61)
Male/female	65/39
Disease risk, low/high	51/53
HLA, match/mismatch	72/32
Donor, unrelated/related	67/37
Graft, BM/PB/CB	89/11/4
Conditioning, conventional/RIST	78/26
Median observation period, mo (range)	13.7 (0.7-120.7)
Acute GVHD	
Stage skin (at the time of biopsy), 1/2/3/4	22/57/25/0
Skin (the maximal severity), 1/2/3/4	16/25/52/11
Gut, 0/1/2/3/4	69/9/8/15/3
Liver, 0/1/2/3/4	82/5/3/9/5
Grade (at the time of the biopsy), I/II/III/IV	58/41/4/1
Grade (the maximal severity), I/II/III/IV	28/44/19/13
Primary steroid treatment, yes/no	46/58
Second treatment, yes/no	18/30
Outcome of GVHD, improved/refractory	84/20
Skin lesion	
Median date of appearance, days (range)	24 (5-81)
Median date of skin biopsy, days (range)	31.5 (6-82)
Median date of highest stage of skin GVHD, days (range)	34 (9-90)
No. of infiltrating $CD8^+$ cells	65 (2-305)
No. of infiltrating $CD163^+$ cells	132.5 (38-372)
No. of infiltrating $CD1a^+$ cells	7 (0-122)

Disease risk low indicates acute leukemia in first remission; CML, in first chronic phase; MDS, refractory anemia or nonmalignant hematologic disease; disease risk high, all other diagnoses; HLA match, identical HLA-A, -B, and -DRB1 loci; HLA mismatch, at least one disparity at one of these loci; BM, bone marrow; PB, peripheral blood; CB, cord blood; and RIST, reduced intensity conditioning regimens.

considerably wide variety (Figure 1B). The median number of infiltrating $CD8^+$ T cells was 65 (range, 2-305), that of infiltrating $CD163^+$ macrophages was 132.5 (range, 38-372), and thatof infiltrating $CD1a^+$ dendritic cells was 7 (range, 0-122). We used 3 skin biopsy specimens of drug rash from autologous transplantation patients as non-GVHD controls; the median numbers of $CD8^+$, $CD163^+$, and $CD1a^+$ infiltrating cells were 11 (range, 6-15), 26 (range, 19-30), and 68 (range, 65-83), respectively. MT was correlated with an HLA mismatch ($P = .047$), grade III-IV acute GVHD ($P = .03$), and MM ($P = .01$), whereas MM was correlated with unrelated donor ($P = .04$), an HLA mismatch ($P = .049$), refractory GVHD ($P = .004$), and MT ($P = .01$) using χ^2 analyses. The sensitivity and specificity of MT for refractory GVHD were 25.0% and 70.5% in all 104 patients, and 25.0% and 73.3% in 46 receiving steroids, whereas those of MM were 43.8% and 82.9%, and 43.8% and 86.7%, respectively.

In 46 patients undergoing steroid treatments, the median date of the appearance of skin lesions was 17.0 days (range, 5-54 days), whereas that of skin biopsy was 27.5 days (range, 6-63 days) and that of the highest skin stage was 32.0 days (range, 9-68 days). Treatments for GVHD were considered for GVHD patients without spontaneous regression and with progression to a higher grade, except for those in which enhanced immunosuppression would not be preferable, such as encephalopathy resulting from calcineurin inhibitor or a pathologic diagnosis of intestinal transplantation-associated microangiopathy.[9,10] The median interval from the initial clinical manifestation of GVHD to the primary treatments was 4.5 days (range, 0-97 days). The dose of prednisolone was 0.5 mg/kg in 4 patients, 1 mg/kg in 20 patients, 2 mg/kg in 19 patients, 500 mg/body in 1 patient, and 1000 mg/body in 2 patients. Only MM was identified as a negative predictive factor for refractory GVHD (Table 2). In 46 patients undergoing steroid treatments, only MM was identified as a negative predictive factor for refractory GVHD (odds ratio, 5.05; 95% confidence interval [CI], 1.19-21.3; $P = .03$).

In the Cox proportional hazard model, age more than 50 years, high risk, and MM were identified as significant risk factors by univariate analyses, with MM and high risk remaining a significant risk in a multivariate analysis (Table 3). OS rates were significantly higher in FM patients compared with those in MM (Figure 1C). Uncontrolled GVHD was the cause of 6 MM patients of 11 (54.5%) who died because of transplantation-related mortality (TRM), whereas in FM patients, 3 of 24 (12.5%) died because of uncontrolled GVHD. The causes of death for the 5 MM patients who did not die of uncontrolled GVHD were infection in 3 patients, intestinal transplantation-associated microangiopathy in 1 patient, and liver failure in 1 patient. In 46 patients who underwent steroid treatments, only MM was identified as a significant risk factor in the Cox proportional hazards model (Hazard ratio 3.25; 95% CI, 1.46-7.26; $P = .004$). OS rates were significantly higher in FM patients compared with those in MM (Figure 1D). Uncontrolled GVHD was the cause of 6 MM patients of 9 (66.7%) who died because of TRM, whereas in FM patients, 3 of 15 (20.0%) died because of uncontrolled GVHD.

Our study suggested that macrophages are involved in a specific type of acute GVHD that tended to be systemic and refractory to conventional therapies, such as corticosteroids or calcineurin inhibitors.[11-13] Differences in treatment efficacy could be explained by the difference in infiltrating cell types. Although efforts have been made at predicting refractory GVHD,[14-17] no confirmed factor has been established to date. Our findings could prove to be a relatively simple and useful method directly related to the prognosis of patients.

Macrophages could not be completely suppressed by current therapies for acute GVHD mainly targeting T cells. Considered together with

Table 2. Analyses of predictive factors for refractory GVHD in all 104 patients

Parameter	Odds ratio (95% CI)	P
More than 50 y old	1.21 (0.35-4.19)	.76
High disease risk	1.29 (0.44-3.76)	.65
Graft PB (vs BM)	1.19 (0.23-6.11)	.83
Unrelated donor	0.91 (0.30-2.73)	.86
HLA mismatch	1.96 (0.66-5.84)	.23
Conventional regimens	0.94 (0.27-3.23)	.92
Skin stage 3 or 4 at biopsy	1.97 (0.69-5.68)	.21
MT (> 100 $CD8^+$ cells)	1.26 (0.37-4.27)	.71
MM (> 200 $CD163^+$ cells)	3.79 (1.22-11.8)	.02