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Effect of major histocompatibility complex haplotype matching by *C4* and *MICA* genotyping on acute graft versus host disease in unrelated hematopoietic stem cell transplantation

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Department of Medicine

The Graduate School, Yonsei University

Effect of major histocompatibility complex haplotype matching by *C4* and *MICA* genotyping on acute graft versus host disease in unrelated hematopoietic stem cell transplantation

Directed by Professor Hyon-Suk Kim

The Doctoral Dissertation
submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree
of Doctor of Philosophy

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June 2015

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ABSTRACT

Effect of major histocompatibility complex haplotype matching by *C4* and *MICA* genotyping on acute graft versus host disease in unrelated hematopoietic stem cell transplantation

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(Directed by Professor Hyon-Suk Kim)

Background: Human leukocyte antigen (HLA) matching between the donor and recipient is an important factor for the prognosis of hematopoietic stem cell transplantation (HSCT) in patients with hematologic malignancies. However, many other genetic factors are related to the outcomes of HSCT. In this study, we explored whether matching of *HLA* haplotypes between the recipient and donor can be predicted by *C4* and *MICA* typing as proxy markers, and whether it is associated with the incidence of acute graft versus host disease (aGVHD).

Methods: DNA preparations collected from a total of 59 recipient and unrelated donor pairs were used for PCR-based *C4* subtyping and *MICA* sequence-based typing. Additional samples from 22 recipient and related donor pairs were also assayed for *C4* subtyping. Medical records and clinicopathologic status including development of aGVHD were compared according to the groups classified by the results of *C4* and *MICA* typing. A single nucleotide polymorphism (SNP) microarray analysis for six

selected recipient/donor pairs was performed to determine matching of various genes located in and around the HLA region between the recipients and donors.

Results: The six most common *MICA* alleles with frequencies greater than 10% in this study were *MICA*008:01*, **010:01*, **002:01*, **004*, **009:01/049*, and **012:01*, and they were significantly associated with *HLA-B*07:02*, **15:01*, **58:01*, **44:03*, **52:01*, and **54:01*, respectively. Among the 59 unrelated pairs, *HLA* alleles were matched in 34 (57.6%) and mismatched in 25 (42.4%). *C4* subtypes were identical between the recipient and donor in 28 (82.4%) *HLA*-matched unrelated pairs, while *MICA* genotypes were matched in all *HLA*-matched unrelated pairs. In the related pairs, all 22 recipients showed the same *C4* subtypes with their respective *HLA*-matched donors. *C4*-mismatched cases showed a higher incidence of aGVHD than the *C4*-matched group regardless of an *HLA* match in the unrelated group (P=0.009). In multivariate analysis, *C4* mismatch was also a significant risk factor associated with the development of aGVHD in unrelated HSCT cases (hazard ratio=3.24, P=0.006). Among more than 3,000 SNPs belonging to the MHC domain, 96.6% and 95.8% were identical in the two *HLA*/*MICA*/*C4*-matched recipient and donor pairs, while 96.4% and 86.3% were identical in the two *HLA*/*MICA*-matched but *C4*-mismatched pairs.

Conclusions: PCR-based *C4* subtyping is a simple method for assessing the genetic identity of the HLA region between a recipient and unrelated donor. *C4* mismatch was also related with an increased incidence of aGVHD. Thus, this test would be useful for donor assessment and prediction of aGVHD in HSCT.

Key words : *C4* gene, MHC class I polypeptide-related sequence A (*MICA*), human leukocyte antigen, graft versus host disease, hematopoietic stem cell transplantation

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I. INTRODUCTION

Human leukocyte antigens (HLAs) play major roles in the immune system including antigen presentation and self-recognition. The combination of *HLA* allele types for different loci—including *HLA-A*, *-B*, *-C*, and *-DRB1*—on one of the chromosome pairs is defined as the 'haplotype'. *HLA* genes are located in a relatively small domain (~3600 kb) on the short arm of chromosome 6; thus, one of the two *HLA* haplotypes from each parent is inherited by the progeny under most circumstances.

Matching of genes that belong to the human major histocompatibility complex (MHC) including *HLA* loci between the donor and recipient for hematopoietic stem cell transplantation (HSCT) is one of the most important factors for graft survival and patient prognosis.[1] However, many other genetic factors—including polymorphisms in the genes encoding various cytokines and their receptors, killer cell immunoglobulin-like receptor (*KIR*), and matching of MHC class I polypeptide-

related sequence A (*MICA*) and B (*MICB*) genes between the donor and recipient—are known to be related to the outcomes of HSCT.[2-9] Therefore, a hematopoietic stem cell donor should be more genetically identical to a patient to improve clinical outcomes after HSCT, although testing and matching of all risk-associated genes for the recipient and donor would not be cost-effective from a practical viewpoint. In the current guidelines from the National Marrow Donor Program, genotyping and matching for at least four *HLA* loci (*HLA-A*, *-B*, *-C*, and *-DRB1*) are recommended.[10]

HLA genotypes in a certain ethnic group or population are known to be relatively conserved along repeated generations.[11] However, irrespective of *HLA* genotype, genes known to be related to immunological functions can be inherited heterogeneously because there is a chance of recombination between the pair of the same chromosome during meiosis.[12] Moreover, it is currently not practical to perform high-resolution sequence-based typing (SBT) for most of the relevant or suspicious genes, including *HLA* loci other than *HLA-A*, *-B*, *-C*, and *-DRB1*. Therefore, a proxy marker on chromosome 6p, which can represent extended haplotypes of genes with immunoregulatory functions other than *HLA*, can be useful to predict the degree of genetic concordance between the recipient and unrelated donor to estimate the risk for developing graft-versus-host disease (GVHD) and other adverse events after HSCT.

In most cases when the *HLA* genotypes of the donor and the recipient are identical, a closely related donor for HSCT; i.e., brother or sister of the recipient, would have identical genotypes for not only *HLA* genes but also other genes located on chromosome 6p, which can be associated with regulating immune function. However, *HLA* zero-mismatched HSCT does not always guarantee matching of both *HLA* haplotypes and other relevant genes on the short arm of chromosome 6 between the recipient and unrelated donor when only *HLA* loci such as *HLA-A*, *-B*, *-C*, and *-DRB1* are considered for matching. In these contexts, genotyping and matching of some

immunoregulatory genes on chromosome 6p between the hematopoietic stem cell (HSC) donor and the recipient can be helpful to ensure more genetically identical transplantation, particularly when the donor is unrelated to the recipient.[13,14] However, few reports have explored the effect of both haplotypes matching on the clinical outcomes of HSCT.[15,16]

GVHD remains one of the significant adverse events after HSCT, and can be a barrier to successful application of allogeneic HSCT. Currently, diagnosis of GVHD depends on the presence of clinical symptoms and findings by biopsy of the involved organs. There have been many studies on the genetic polymorphisms associated with the risk of developing GVHD.[2,7] However, proxy markers for the identification of genetic identity between HSC donor and recipient pairs and for prediction of the probability developing GVHD have not been well-assessed. The *C4* gene belongs to the MHC class III (gamma block of MHC), which is located between the MHC class I and II domains, and is a possible marker for identifying the degree of genetic identity between a recipient and a donor. The *MICA* gene is located on the proximal region of the MHC beta block (chromosome 6p 31.37-31.38) and can also be used as a proxy marker.

In this study, we explored whether matching of both *HLA* haplotypes for ensuring more genetically identical HSCTs between the recipient and unrelated donor can be predicted by determining matching of proxy genes, such as *C4* and *MICA*. To assess the genetic concordance between the recipient and donor according to the matching status of *C4* and *MICA* genes, single nucleotide polymorphisms (SNPs) of the genes located in and around the human MHC region were detected using the SNP microarray for selected cases. We also evaluated whether matching of these proxy markers between the unrelated HSCT donor and recipient are related to the incidence of acute GVHD (aGVHD).

II. MATERIALS AND METHODS

1. Samples and subjects

During 2008 and 2013, a total of 162 DNA preparations were collected at Severance Hospital from 22 recipients and related donors, as well as 59 unrelated recipient/donor pairs, and were stored at -70°C for later use. DNA from whole blood samples was extracted using a QuickGene-Mini 80 nucleic acid isolation instrument with the QuickGene DNA whole blood kit S (Kurabo Industries LTD., Osaka, Japan). Only samples with a sufficient quantity and those collected from HSC donors and their respective recipients suffering from acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), aplastic anemia, and other hematologic diseases excluding chronic myelogenous leukemia (CML) were included in this study. This study was approved by the Institutional Review Board of Severance Hospital (no. 4-2013-0300).

2. Review of medical records

Medical records including sex and age of the patients, clinical and genetic characteristics of the disease, symptoms and clinical grades of GVHD, and other clinicopathologic conditions as well as ABO blood group types and *HLA* genotypes of the donor and the recipient, and the number of mismatched *HLA* alleles were reviewed. Diagnosis of aGVHD was referred to the clinicians including the hemato-oncologist, ophthalmologist, and dermatologist or to the results of skin or mouth mucosa biopsies, when the first symptom of GVHD developed within 100 days after HSCT.

3. *C4A* and *C4B* subtyping

We performed PCR-based *C4A* (Rodgers blood group antigen) and *C4B* (Chido blood group antigen) subtyping using sequence-specific primers as described previously.[17] Isotype-specific *C4A*/*C4B* primers were as follows: A-up, GCATG CTCCT GTCTA ACACT GGAC; A-down, AGGAC CCCTG TCCAG TGTTA GAC; B-up, TGCTC CTATG TATCA CTGGA GAGA; B-down, AGGAC CTCTC TCCAG TGATA CAT. In addition, sequence-specific Rg/Ch primers were as follows: Ch-5, TGCGG CTTGG TTGTC ACGGG A; Ch5, TGCGG CTTGG TTGTC ACGGG G; Rg1, AGGTT GTTGT GGGCA ACACC GA; Ch1, AGGTT GTTGT GGGCA ACACC CC; Rg3, AGCCT CCATC TCAAA GGCAA A; Ch6, AGCCT CCATC TCAAA GGCAA G. Primers for the 780-bp fragment from the *C4* gene (7b, TGAGG GGACC AGCTG GAAGA GTC; 8, CAAGC GCCGC CACCT GTGCC CTA) were used for all PCR reactions as an internal control. A total of six combinations of forward and reverse primers were respectively used for *C4A* and *C4B* subtyping. Primer combinations for *C4A* subtyping included A-up/Ch-5, A-up/Ch5, A-down/Rg1, A-down/Ch1, Rg1/Rg3, and Rg1/Ch6, and those for *C4B* were B-up/Ch5, B-up/Ch-5, B-down/Ch1, B-down/Rg1, Ch1/Ch6, and Ch1/Rg3. PCR was performed with a mixture of 30-50-ng genomic DNA, 10 pmol of each control primer and respective forward and reverse primers, and PCR premix (BioSewoom Inc., Seoul, Korea) with a final volume of 20 μL using the C1000^{TM} Thermal Cycler (Bio-Rad Laboratories Inc., Hercules, CA, USA). Cycle conditions for primer pairs of A-up/Ch-5, A-up/Ch5, B-up/Ch5, and B-up/Ch-5 were as follows: initial denaturation at 94°C for 3 min, denaturing at 94°C for 20 s, annealing at 66°C for 45 s, extension at 72°C for 1 min for 35 cycles, and a final extension at 72°C for 10 min. Annealing temperature of 67°C was applied to PCR with primer pairs A-down/Rg1, A-down/Ch1, B-down/Ch1, and B-down/Rg1,

and that of 68°C was used for PCR with Rg1/Rg3, Rg1/Ch6, Ch1/Ch6, and Ch1/Rg3. PCR products were separated on a 1% agarose gel stained with ethidium bromide, and any results without amplification of both the internal control fragment and allele-specific product were repeated. Detailed procedures for PCR-based *C4A* and *C4B* subtyping were based on previous studies.[17,18] Results for a HSCT recipient and his/her respective donor were compared, and the pair was defined to have identical *C4* subtypes when the banding patterns for PCR products were identical between the recipient and donor (Fig. 1).

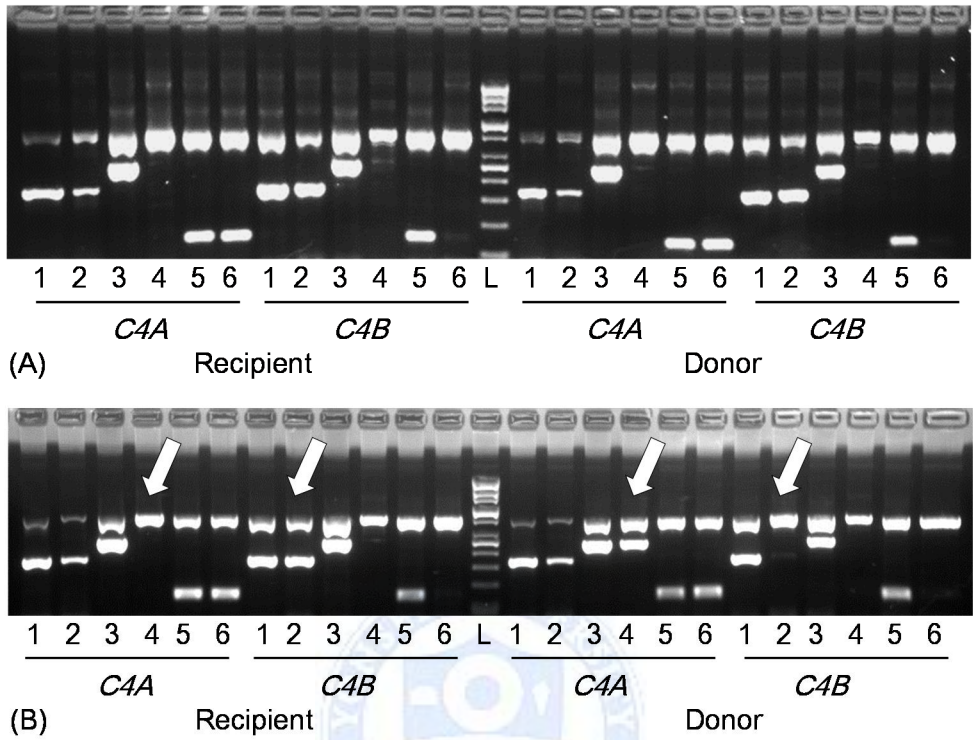

Fig. 1. Examples of *C4A* and *C4B* subtyping results using sequence-specific primed PCR. **(A)** The recipient and donor showed identical PCR banding patterns, and were considered *C4*-matched recipient and donor pairs. **(B)** There were two different PCR banding patterns (white arrows) between the recipient and donor; thus, they were defined as a *C4*-mismatched pair. Primers and respective product sizes were as follows: ***C4A*** **1**, A-up/Ch-5, 349 bp; **2**, A-up/Ch5, 348 bp; **3**, A-down/Rg1, 499 bp; **4**, A-down/Ch1, 498 bp; **5**, Rg1/Rg3, 151 bp; **6**, Rg1/Ch6, 151 bp; ***C4B*** **1**, B-up/Ch5, 349 bp; **2**, B-up/Ch-5, 350 bp; **3**, B-down/Ch1, 502 bp; **4**, B-down/Rg1, 503 bp; **5**, Ch1/Ch6, 150 bp; **6**, Ch1/Rg3, 150 bp. The PCR product size of the internal control target was 780 bp. L: ladder with DNA sizes of 100, 200, 300, 400, 500 (first thick band), 650, 850, and 1000 bp and over.

4. *MICA* genotyping

SBT for *MICA* gene was performed as described previously.[19] PCR primers were designed to amplify the *MICA* gene from exon 2 to exon 5: forward (MICA-F), CCACC ATCTC CTCTG GCAAG ACTAA TATTC CTCCA CC; reverse (MICA-R), CCAAA TTCCC CAACT TTCAT CCTAA TATAC GGAAG CC. PCR reactions were performed with 6 μL of PCR premix (BioSewoom Inc.), 50-ng genomic DNA, and distilled water in a final volume of 10 μL. The following conditions were used for PCR: 95°C for 4 min, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 90 s; and a final extension at 72°C for 5 min. Purified PCR products were sequenced with the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) using eight *MICA* gene-specific primers: *MICA* exon 2F, ATTTC CTGCC CCAGG AAGGT TGG; *MICA* exon 2R, GTGCC GGCTC ACCTC CCCTG CT; *MICA* exon 3F, GTGAG GAATG GGGTC AGTGG AA; *MICA* exon 3R, CAACT CTAGC AGAAT TGGAG GGAG; *MICA* exon 4F, AAGAG AAACA GCCCT GTTCC TCTCC; *MICA* exon 4R, TCCCT GCTGT CCCTA CCCTG; *MICA* exon 5F, GGTAG GGACA GCAGG GAT; *MICA* exon 5R, ACCAA CATGC CTATC TTTGC. The sequencing PCR conditions were as follows: 95°C for 1 min, followed by 25 cycles of 96°C for 10 s, and 50°C for 5 s, and a final extension at 60°C for 4 min. The reaction products were purified and analyzed using an ABI 3730xl DNA analyzer (Applied Biosystems). The sequence data were compared with previously reported sequences for *MICA* alleles using the HLA Analysis program (Biosewoom Inc., Seoul, Korea). All homozygous results for *MICA* genotyping were tested for the presence of a null allele using sequence-specific PCR primers, as described previously.[20]

5. SNP microarray

Selected samples collected from the recipient and unrelated donor pairs were used to determine the SNP genotypes of various genes that belong to and are outside of the human MHC domain. The SNP microarray experiments were performed using Infinium HumanExome BeadChip kits (Illumina Inc., San Diego, CA, USA) according to the manufacturer's instructions. This method is designed for genotyping of more than 250,000 SNPs in the human exome. After excluding SNPs showing less than 95% call rate and deviation from Hardy-Weinberg equilibrium (P<0.001), SNPs in the region spanning the MHC (throughout the 7.0-Mb region around human MHC domain in chromosome 6p, nucleotides 26,156,752 to 33,185,918) were analyzed to evaluate the match of various genes in the MHC region between the donor and recipient.

6. *HLA* SBT

PCR and sequencing were performed for exon 2 of the *HLA-DRB1* gene, as well as exons 2 to 4 of the *HLA-A*, *-B*, and *-C* genes using AlleleSEQR® *HLA-A*, *-B*, *-C*, and *-DRB1* PCR kits (Celera Co., Alameda, CA, USA) according to the manufacturer's instructions. The resulting nucleic acid sequences were read using an ABI 3100 DNA analyzer (Applied Biosystems). All sequenced results were analyzed using Assign™ SBT software ver. 3.5.1.45 (Conexio Genomics Pty Ltd., Fremantle, Australia).

7. Statistical analysis

All loci were estimated for Hardy-Weinberg equilibrium using Arlequin ver 3.5.1 software.[21] *MICA* alleles and *MICA-HLA* haplotypes were also analyzed, and the haplotypes were assigned using the EM algorithm with the same software.

Association of loci was analyzed using the chi-squared test. Baseline characteristics of the patient groups according to the development of aGVHD were compared using the Mann–Whitney U-test for continuous variables and the Fisher's exact test or chi-squared test for categorical variables. The incidence of aGVHD according to the matching of *HLA* genotypes, *C4* subtypes, *MICA* genotypes, and other factors was analyzed by Cox proportional hazard regression and Kaplan–Meier survival analysis with the log-rank test, and the hazard ratio for each independent variable was calculated by stepwise backward elimination. Statistical analyses were performed using the Analyse-it Method Validation edition version 3.76 software (Analyse-it Software Ltd, City West Business Park, Leeds, UK) and IBM SPSS Statistics version 22 (IBM Co., Armonk, NY, USA). *P*-values less than 0.05 were considered to indicate statistical significance in all analyses.

III. RESULTS

1. Allele frequency and association of the *MICA* gene with *HLA-B* and *-DRB1* loci

Frequencies of *MICA* alleles in our study subjects are shown in Table 1. To minimize selection bias, *MICA* allele types of the recipients were excluded in the estimation of allele frequency and haplotype association, and only those of unrelated donors (2n=118) were assessed. The six most common MICA alleles with frequencies greater than 10% in our subjects were *MICA*008:01*, **010:01*, **002:01*, **004*, **009:01/049*, and **012:01*. *MICA* alleles were significantly associated with *HLA-B* and *-DRB1* alleles (Table 2).

Table 1. *MICA* allele frequency of unrelated hematopoietic stem cell donors (2n=118)

Allele	Allele frequency (%)		*P*-value[1]
	This study (2n=118)	Sohn et al.[19] (2n=278)	
*MICA*008:01*	23.7	14.7	0.031
*MICA*010:01*	17.8	19.4	0.705
*MICA*002:01*	16.1	17.6	0.713
*MICA*004*	15.3	8.3	0.037
*MICA*009:01/049*[2]	10.2	11.5	0.698
*MICA*012:01*	10.2	9.4	0.801
*MICA*007:01*	4.2	4.0	0.897
*MICA*016*	0.8	-	0.124
*MICA*011*	0.8	1.1	0.833
*MICA*006*	0.8	1.4	0.630

[1] *P*-value was calculated using the chi-squared test.

[2] *MICA*009:01* and **049* have an identical DNA sequence from exon 2 to exon 5; thus, these two alleles could not be discriminated in our study.

Table 2. Haplotype association of *MICA* alleles with *HLA-B* and *-DRB1* alleles with frequencies greater than 1.0% in this study (2n=118)

Haplotype	HF (%)	LD (%)	RLD	χ^2	*P*-value	HF in a previous study[19] (%)
HLA-B - MICA association						
*B*44:03-MICA*004*	15.3	12.9	1.00	118.00	<0.001	8.3
*B*15:01-MICA*010:01*	9.3	7.7	0.47	56.03	<0.001	10.4
*B*58:01-MICA*002:01*	7.6	6.4	0.43	50.77	<0.001	7.6
*B*46:01-MICA*010:01*	5.1	4.2	0.25	29.20	<0.001	5.8

*B*52:01-MICA*009:01/049*[1]	5.1	4.6	0.47	55.84	<0.001	2.2
*B*07:02-MICA*008:01*	4.2	3.2	0.14	16.78	<0.001	3.2
*B*54:01-MICA*012:01*	4.2	3.7	0.31	37.04	<0.001	5.4
*B*13:02-MICA*008:01*	3.4	2.6	0.11	13.31	<0.001	3.6
*B*35:01-MICA*002:01*	3.4	2.8	0.18	21.57	<0.001	6.1
*B*51:01-MICA*009:01/049*[1]	3.4	2.8	0.15	17.97	<0.001	7.9
*B*13:01-MICA*007:01*	2.5	2.4	0.59	69.57	<0.001	-
*B*38:02-MICA*002:01*	2.5	2.1	0.14	16.04	<0.001	-
*B*40:01-MICA*008:01*	2.5	1.7	0.05	6.01	0.014	2.9
*B*40:02-MICA*008:01*	2.5	1.9	0.08	9.89	0.002	-
*B*40:06-MICA*008:01*	2.5	1.7	0.05	6.01	0.014	-
*B*55:02-MICA*012:01*	2.5	2.3	0.23	27.19	<0.001	-
*B*15:11-MICA*010:01*	1.7	1.4	0.08	9.40	0.002	-
*B*27:05-MICA*007:01*	1.7	1.6	0.39	45.98	<0.001	3.2
*B*37:01-MICA*008:01*	1.7	1.3	0.06	6.54	0.011	-
*B*59:01-MICA*012:01*	1.7	1.5	0.15	17.97	<0.001	-
*B*67:01-MICA*002:01*	1.7	1.4	0.09	10.60	0.001	-
MICA - *HLA-DRB1* association						
*MICA*004-DRB1*13:02*	9.3	7.3	0.35	40.98	<0.001	4.5
*MICA*009:01/049*[1]*-DRB1*15:02*	5.1	4.3	0.29	34.04	<0.001	-
*MICA*012:01-DRB1*04:05*	5.1	4.1	0.22	26.15	<0.001	3.8
*MICA*002:01-DRB1*13:02*	4.2	2.1	0.03	3.14	0.076	3.6
*MICA*004-DRB1*07:01*	4.2	2.9	0.09	10.20	0.001	2.3
*MICA*010:01-DRB1*08:03*	4.2	2.3	0.04	4.26	0.039	5.0
*MICA*002:01-DRB1*15:01*	3.4	1.9	0.03	3.69	0.055	-
*MICA*008:01-DRB1*01:01*	3.4	2.2	0.05	6.44	0.011	2.7
*MICA*008:01-DRB1*07:01*	3.4	1.4	0.01	1.60	0.206	2.5
*MICA*010:01-DRB1*04:06*	3.4	2.6	0.12	13.81	<0.001	5.8
*MICA*010:01-DRB1*15:01*	3.4	1.7	0.02	2.86	0.091	2.6
*MICA*002:01-DRB1*03:01*	2.5	2.1	0.14	16.04	<0.001	2.0
*MICA*007:01-DRB1*12:02*	2.5	2.3	0.28	32.63	<0.001	-

*MICA*008:01-DRB1*04:05*	2.5	0.3	0.00	0.08	0.772	-
*MICA*008:01-DRB1*08:03*	2.5	-0.1	0.00	0.00	0.953	-
*MICA*010:01-DRB1*09:01*	2.5	1.8	0.05	6.36	0.012	2.0
*MICA*012:01-DRB1*08:03*	2.5	1.4	0.02	2.66	0.103	-
*MICA*002:01-DRB1*15:02*	1.7	0.5	0.00	0.27	0.603	-
*MICA*002:01-DRB1*16:02*	1.7	1.3	0.05	5.83	0.016	-
*MICA*008:01-DRB1*09:01*	1.7	0.7	0.01	0.76	0.382	-
*MICA*008:01-DRB1*10:01*	1.7	1.3	0.06	6.54	0.011	-
*MICA*009:01/049*[1]*-DRB1*14:03*	1.7	1.5	0.15	17.97	<0.001	-
*MICA*010:01-DRB1*04:05*	1.7	0.0	0.00	0.00	0.972	-
*MICA*010:01-DRB1*14:06*	1.7	1.4	0.08	9.40	0.002	-

[1] *MICA*009:01* and **049* have an identical DNA sequence from exon 2 to exon 5; thus, these two alleles could not be discriminated in our study.

Abbreviations: HF, haplotype frequency; LD, linkage disequilibrium value; RLD, relative linkage disequilibrium value.

2. Match of *HLA*, *C4*, and *MICA* genes

A total of 59 recipient and unrelated donor pairs were assessed for matching of *HLA*, *C4*, and *MICA* genes. The distribution of recipient and donor pairs according to the estimated genes is summarized in Table 3. Among all unrelated pairs, *HLA* loci were matched in 34 (57.6%) and mismatched in 25 (42.4%). *C4* subtypes were identical between the recipient and donor in 28 (82.4%) of these *HLA*-matched pairs, while *MICA* genotypes were matched in all *HLA*-matched pairs. In addition, 22 recipient and related donor pairs were evaluated for matching *HLA* and *C4* genes, and all 22 recipients showed the same PCR-based *C4* gene subtypes with their respective *HLA*-matched related donors. Among those recipients, five (22.7%) developed aGVHD, and no significant difference was found between the incidences of aGVHD in the unrelated and related pairs with matching *HLA* and *C4* genes (P=1.000).

Incidences of aGVHD according to the groups classified by matching *HLA*, *C4*, and *MICA* genes were compared between groups and are summarized in Table 4. *C4*-matched unrelated cases showed a lower incidence of aGVHD than *C4*-mismatched unrelated pairs (*P*=0.009). In *HLA*-matched unrelated cases, aGVHD was also less frequently developed in the *C4*-matched group than the *C4*-mismatched (*P*=0.048).

Table 3. Matches of assessed genes between the recipient and respective donor pairs and the incidence of acute graft-versus-host disease

Donor type	Genes			No (% among each donor type)	No of aGVHD	% aGVHD[1]	*P*-value[2]
	HLA	*C4*	*MICA*				
Unrelated	M	M	M	28 (47.5)	6	21.4	1.000
	M	mm	M	6 (10.2)	4	66.7	0.064
	mm	M	M	10 (16.9)	3	30.0	0.681
	mm	M	mm	2 (3.4)	1	50.0	0.446
	mm	mm	M	11 (18.6)	7	63.6	0.052
	mm	mm	mm	2 (3.4)	1	50.0	0.446
	Subtotal			59 (100.0)	22	37.3	0.292
Related	M	M	ND	22 (100.0)	5	22.7	-

[1] Proportion of patients who developed aGVHD in the respective groups according to the matches of genes

[2] *P*-values calculated by comparing the proportion of recipients who developed aGVHD in each group with that in the related pairs using the Fisher's exact test.

Abbreviations: aGVHD, acute graft-versus-host disease; M, matched; mm, mismatched; ND, not done.

Table 4. Comparison between the incidences of acute graft versus host disease according to the matches of assessed genes

Group 1		Group 2		*P*-value[1]
Cases (n)	No of aGVHD (%)	Cases (n)	No of aGVHD (%)	
HLA- / *C4*-matched unrelated (28)	6 (21.4)	*HLA*-matched / *C4*-mismatched unrelated (6)	4 (66.7)	0.048
HLA-mismatched / *C4*-matched unrelated (12)	4 (33.3)	*HLA*- / *C4*-mismatched unrelated (13)	8 (61.5)	0.238
C4-matched unrelated (40)	10 (25.0)	*C4*-mismatched unrelated (19)	12 (63.2)	0.009
HLA-mismatched, *MICA*-matched unrelated (21)	10 (47.6)	*HLA*- / *MICA*-mismatched unrelated (4)	2 (50.0)	1.000
HLA-matched unrelated (34)	10 (29.4)	*HLA*-mismatched unrelated (25)	12 (48.0)	0.179
HLA-matched related (22)	5 (22.7)	All unrelated (59)	22 (37.3)	0.292
HLA-matched related (22)	5 (22.7)	*HLA*-matched unrelated (34)	10 (29.4)	0.759

[1] *P*-values were calculated using the Fisher's exact test.

Abbreviations: aGVHD, acute graft-versus-host disease.

3. Characteristics of the recipient and unrelated donor pairs

Clinicopathologic characteristics and matched *HLA*, *C4*, and *MICA* genes between the recipient and unrelated donor pairs are summarized in Table 5 according to the group with or without aGVHD. There were 7 (18.9%) *C4*-mismatched cases in the group without aGVHD (n=37), while 12 cases (54.5%) were *C4* mismatched in the aGVHD group (n=22). The proportions of '*C4*-mismatched' and '*C4*- or *MICA*-mismatched' cases were different between the two groups (*P*=0.005 and 0.004, respectively). In addition, the numbers of sex-mismatched, ABO-mismatched, and *HLA*-mismatched pairs were 10 (27.0%), 19 (51.4%), and 13 (35.1%), respectively, in the group without aGVHD, and were 10 (45.5%), 14 (63.6%), and 12 (54.5%), respectively, in the aGVHD group. The proportions of sex-mismatched, ABO-mismatched, and *HLA*-mismatched cases were higher in the aGVHD group, but the differences were not statistically significant (*P*=0.148, 0.358, and 0.115, respectively).

Table 5. Characteristics of the recipient and unrelated donor pairs according to the groups classified by the development of acute graft-versus-host disease

Characteristic	No aGVHD (n=37)	aGVHD (n=22)	*P*-value[1]
Age of recipient	36.0 (30.0 to 46.3)	29.0 (23.7 to 45.0)	0.170
Male recipient	24 (64.9%)	12 (54.5%)	0.432
Sex mismatch	10 (27.0%)	10 (45.5%)	0.148
Female donor to male recipient	6 (16.2%)	3 (13.6%)	0.790
Disease			0.232
AML	18 (48.6%)	13 (59.1%)	
ALL	10 (27.0%)	8 (36.4%)	
Aplastic anemia	5 (13.5%)	0 (0.0%)	
Others	4 (10.8%)	1 (4.5%)	
Stem cell source			0.180

Bone marrow	6 (16.2%)	1 (4.5%)	
Peripheral blood	31 (83.8%)	21 (95.5%)	
Total body irradiation	5 (13.5%)	3 (13.6%)	0.989
GVHD onset after HSCT (days)	-	39.5 (10.9 to 76.5)	-
Site of the first symptom			-
Skin	-	18 (81.8%)	
Mouth	-	2 (9.1%)	
Eye	-	1 (4.5%)	
Gastrointestinal tract	-	1 (4.5%)	
GVHD grade after follow-up			-
I	-	4 (18.2%)	
II	-	5 (22.7%)	
III	-	11 (50.0%)	
IV	-	2 (9.1%)	
Any ABO mismatch	19 (51.4%)	14 (63.6%)	0.358
Major	4 (10.8%)	6 (27.3%)	0.103
Minor	7 (18.9%)	7 (31.8%)	0.260
Major and minor	8 (21.6%)	1 (4.5%)	0.078
Major + major and minor	12 (32.4%)	7 (31.8%)	0.961
Minor + major and minor	15 (40.5%)	8 (36.4%)	0.750
HLA genotype mismatch	13 (35.1%)	12 (54.5%)	0.145
No of mismatched alleles	0.0 (0.0 to 1.0)	1.0 (0.0 to 1.1)	0.127
C4 subtype mismatch	7 (18.9%)	12 (54.5%)	0.005
MICA genotype mismatch	2 (5.4%)	2 (9.1%)	0.586
C4 and *MICA* mismatch	1 (2.7%)	1 (4.5%)	0.705
C4 or *MICA* mismatch	8 (21.6%)	13 (59.1%)	0.004

[1] *P*-values calculated using the Mann-Whitney U-test for continuous variables and the chi-squared test for categorical variables.

Data are shown as 'n (%)' or 'median (first to third quartiles)'.

Abbreviations: aGVHD, acute graft-versus-host disease; AML, acute myelogenous leukemia; ALL, acute lymphoblastic leukemia; HSCT, hematopoietic stem cell transplantation.

4. Association of *C4* or *MICA* mismatch with the incidence of aGVHD

The incidences of aGVHD according to the groups classified by matched *C4* and *MICA* genes between the recipients and donors were compared using Kaplan-Meier survival analysis (Fig. 2). In the unrelated HSCT, aGVHD developed more frequently in the *C4*-mismatched pairs than in *C4*-matched cases, regardless of *HLA* match or mismatch (P=0.018 by log rank test). In addition, the incidence of aGVHD was higher in cases with either *C4* or *MICA* mismatch than in the group with both *C4* and *MICA* match (P=0.016 by log-rank test). However, there was no significant difference in the incidence of aGVHD between the *MICA* match and mismatch groups (P=0.777 by log-rank test). Meanwhile, no significant difference in the incidence of aGVHD was found between the unrelated and related pairs with matched *HLA* and *C4* genes (P=0.917 by log-rank test).

To identify independent factors associated with the development of aGVHD in unrelated HSCT cases, Cox proportional hazard regression was performed with covariates including age of recipients, receiving total body irradiation or not, bone marrow or peripheral blood as the HSC source, match of sex, and ABO, *HLA*, *C4*, and *MICA* types between the recipient and donor. As a result, *C4* mismatch was the only significant risk factor associated with the risk of developing aGVHD [P=0.006, hazard ratio (HR)=3.24, 95% confidence interval (CI)=1.39 to 7.52]. When 'either *C4* or *MICA* mismatch' was added to the covariables, it was the only significant factor associated with aGVHD (P=0.006, HR=3.32, 95% CI=1.41 to 7.80).

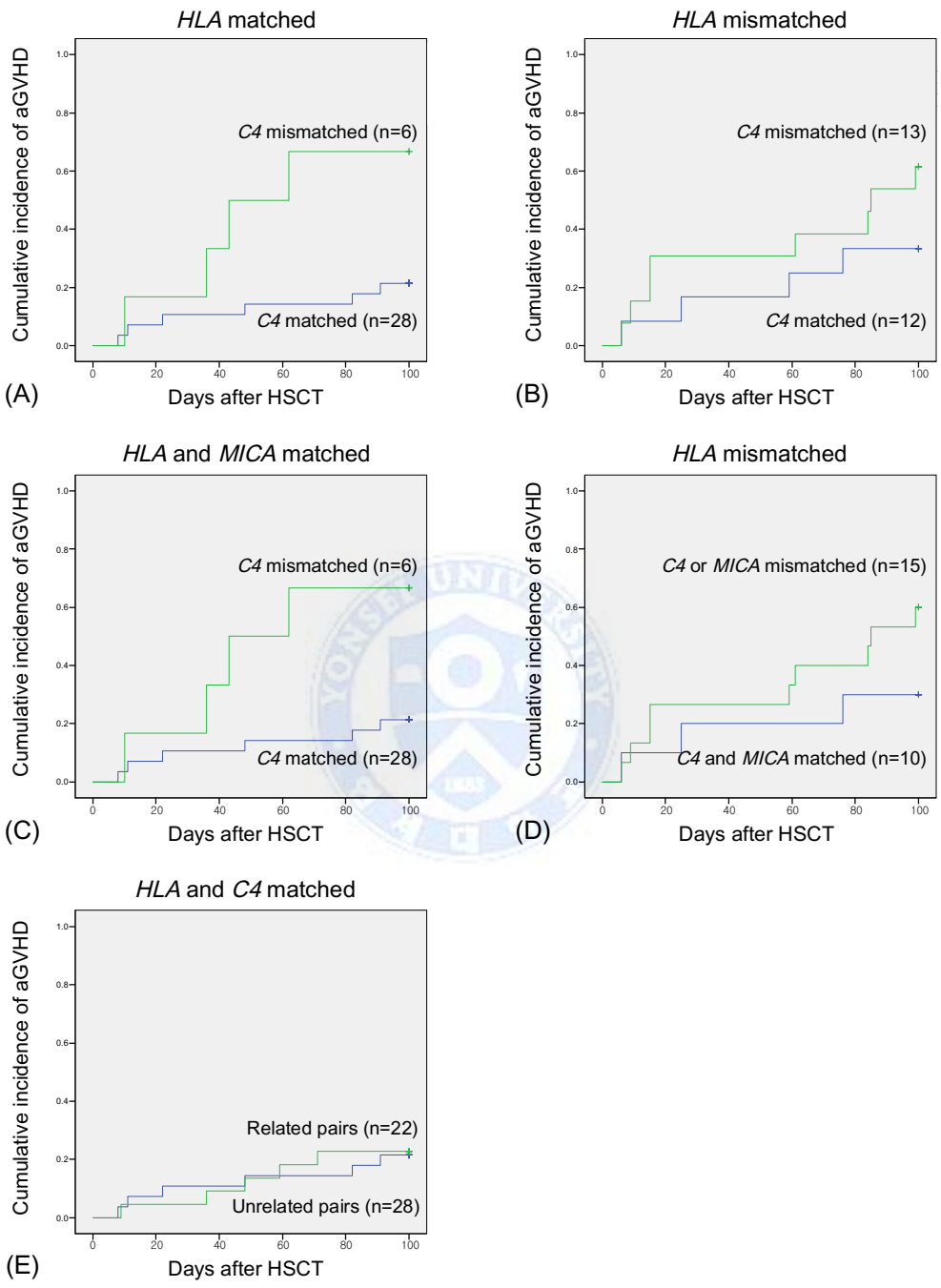

Fig. 2. Cumulative incidence of acute graft versus host disease (aGVHD) according to

the groups classified by matching *C4* and *MICA* genes between the recipients and donors. (A and B) The incidence of aGVHD in unrelated hematopoietic stem cell transplantation was higher in the *C4*-mismatched group compared to the *C4*-matched cases, regardless of *HLA* match or mismatch (P=0.018 by log-rank test). (C) All *HLA*-matched pairs showed *MICA* match, and (D) aGVHD developed more frequently in the *C4*- or *MICA*-mismatched group than in the *C4*- and *MICA*-matched group, regardless of *HLA* match (P=0.016 by log-rank test). (E) No significant difference in the incidence of aGVHD was found between the unrelated and related pairs with *HLA* and *C4* matches (P=0.917 by log-rank test).

5. Matches of SNPs between recipients and donors

A total of 12 DNA samples from six recipients and their respective unrelated donors were selected for SNP microarray according to matched *HLA*/*C4*/*MICA* types. All 12 samples showed call rates greater than 99.69% for control probes. Since the call rate for each specific SNP differed according to the sample used, results for SNPs that were valid in both the recipient and respective donor were analyzed (Fig. 3). Consequently, 3,053 to 3,062 SNPs in the MHC domain (nucleotides 29,691,278 to 33,096,766, 3.4 Mb in the chromosome 6p) of the recipients were compared with those of respective donors, and 96.6% and 95.8% of the SNPs were respectively identical in two *HLA*/*C4*/*MICA*-matched pairs, while 96.4% and 86.3% were respectively identical in two *HLA*/*MICA*-matched but *C4*-mismatched pairs (Table 6). For the *HLA*/*C4*/*MICA*-mismatched pair, the percent concordance of SNPs was as low as 81.9%, and 83.4% of SNPs in our results for the MHC domain were identical between the recipient and donor with *C4* match but *HLA*/*MICA* mismatch.

In addition, 3,949 to 3,960 SNPs in the 7.0-Mb region (nucleotides 26,156,752 to

33,185,918) including the MHC domain and genes located in the region distal from the MHC domain were also analyzed for match per recipient/donor pairs. As a result, 82.9% to 97.0% of the SNPs were identical and similar to the percent concordance of SNPs in the MHC domain for the same pair.

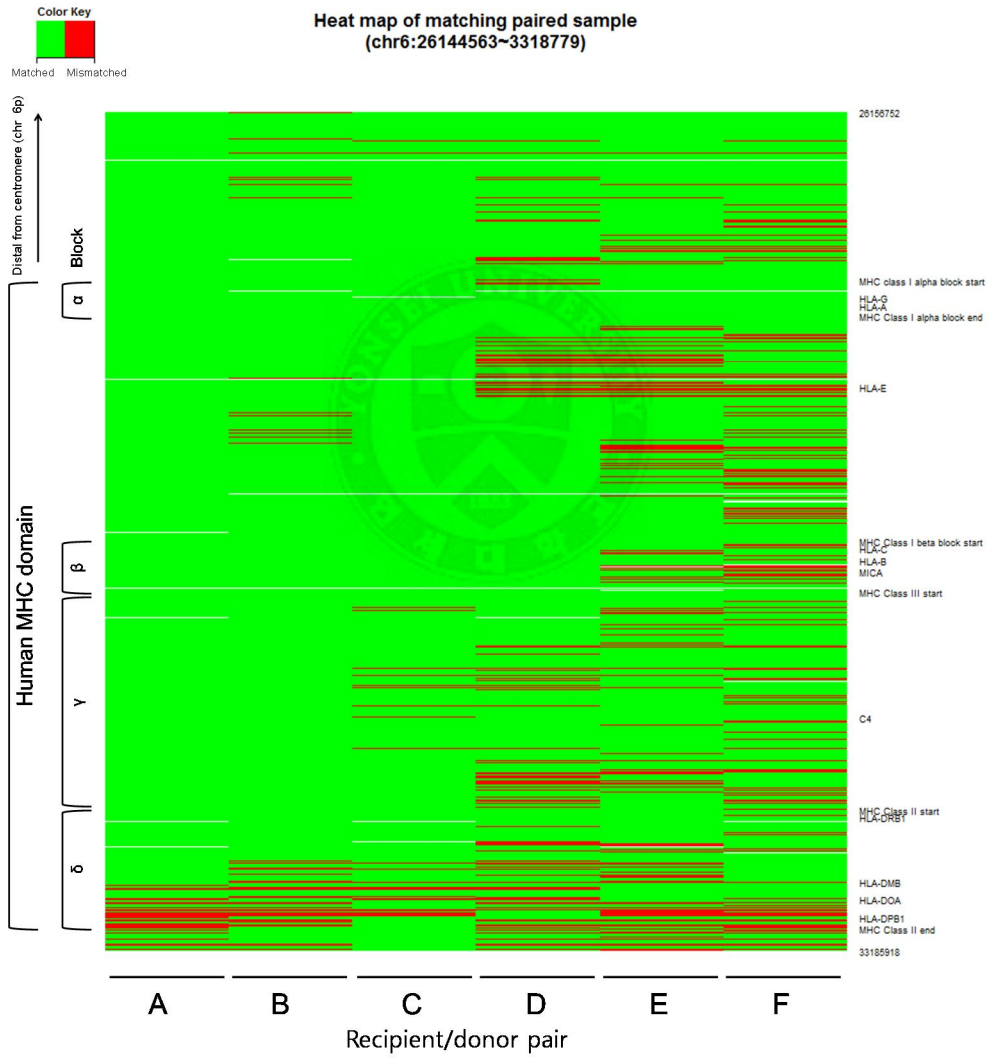

Fig. 3. Match map of single nucleotide polymorphisms (SNPs) in and around the human major histocompatibility complex region. Genotypes for 3,949 to 3,960 SNPs in chromosome 6p were compared between the recipient and unrelated donor. (A and B) *HLA*-/*MICA*-/*C4*-matched pairs. (C and D) *HLA*-/*MICA*-matched but *C4*-mismatched pairs. (E) *HLA*-/*MICA*-mismatched but *C4*-matched pair. (F) *HLA*-/*MICA*-/*C4*-mismatched pair. Green and red lines in each column indicate matched and mismatched SNPs, respectively, and white lines show the positions of invalid SNPs for either or both the recipient and donor.

Table 6. Concordance of SNPs between the recipients and their respective unrelated donors according to the matching of *HLA*, *MICA*, and *C4* genes

Target region (nucleotides # in Chr 6p)	Parameter	*HLA*-/*MICA*-/*C4*-matched		*HLA*-/*MICA*-matched *C4*-mismatched		Only *C4*-matched	All mismatched
		Pair #1 (A in Fig. 3)	Pair #2 (B in Fig. 3)	Pair #3 (C in Fig. 3)	Pair #4 (D in Fig. 3)	Pair #5 (E in Fig. 3)	Pair #6 (F in Fig. 3)
29,691,278 to 33,096,766 (3.41 Mb)	Compared SNPs (n)	3,059	3,062	3,053	3,055	3,059	3,053
	Identical SNPs (n)	2,956	2,932	2,943	2,637	2,552	2,499
	% identical SNPs	96.6	95.8	96.4	86.3	83.4	81.9
26,156,752 to 33,185,918 (7.03 Mb)	Compared SNPs (n)	3,956	3,960	3,949	3,954	3,956	3,952
	Identical SNPs (n)	3,823	3,790	3,831	3,461	3,370	3,277
	% identical SNPs	96.6	95.7	97.0	87.5	85.2	82.9

Abbreviation: SNP, single nucleotide polymorphism; Chr, chromosome.

IV. DISCUSSION

HLA matching is one of the most important processes in the screening and selection of unrelated HSC donors to improve clinical outcomes of patients with hematologic malignancies. However, *HLA-A*, *-B*, *-C*, and *-DRB1* matched unrelated donors are not always available, and the clinicians are recommended to select best-matched donors considering matches of other genes (including *HLA-DQB1*, *-DRB3/4/5*, and *–DP*) between the recipient and donor when an *HLA* 8/8-matched unrelated donor is not available.[10] In this context, additional proxy markers that reflect genetic concordance of the MHC domain between the recipient and unrelated donor would assist donor selection in terms of cost-effectiveness and convenience. Hence, we evaluated whether *C4* and *MICA* typing reflect the degree of genetic identity between the recipient and donor, and thus are useful in predicting the development of aGVHD.

The *MICA* gene is located on the proximal region of the MHC beta block (chromosome 6p 31.37-31.38), about 46.4 kb centromeric to the *HLA-B* gene. According to the international immunogenetics (IMGT)/HLA database (January 2015), 100 *MICA* alleles have been identified to date.[22] In this study, we analyzed the frequencies and association of *MICA* alleles with other *HLA* alleles. Consequently, the six most common *MICA* alleles in our subjects were *MICA*008:01*, **010:01*, **002:01*, **004*, **009:01/049*, and **012:01* (Table 1), and were similar to those of previous studies on the *MICA* gene in Korean populations.[19,23] In addition, *MICA* alleles were significantly associated with *HLA-B* and *-DRB1* alleles showing strong linkage disequilibrium (Table 2). This indicates that *MICA* allele types are highly conserved in Korean populations, and thus could be a proxy marker for genetic concordance between the recipient and a possible unrelated donor. However, we did not observe a

significant association of *MICA* genotype matching with the incidence of aGVHD. This may be caused by the small sample size of our study (n=59, unrelated pairs). *MICA* genotypes of the recipients and respective unrelated donors were identical in all *HLA*-matched cases in our results (Table 3). On the contrary, a previous study suggested that patients (n=44) who were *HLA-B* and *-C* matched had significantly improved survival when they were additionally matched for MHC beta block or *MICA*/*MICB* genes.[3] The difference in the results between studies may be caused by ethnic differences of genes that belong to and are around the human MHC region. Further studies on large Korean populations are required to identify the usefulness of *MICA* allele typing in the prediction of haplotype matching and aGVHD development. Meanwhile, C4A and C4B protein allotypes can be defined at the functional level by determining their hemolytic activity.[24] However, this procedure includes high-voltage immunofixation agarose gel electrophoresis, hemolytic activity assay using sheep RBCs, and immunoblotting, and is not easily applicable to simple *C4* gene typing and determining matching of the *C4* gene between recipient and donor pairs. Instead, we performed PCR-based *C4A* and *C4B* subtyping based on previously described methods.[17] Consequently, *C4* subtype mismatch was significantly associated with an increased incidence of aGVHD. Regardless of *HLA* match or mismatch, the incidence of aGVHD was higher in the *C4*-mismatched cases (P=0.009) in the unrelated HSCT. Multivariate analysis also showed that *C4* mismatch was a significant risk factor associated with aGVHD (P=0.006, HR=3.24). Petersdorf et al. reported that MHC haplotype mismatching was associated with a significantly increased risk of aGVHD (odds ratio 4.51, P<0.001) in 246 HSCT recipients and their *HLA-A*, *-B*, *-C*, *-DRB1*, and *-DQB1* allele-matched unrelated donors.[16] They used a novel DNA microarray based method to determine the physical linkage of *HLA* alleles. Their method could assess the real linkage of *HLA* alleles; i.e., exact *HLA* haplotypes, but could be labor-

intensive and not cost-effective for routine donor screening. In practical terms, simple testing for specific proxy markers instead of the complex assay could be used for the estimation of genetic appropriateness of possible unrelated donors for a certain recipient. In our results using the SNP microarray, *C4*-matched unrelated pairs tended to show higher concordance rates for SNPs that belong to the MHC domain than *C4*-mismatched pairs with the same *HLA*/*MICA* match status (Table 6). This result may support the usefulness of *C4* subtyping as a proxy marker for assessing concordance of the genes in the MHC region between a recipient and respective unrelated donor. In addition, all related HSCT cases showed identical *C4* subtypes between the recipients and donors. Thus, *C4* subtyping is useful only for unrelated donor assessment. Further studies on a large population using a SNP microarray is required for the exact estimation of the performance of PCR-based *C4* subtyping in predicting genetic concordance or HLA haplotype matching between the recipient and an unrelated donor of HSCT. In addition, we could not estimate the direct association of differences in the C4 allotypes between the recipient and a donor with the development of aGVHD. The results of PCR-based *C4* subtyping used in our study are not directly related with conventional C4 allotypes. Thus, the association of *C4* mismatch and higher incidence of aGVHD in our results may be due to differences in C4 allotypes between the recipient and unrelated donor and/or differences in other genes around the MHC domain based on *C4* subtyping as a proxy test.

Interestingly, *HLA* mismatch was not significantly related to the incidence of aGVHD in the multivariate analysis in our study. In addition, the incidence of aGVHD was not significantly different between the *HLA*-matched and -mismatched unrelated groups (P=0.179). Since the average number of mismatched *HLA* alleles in the *HLA*-mismatched cases was only 1.5, and the mismatched alleles could be tolerable, the *HLA* allele mismatch between the recipient and unrelated donor may not significantly

affect the probability of developing aGVHD in our subjects. In addition, the number of subjects in our study may not be sufficiently large to detect statistically significant differences in the incidence of aGVHD between the *HLA*-matched and -mismatched unrelated groups. Nevertheless, *C4* mismatch could be a risk factor for the development of aGVHD with a similar or greater impact than one or two *HLA* allele mismatches. Further studies are required to estimate the usefulness of *C4* subtyping as a proxy test in assessing the degree of genetic identity between the recipient and a possible unrelated HSC donor, because ethnic differences in the MHC haplotypes may affect our results. Additional studies are also required to evaluate the effect of *C4* mismatch in comparison with *HLA* mismatch on the development of aGVHD.

V. CONCLUSION

MICA alleles were significantly associated with *HLA-B* and *-DRB1* alleles in Korean. PCR-based *C4* subtyping would be a simple method of assessing the genetic identity of the MHC region between the recipient and an unrelated donor in HSCT. *C4* mismatch between the recipient and unrelated donor was also associated with an increased incidence of aGVHD. Thus, these tests can be used for HSC donor assessment and prediction of aGVHD.

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ABSTRACT(IN KOREAN)

비혈연 조혈모세포이식에서 급성 이식편대숙주병 발생에 대한 *C4* 및 *MICA* 유전자형 결정을 통한 주조직적합복합체 일배체형 적합성 판정의 효과

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박 용 정

배경 : 조혈모세포이식에서 환자-공여자간 사람백혈구항원(HLA) 적합성은 혈액암 환자의 예후와 관련된 중요한 요인 중 하나이다. 하지만, 사람백혈구항원 이외에도 다양한 유전적 요인이 조혈모세포이식의 결과와 연관되어 있을 수 있다. 본 연구에서는 환자-공여자간의 사람백혈구항원 일배체형 수준 적합성 판정을 위한 *C4* 및 *MICA* 유전자형 검사의 효용성을 평가하고 이들 유전자형 적합성과 급성 이식편대숙주병 발생과의 연관성을 알아보고자 하였다.

방법 : 총 59쌍의 비혈연 공여자와 환자로부터 수집된 DNA 검체를 이용하여 PCR기반 *C4* 형별검사와 *MICA* 염기서열분석을 시행하였다. 추가적으로 22쌍의 혈연 공여자-환자에서 *C4* 형별을 검사하였다. 환자-공여자간 *HLA*, *MICA* 및 *C4* 유전자 적합성에 따라 대상군을 분류하여 급성

이식편대숙주병 발생을 포함한 대상자의 임상적 상태를 비교하였다. 단일뉴클레오티드다형태(single nucleotide polymorphism, SNP) 마이크로어레이 방법을 사용하여 6쌍의 환자-공여자에서 *HLA* 유전자 주위 다양한 유전자의 일치 정도를 평가하였다.

결과 : 본 연구에서 흔하게 발견된 6종의 *MICA* 대립유전자형은 *MICA*008:01*, **010:01*, **002:01*, **004*, **009:01/049* 및 **012:01*이었고, 각각 *HLA-B*07:02*, **15:01*, **58:01*, **44:03*, **52:01* 및 **54:01* 대립유전자형과 유의하게 연관되어있었다. 비혈연 공여자-환자 59쌍 중, *HLA* 대립유전자형은 34쌍(57.6%)에서 환자-공여자간에 일치하였고 25쌍(42.4%)에서 불일치하였다. *HLA* 형별이 일치하는 쌍 중 28례(82.4%)에서 *C4* 유전자형이 환자-공여자간에 일치하였으나, *MICA* 유전자형은 모두 일치하였다. *HLA* 적합 혈연 공여자-환자 22쌍의 경우 *C4* 유전자형은 환자-공여자간에 모두 일치하였다. 비혈연 이식의 경우, *HLA* 적합성에 관계없이 *C4* 유전자가 불일치하는 군에서 *C4* 유전자 일치군보다 급성 이식편대숙주병의 발생 빈도가 유의하게 높았다(P=0.009). 다변량분석 결과에서도 *C4* 유전자 불일치는 급성 이식편대숙주병 발생과 독립적으로 연관된 유의한 위험요인이었다(hazard ratio=3.24, P=0.006). *HLA* 유전자 주위 약 3000종 이상의 SNP 중, 각각 96.6% 및 95.8%가 두 예의 *HLA*/*MICA*/*C4* 일치 환자-공여자 쌍에서 일치하였고, 각각 96.4% 및 86.3%가 두 예의 *HLA*/*MICA* 일치, *C4* 불일치 쌍에서 환자-공여자간에 동일하였다.

결론 : PCR 기반 *C4* 유전자형 검사는 *HLA* 유전자 주위 다양한 유전자의 환자와 비혈연 공여자 간 일치 정도를 평가하기 위한 간단한 방법으로

사용될 수 있을 것이다. *C4* 유전자형이 환자-공여자간에 불일치 하는 경우 급성 이식편대숙주병이 더 흔하게 나타났다. 따라서, 본 검사는 조혈모세포 이식 전 공여자 평가 및 급성 이식편대숙주병 발생 예측에 유용하게 사용될 수 있을 것으로 사료된다.

핵심되는 말 : *C4* 유전자, 주조직적합복합체 class I 폴리펩티드 연관 서열 A, 사람백혈구항원, 이식편대숙주병, 조혈모세포 이식