Association between single nucleotide polymorphisms within HLA region and disease relapse for patients with hematopoietic stem cell transplantation

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Disease relapse occurs in patients with leukemia even hematopoietic stem cell transplantation (HSCT) was performed with human leukocyte antigen (HLA)-matched donors. As revealed previously by Petersdorf et al., there are nine single nucleotide polymorphisms (SNPs) located in the HLA region that potentially modulate the efficacy of HSCT. In this study, we investigated whether or not the genomic variants 500 base pairs flanking the nine transplantation-related SNPs were related to the risk of post-HSCT relapse for patients with leukemia (n = 141). The genomic DNAs collected from 85 patients with acute myeloid leukemia (AML), 56 patients with acute lymphocytic leukemia (ALL), and their respective HLA-matched donors were subject to SNPs analysis, conferred by the mode of mismatch between donor-recipient pair or by recipient or donor genotype analysis. Seven SNPs were revealed to associate with the risk of relapse post-HSCT. For patients with AML, the increased risk of post-HSCT relapse was associated with the donor SNP of rs111394117 in the intron of NOTCH4 gene, and the recipient SNPs of rs213210 in the ring finger protein 1 (RING1) gene promoter, and rs17220087 and rs17213693 in the intron of HLA-DOB gene. For patients with ALL, the increased risk of post-HSCT relapse was associated with the donor SNP of rs213210 in the RING1 gene promoter, and the recipient SNPs of rs79327197 in the HLA-DOA gene promoter, rs2009658 in the telomeric end of lymphotoxin-alpha (LTA) gene, rs17220087 and rs17213693 in the intron of HLA-DOB gene, and rs2070120 in the 3′-UTR of HLA-DOB gene. This study sheds new insight into selecting better candidate donors for performing HSCT in patients with AML and ALL.

The human leukocyte antigen (HLA) region located on chromosome 6p21.3 represents the most polymorphic region of the human genome. With the high density distribution of genes related to immune function, identification of clinically important genetic variants located in HLA region is crucial in stem cell transplantation1. Allogeneic hematopoietic stem cell transplantation (HSCT) is an approach to treat different types of hematologic disorders2–5. Patients with HSCT are able to receive high dose of therapeutic regimens to increase the cure rate. Disease relapse represents the major cause of transplant failure, while post-HSCT death is mainly caused by infection and graft-versus-host disease (GVHD). High risks of disease relapse and GVHD and high mortality are caused by transplantation of recipients with mismatched HLA donors when compared with HLA-matched donors6,7. The outcome of transplantation is usually better for related donor-recipient pairs when compared to the

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unrelated pairs. Accordingly, related HLA-matched donors are the priority choice when allo-transplantation is performed. Patient death may still occur even when HLA-matched donors are used in HSCT. This implies that the outcomes of HSCT are likely related to other genetic factors in addition to the HLA alleles. A recent study conducted by Petersdorf et al. demonstrated that within the HLA region, there are nine single nucleotide polymorphisms (SNPs) (rs2244546, rs915654, rs429916, rs2242656, rs209130, rs2075800, rs394657, rs2071479 and rs107822) related to the occurrence of adverse effects associated with HSCT, including the patient death, transplant-related death, disease-free survival, relapse, and acute and chronic GVHD. These SNPs in the form of donor DNA, recipient DNA, or mismatch between donor-recipient pair DNA lead to unfavorable or favorable outcome of patient post-HSCT. These studies indicate that the efficacy of HSCT is affected by the genetic variants in the HLA region.

We speculated that the relapse for patient with HLA-matched HSCT might be conferred by undefined genetic variants located at the HLA region of donor and recipient genome. The issue was investigated in this study by determining and analyzing the genomic sequences 500 base pair (bp) flanking the nine HSCT-related SNPs.

Results

Patients (n = 141) with acute myeloid leukemia (AML, n = 85) and acute lymphocytic leukemia (ALL, n = 56) receiving HSCT from HLA-matched donors were recruited to determine whether or not the risk of relapse is related to the SNPs within the HLA region (Table 1). Genomic DNA of the donor-recipient pairs were subject to PCR amplification of the genomic regions 500 bp flanking the 9 sourced SNPs (Table 2) using the forward and reversed primers (Table 3). Candidate SNPs that were related to the post-HSCT relapse were searched by sequencing the PCR amplicons. Collectively 34 SNPs in the HLA region were defined to associate with post-HSCT relapse. These SNPs were classified into group 1 (donor genotype, n = 5), group 2 (recipient genotype, n = 11),

| Sourced SNP | Model | SNP under analysis |
|-------------|-------|-------------------|
| rs2244546   | Donor genotype | rs2523675 rs2518028 rs141341529 |
| rs394657    | Donor genotype | rs2518028 rs111394117 |
| rs429916    | Recipient genotype | rs9276982 rs71565361 rs79327197 rs151190962 |
| rs915654    | Recipient genotype | rs2009658 rs736160 rs915654 |
| rs2075800   | Recipient genotype | rs2844464 rs2227956 |
| rs2242656   | Mismatch | rs3130048 rs213210 |
| rs107822    | Mismatch | rs19791445 rs2071479 |
| rs209130    | Mismatch | rs209132 rs209131 rs209130 rs2070120 |
| rs2071479   | Mismatch | rs11244 rs2070120 rs41258084 rs17220087 |

Table 2. The SNPs that are 500 bps flanking the sourced SNPs. *The table was partially reproduced from the reference.
The table was partially reproduced from reference \(^*\). \(^*\)BAG6: BCL2 associated athanogene 6; HCP5: HLA complex P5; HLA-DOA: major histocompatibility complex, class II, DO alpha; HLA-DOB: major histocompatibility complex, class II, DO beta; HSPA1L: heat shock protein family A member 1 like; LTA: lymphotoxin alpha; RING1: ring finger protein 1; TRIM27: tripartite motif containing 27.

The primer sequences for amplification of gene fragments by PCR \(^*\). (Table 3).

| Gene          | Primer Sequences                                                                 |
|---------------|----------------------------------------------------------------------------------|
| BAG6\(^*\)    | F: 5'-ATTGATTCTCACGGGGCACAGGGCC-3\(^{\prime}\)  
|               | R: 5'-GCGGAGGTGGAGAAGATAAGGAGGACG-3\(^{\prime}\)  |
| HCP5\(^*\)    | F: 5'-GGCGACCTAATGCGTGTAGTACG-3\(^{\prime}\)  
|               | R: 5'-CTTGACGCTCTTCTCAGGAAGAG-3\(^{\prime}\)  |
| HLA-DOA\(^*\) | F: 5'-CAACAGGAGAAGGTACCGTCTG-3\(^{\prime}\)  
|               | R: 5'-GGCCACCTCTTCTTGTATTATATAACG-3\(^{\prime}\)  |
| HLA-DOB\(^*\) | F: 5'-CTCTTCTGAAAGGCTGACAGTGC-3\(^{\prime}\)  
|               | R: 5'-TTCCATAGGAGGTGCATGATATGA-3\(^{\prime}\)  |
| HSPA1L\(^*\)  | F: 5'-TCCCCTTCTAACGTACCTCAGGGCC-3\(^{\prime}\)  
|               | R: 5'-TGATCCAGGTGATGAGGGCGAGAG-3\(^{\prime}\)  |
| LTA\(^*\)     | F: 5'-AACATAAAAGCGAACAGGGGCG-3\(^{\prime}\)  
|               | R: 5'-TTAGGATGAGGGGACACCTC-3\(^{\prime}\)  |
| NOTCH4\(^*\)  | F: 5'-GATTGTCGTGTGGTTGGAGCTGTG-3\(^{\prime}\)  
|               | R: 5'-TGAGGCTGTATCAGAATGAGGTGCTCCT-3\(^{\prime}\)  |
| RING1\(^*\)   | F: 5'-TTATCGACTTCTGCGCGCCTTAT-3\(^{\prime}\)  
|               | R: 5'-AACAACCTTCTAGCCTGCCCTTCCCT-3\(^{\prime}\)  |
| TRIM27\(^*\)  | F: 5'-AGTCGGGATTACAGAAATGCACC-3\(^{\prime}\)  
|               | R: 5'-GCAAGGACATTTTGAGGTAACC-3\(^{\prime}\)  |

Table 3. The primer sequences for amplification of gene fragments by PCR \(^*\). 

and group 3 (donor-recipient pair mismatch type, n = 18) SNPs according to the category of and the relative position to the sourced SNPs (Table 2). The association between these SNPs and the risk of disease relapse were analyzed and conferred by donor SNP (mode of donor genotype analysis), recipient SNP (mode of recipient genotype analysis) or mismatched of donor-recipient pair SNP (mode of mismatch between donor-recipient pair defined by having a specific combination of different SNP alleles between the donor and recipient).

Of the 5 SNPs in group 1, three SNPs were located at the telomeric end of HLA class I histocompatibility antigen protein P5 (HCP5) gene and two SNPs were located in the intron of NOTCH4 gene, respectively. Donor genotype analysis demonstrated that none was related to the risk of relapse for patients with AML (Supplementary Table 1). However, the SNP of rs111394117 in the NOTCH4 intron was related to the risk of relapse for patients with ALL (genotypic test: \(P = 0.0166; \) Table 4). A greater risk of relapse for patients with ALL was associated with the donors who carried the polymorphism of A at rs111394117 when compared to the donors who carried the polymorphism of G at the same SNP position.

Of the 11 SNPs in group 2, five SNPs were located at the HLA-DOA gene promoter, three SNPs were located at the telomeric end of lymphotoxin-alpha (LTA) gene, and three SNPs were located in the intron of the heat shock protein family A member 1 like (HSPA1L) gene, respectively.Recipient genotype analysis revealed that none was associated with the risk of relapse for patients with AML (Supplementary Table 2). On the other hand, the risk of relapse was associated with the SNPs of rs79327197 located at the HLA-DOA promoter and rs2009658 located at the telomeric end of LTA gene for patients with ALL (rs79327197: genotypic test, \(P = 0.0148; \) Table 4). A greater risk of relapse for patients with ALL was associated with the recipients who had the polymorphism of G at rs79327197 and G at rs2009658 than the recipients who had the polymorphism of A and C at the corresponding position of SNP respectively.

Of the 18 SNPs in group 3, three SNPs were located in the intron of BCL2 associated athanogene 6 (BAG6) gene, two SNPs were located at the ring finger protein 1 (RING1) gene promoter, six SNPs were located at the telomeric end of tripartite motif containing 27 (TRIM27) gene, seven SNPs were located in the exon, intron, or 3'-untranslated region (UTR) of HLA-DOB gene, respectively. None of the SNPs with donor-recipient pair mismatched genotype was related to the risk of relapse (Supplementary Table 3 and 4). On the other hand, the SNP of rs213210 was related the risk of disease relapse for patients with AML as revealed by recipient genotype analysis (genotypic test \(P = 0.0444, \) Table 4 and Supplementary Table 5). A greater risk of relapse was associated with the patients who had the polymorphism of G at rs213210 than the patients who had the polymorphism of A at the same SNP position. Donor genotype analysis of these SNPs revealed that rs213210 located at the RING1 promoter was related to the risk of relapse for patients with ALL (genotypic test \(P = 0.0285; \) Table 4 and Supplementary Table 6). Donors with G/G genotype of rs213210 resulted in lower risk of relapse for recipients. Additional analysis by Chi-square test revealed that the recipient SNPs of rs17220087 and rs17213693 located in the HLA-DOB intron were related to the risk of relapse for patients with AML (rs17220087: Chi-square test \(P = 0.0465; \) rs17213693: Chi-square test \(P = 0.0465; \) Table 4) and ALL (rs17220087: Chi-square test \(P = 0.0311; \) rs172213693: Chi-square test \(P = 0.0311; \) Table 4). One additional recipient SNP of rs2070120 located in the HLA-DOB gene 3'-UTR was related to the risk of relapse for patients with ALL (Chi-square test \(P = 0.0311; \) Table 4).

There was no association between the new 7 SNPs identified in this study and the previous 9 SNPs reported by Petersdorf et al.\(^*\) as revealed by the pair-wise linkage disequilibrium (LD) analysis. The newly identified rs213210 and rs17213693 was in high LD with rs107822 (\(D^2 = 0.96\)) and rs2070120 (\(D^2 = 0.96\)). In the analysis, \(D^2\) was the parameter for normalized standard measurement of LD which compares the observed and expected frequencies.
Discussion

By analysis of the DNA from 141 patients with leukemia and their respective HSCT donors, a panel of 7 SNPs in the HLA region was defined to associate with the risk of relapse for patients with leukemia post-HSCT. These SNPs (rs111394117, rs79327197, rs2009658, rs213210, rs17220087, rs17213693, and rs2070120) were located mainly in the NOTCH4, HLA-DOA, HLA-DOB, LTA and RING1 genes (Fig. 1 and Table 4). The disease- and donor/recipient type-specific impacts of these SNPs on the risk of relapse are also demonstrated in this study that can be related to the different mechanistic insight in the pathogenesis of AML and ALL.

The donor type SNP of rs111394117 is located in the NOTCH4 intron 9, 156 bp from the 3′ of exon 8 (Fig. 2).

**Table 4.** The relapse-associated SNPs for patients with HSCT. *Statistical analyses were performed by using genotypic test or Chi-square test.*

| SNP       | Gene/location | Source | Disease/Status | Number of patients (%) | P*     |
|-----------|---------------|--------|----------------|------------------------|--------|
| **Donor genotype** |               |        |                |                        |        |
| rs111394117 | NOTCH4, intron | rs394657 | AML            | A/A 1 (6.7) 2 (13.3) 12 (80.0) | 0.0166 |
|           |               |         |                | A/G 0 (0.0)            |        |
|           |               |         |                | G/G 0 (0.0)            |        |
| rs213210  | RING1, promoter | rs107822 | ALL            | A/A 3 (25.0) 8 (66.7) 1 (8.3) | 0.0285 |
|           |               |         |                | A/G 0 (0.0)            |        |
|           |               |         |                | G/G 10 (25.0) 12 (30.0) 18 (45.0) |        |
| **Recipient genotype** |       |        |                |                        |        |
| rs79327197 | HLA-DOA, promoter | rs429916 | ALL            | A/A 12 (80.0) 1 (6.7) 2 (13.3) | 0.0150 |
|           |               |         |                | A/G 35 (94.6) 2 (5.4) 0 (0.0) |        |
| rs2009658 | 1.6 kb telomeric of LTA | rs915654 | ALL            | C/C 25 (67.6) 12 (32.4) 0 (0.0) | 0.0148 |
| rs213210  | RING1, promoter | rs107822 | AML            | A/A 2 (8.3) 13 (54.2) 9 (37.5) | 0.0444 |
| rs17220087 | HLA-DOB, intron | rs2071479 | AML            | A/A 1 (2.6) 37 (97.4) | 0.0465 |

of one haplotype comprised by alleles at different loci. This implies that the effects of these SNPs on the risk of post-HSCT relapse might not be independent.
antigen loading on HLA class II proteins mediated by HLA-DM. Impaired function or down-regulation of HLA-DO leads to less-restrained antigen presentation. The C polymorphism at the SNPs of rs17220087 and the G polymorphism at the SNPs of rs17213693 which are located in HLA-DOB intron are likely to cause aberrant mRNA splicing and produce HLA-DOB protein variants with abnormal activity for accurate antigen presentation. This may cause an increased in relapse for patients with AML and ALL post-HSCT. The SNPs of rs2070120 was located in the 3′-UTR of HLA-DOB gene. With 3′-UTR of RNA transcript usually containing the target sites of regulatory RNA such as miRNA, the polymorphism of rs2070120 may be related to the relapse of patients with ALL by modulating the levels of HLA-DOB expression and affecting the normal function of HLA-DO in antigen presentation. On the other hand, the SNP of rs79327197 is located in the HLA-DOA promoter. It is likely that the polymorphism of this SNP causes a decrease in HLA-DOA promoter activity leading to a decrease in HLA-DOA expression, thereby relates to the relapse of patients with ALL.
The recipient type SNP of rs2009658 is related to the risk of relapse for patients with ALL and is located in the telomeric end of LTA gene, 1.6 kb from the 5′-end of exon 1 (Fig. 2). LTA encodes a cytokine produced by lymphocytes and attributes to the risk of relapse for patients with ALL. The LTA protein induces a variety of inflammation, immune stimulation and anti-viral responses involved in the development of secondary lymphoid organ formation and apoptosis. LTA expression contributes to the development of T-cell acute lymphoblastic leukemia. With the SNP of rs2009658 located at 200 bp from the 5′-end of the reported promoter region and LTA is an autocrine growth factor for leukemic cells, patients with the polymorphism of G at rs2009658 may enhance the LTA promoter activity by altering the binding affinity or binding pattern of transcription factor(s) to the promoter leading to an increase in LTA expression and contribute to the greater risk of relapse of patients with ALL post-HSCT.

Relapse risk for patients with ALL and AML are associated with the donor genotype and recipient genotype of rs213210, respectively. The SNP is located in the promoter region, 462 bp from exon 1 of RING1 gene (Fig. 2). RING1 belongs to the PcG family proteins which functions in self-renewal and proliferation of normal cells. The study by Xu’s et al. revealed that RING1 is expressed in AML and various subsets of myelodysplastic syndrome. RING1 overexpression drives tumorigenesis and links to poor prognostic scoring for patients with cancer. With rs213210 located in the promoter region of RING1, patients who carried the polymorphism of G may enhance the RING1 promoter activity by altering the binding affinity or binding pattern of transcription factor(s) to the promoter leading to an increase in RING1 expression. The interaction of RING1 and the oncogenic proteins such as MLL-AF9 and MLL-ENL in the leukemic cells may contribute to the growth of cancer cells and increase the risk of relapse post-HSCT.

In this study, the association between SNPs and post-HSCT relapse was analyzed according to the findings by Petersdorf et al. Nevertheless, different SNPs were unveiled as the risk factors for relapse post-HSCT. Difference in ethnicity is a likely explanation for these findings. On the other hand, SNPs beyond the HLA regions have been revealed as the risk factors for relapse post-HSCT. For example, improved survival of HSCT was associated with the SNPs within the tumor necrosis factor II receptor superfamily member 1B (TNFRSF1B) and the interleukin 10 gene. Whether or not the risk of relapse post-HSCT is related to the SNPs outside the HLA region remains to be a topic of research interest.

Conclusions

In regard to the data obtained from this study, seven SNPs located in the HLA region contribute to an increase in the risk of post-HSCT relapse for patients with AML and ALL. The study may have clinical impacts on searching and selecting appropriate donor-recipient pair for HSCT. The genes associated with these SNPs mostly have pathophysiological functions in the immunological disorders. Future investigations are required to demonstrate how these SNPs elucidate their biological effects on the adjacent genes leading to transplantation failure.

Methods

Patients and laboratory tests. The Institutional Review Board of Chang Gung Memorial Hospital (CGMH) has reviewed and approved the study. The approval ID was 102-4949B. All methods were performed in accordance with the relevant guidelines and regulations. Patients (n = 141) with AML (n = 85) and ALL (n = 56) receiving HSCT from their donors were recruited at CGMH (Table 1). Written informed consent was provided by all 141 recipients before enrollment in this study.

The sequence-specific oligonucleotide probes-based method, LABType SSO Typing Test (Thermo Fisher, Waltham, MA), was used for HLA typing of HLA-A, -C, -B, -DRB1, and -DQB1 alleles for donors and recipients prior to transplantation. High-resolution HLA typing by the SeCore kit (Thermo Fisher, Waltham, MA) was then performed to obtain more detailed allele information. Allele ambiguity of the SeCore typing was resolved by using sequence-specific primers-based method, the MicroSSP Allele Specific Typing Tray (Thermo Fisher, Waltham, MA).

Chimerism test and relapse assessment post-HSCT. The AmpFISTR Identifiler amplification kit (Thermo Fisher, Waltham, MA) for analysis of short tandem repeats (STR) was used as the chimerism test to evaluate HSCT engraftment as described previously. Briefly, the following tetranucleotide STR loci were included in the STR analysis: D8S1179, D21S11, D7S820, and CSF1PO (all labeled with 6-FAM blue dye); D3S1358, TH01, D13S317, D16S539, and D21S1338 (all labeled with VIC green dye); and D19S3433, vWA, TPOX, and D18S51 (all labeled with NED yellow dye). Manufacturer’s instruction was followed to set up the cycle conditions of PCR and the analysis of PCR product. Recurrence of malignancy as defined by relapse was based on one or more of the following laboratory findings: reappearance of leukemia blasts in the peripheral blood or >5% blasts in the bone marrow, >0.01% of the peripheral blood cells as analyzed by flow cytometry carrying the individualized minimal residual disease panel for each patient according to the initial CD markers at diagnosis, abnormal karyotypes with the changes for the number of chromosomes or with structural variants of translocation, insertion, and deletion in cytogenetic analysis, and the presence of >5% recipient STR alleles in chimerism test.

Selection of SNPs. The risk of post-HSCT disease-free survival, patient death, transplant-related death, relapse, and acute and chronic GVHD has been related to the nine SNPs (rs2244546, rs915654, rs429916, rs2242656, rs209130, rs2075800, rs394657, rs2071479, rs107822) within the HLA region in a previous study. These SNPs were considered as the sourced SNPs in this study and were classified into three groups including donor genotype, recipient genotype, and mismatched donor-recipient pair genotype. The classification was determined according to whether donor or recipient SNP or mismatch of donor-recipient pair SNP conferred the relapse-associated risks.
**PCR and sequencing.** The PCR and sequencing was performed as described previously. Briefly, the QIAamp DNA Blood mini Kit (Qiagen, Valencia, CA) was used to extract the genomic DNA from 3 ml peripheral blood. The DNA fragments that flanking 500 bps of the 9 sourced SNPs were amplified by using 9 different primer pairs (Table 3), respectively. In a reaction volume of 50 μl containing 1X reaction buffer, 10 nmol of dNTP, 6 pmol of forward and reversed primers, 300 ng of genomic DNA, and 1 μl of Pfu Turbo Hotstart DNA Polymerase (Agilent, Santa Clara, CA), PCR was performed with the following cycling condition: 4 min at 94 °C for 1 cycle, 30 sec at 94 °C, 30 sec at 58 °C, and 45 sec at 72 °C for 30 cycles, and 10 min at 72 °C for 1 cycle. When PCR was completed, 5μl of PCR products were fractionated on a 2% agarose gel and analyzed by ethidium bromide staining. The Big Dye Terminator Cycle Sequencing Kit (Thermo Fisher, Waltham, MA) and an ABI PRISM Genetic Analyzer (Thermo Fisher, Waltham, MA) were used for direct sequencing of the remaining PCR product based on the instruction of the manufacturer. SNPs data were not available for all donor-recipient pairs because of insufficient amount of genomic DNA and failure of PCR reaction.

**Statistical analysis.** The analysis was performed as previously described. Briefly, the quality of SNPs testing experiments was examined by using the Hardy-Weinberg equilibrium (HWE) test. Those SNPs which infringed on the HWE test were excluded from the analysis. The association of disease relapse with candidate SNPs was evaluated by calculating and comparing the allele and genotype frequencies between the non-relapse and relapse groups. Whether the specific SNP genotypes related to the risk of post-HSCT relapse was evaluated by a genotypic test. Chi-square test was further used to search for additional SNP genotypes that were related to the risk of relapse. For the mode of donor-recipient pair analysis, the association for the risk of disease relapse with the mismatch status of SNP genotypes was evaluated by the chi-square and Fisher’s exact tests. HaploView 4.2 software (https://www.broadinstitute.org/haploview/haploview) was used for pair-wise LD analysis of SNPs.

**Data Availability**
The raw data and statistical data used in this study were included in the Supplemental Files.

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Author Contributions
D.P.C. conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or tables, and wrote draft of the manuscript. S.W.C. and P.N.W. analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or tables, and reviewed drafts of the manuscript. F.P.H. performed the experiments and approved the final draft. C.P.T. reviewed literature, analyzed and interpreted data, wrote draft of the manuscript, and critically revised the manuscript. All authors read and approved the final manuscript.

Additional Information
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