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ORIGINAL ARTICLE

Impact of amino acid substitution at residue 9 of HLA-A2 on the development of acute GVHD in Korean pediatric patients receiving unrelated hematopoietic stem cell transplantation

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Summary

Incompatibility of human leukocyte antigen (HLA) alleles between donors and recipients of unrelated hematopoietic stem cell transplantation (UHSCT) increases the risk of acute graft-versus-host disease (GVHD). We evaluated the positional effect of amino acid substitutions in HLA molecules on severe acute GVHD in Korean pediatric recipients of UHSCT. All of 64 donor–recipient pairs were serologically matched for HLA-A, -B, and -DR loci. Only substitution at residue 9 resulting from an HLA-A*02 polymorphism was significantly associated with the risk of severe acute GVHD in patients (OR = 7.0, $P = 0.033$) on multivariate analysis. Recipients of this mismatched HLA also showed shortened overall survival (HR = 9.7, $P < 0.001$) and increased risk for transplant-related mortality (HR = 9.1, $P = 0.027$). Structural modeling showed that the amino acid substitution could alter the peptide preference of the ligand-binding pocket. A single amino acid substitution at position 9 was a major predictor of severe acute GVHD in Korean pediatric patients.

Introduction

Unrelated hematopoietic stem cell transplantation (UHSCT) has become a mainstay of treatment for patients with hematopoietic malignancies [1]. Despite developments in immunosuppressive therapy, however, acute graft-versus-host disease (GVHD) remains a major cause of morbidity and mortality in UHSCT recipients. Incompatibility between donor and recipient human leukocyte antigen (HLA) alleles at high resolution has been found to increase post-transplant complications, including engraftment failure [2], acute and chronic GVHD [3,4], and mortality [5].

Even if an unrelated donor–recipient pair has serologically identical HLA, it is highly unlikely that all of their HLA alleles are the same. Thus, it has been speculated that a number of Korean patients could receive allogeneic hematopoietic stem cells mismatched at the allelic level even though serologically matched with donors [6,7]. The patients receiving such transplants suffer a greater incidence of acute GVHD and exhibit lower survival rates [8]. Thus, the identification of nonpermissive mismatches is important to the success of UHSCT, especially in light of ethnic-specific differences in the distribution of HLA alleles.

The alloreactivity of donor T cells to recipient peptide-allogeneic-major histocompatibility complex (MHC)

Table 1. Patient characteristics (n = 64).

Characteristics	Frequency	Percent
Sex		
Male	39	60.9
Female	25	39.1
Diagnosis of underlying disease		
Acute lymphoid leukemia	23	35.9
Acute myeloid leukemia	24	37.5
Chronic myelogenous leukemia	2	3.1
Myelodysplastic syndrome	3	4.7
Severe aplastic anemia	3	4.7
Other disease	9	14.1
Source of hematopoietic cell graft		
PBSC	22	34.4
Cord blood	3	4.7
BM	39	60.9
Disease status at transplantation		
Standard risk	44	69.8
High risk	19	30.2
Sex match between recipient and donor		
Sex-match	34	53.1
Sex-mismatch	30	46.9
ATG		
No	45	70.3
Yes	19	29.7
Total body irradiation		
No	45	70.3
Yes	19	29.7
Number of allele mismatch		
0	28	43.8
1	21	32.8
2	13	20.3
3	2	3.1
HLA antigen mismatch		
Full match	48	75.0
One-antigen mismatch	16	25.0
Conditioning regimen		
Bu-Cy or variant	34	53.1
Cy-ATG or variant	21	32.8
Bu-Flu-ATG or variant	6	9.4
Others	3	4.7
Acute GVHD		
Not severe (≤ 2)	52	83.9
Severe (>3)	10	16.1
ANC_500* median (range)	16 (10–48)	
PLT_20K† median (range)	26 (13–115)	

PBSC, peripheral blood stem cells; BM, bone marrow; ATG, antithymocyte immunoglobulin; Bu, busulfan; Cy, cyclosporine A; Flu, fludarabine; ANC, absolute neutrophil count; PLT, platelets.

*The day of neutrophil engraftment was the first of three consecutive days with absolute neutrophil counts >500/μL.

†The day of platelet engraftment was the last of seven consecutive days with a platelet count >20 000/μL with no requirement for platelet transfusion.

complexes is thought to be proportional to the structural dissimilarity between alleles, with a higher degree of dissimilarity leading to the development of acute GVHD after UHSCT [9,10]. The degree of structural dissimilarity, in turn, is related to the number, type, and position of HLA amino acid differences between a donor and a recipient [11]. Amino acid differences in positions directly related to peptide binding or T-cell receptor contacts, in particular, may have more deleterious effects on the outcome of UHSCT [12].

In this study, after identifying nonpermissive mismatches at the amino acid level in Korean patients who underwent UHSCT, we investigated the molecular effects of the observed nonpermissive mismatches by structural modeling.

Materials and methods

Patients

Sixty-four consecutive pediatric patients who had undergone hematopoietic stem cell transplantation at Asan Medical Center, Seoul, Korea, between 2005 and 2008, and their paired donors, whose HLA-A, -B, -C, and -DRB1 alleles had been analyzed by high-resolution genotyping, were enrolled in the study. Each donor–recipient pair was serologically matched for HLA-A, -B, and -DR antigens. The HLA-A, -B, -C, and -DRB1 loci were genotyped by sequence-based typing [13]. Each patient received cyclosporin A and short-course methotrexate for GVHD prophylaxis. Acute GVHD was scored from 0 to 4, as described previously [14]. The risk assessment for leukemia relapse was defined according to Kawase *et al.* [15]. Amino acid sequences of HLA-A, -B, -C and -DR molecules were retrieved from the IMGT/HLA sequence database [16], and amino acid substitutions between donor–recipient pairs were evaluated.

Molecular modeling and structural analysis

Insight II (Accelrys, San Diego, CA, USA) was used for structural analysis and modeling, using the coordinates of the crystal structures of HLA-A2 [17] and HLA-Cw [18,19].

Statistical analysis

The positional effect of amino acid substitution on severe acute GVHD (grade ≥3) was evaluated by logistic analysis. Amino acid sequences of HLA class I and II molecules were obtained from the IMGT/HLA Sequence Database [20]. The effect of amino acid substitution on severe acute GVHD was adjusted by the following clinical variables: donor and recipient age, risk of leukemic relapse, source of hematopoietic cell graft, sex mismatch, use of antithymocyte immunoglobulin (ATG), total body irradiation (TBI), HLA antigen mismatch, conditioning regimens, and underlying diseases. For the initial univariate

analysis, survival analysis and comparison were made using the Kaplan–Meier and the log-rank tests. The effects of the position of an amino acid substitution on transplant-related mortality (TRM) and overall survival (OS) were also evaluated using a multivariate Cox regression model. All analyses were performed using SPSS version 10 (SPSS Inc., Chicago, IL, USA). A P-value <0.05 indicated statistical significance.

Results

Patient characteristics

The median age of the 64 children was 6 years (range, 0–18 years) and 60% (39/64) were male patients. The clinical characteristics of patients are summarized in Table 1. Forty-four pediatric patients showed standard-risk features for relapse, including the first complete remission of acute leukemia and the first chronic phase of chronic myelogenous leukemia (CML) at transplant; 19 pediatric patients were considered at high leukemia and CML risk, defined as a more advanced acute leukemia and CML risk status, or had diseases other than acute leukemia and CML. Most patients (75%, 48/64) had no antigen mismatches, and the remaining patients (16/64) had one antigen mismatch.

Positional effect of amino acid substitution on severe acute GVHD

The frequencies of amino acid substitutions at individual positions in the HLA molecules are shown in Table 2. Mutations in HLA class I molecules often involved positions 9, 97, 99, and 156. A univariate analysis showed that substitutions at positions 9 (Tyr – Phe) of the HLA-A molecule and position 71 of the HLA-DR molecule were significantly associated with the risk of acute GVHD (Table 3).

However, after adjusting for clinical factors, a multivariate logistic regression analysis showed that only the mismatch at residue 9 of the HLA-A locus was significantly associated with severe GVHD (OR = 7.0, $P = 0.033$, Table 4). All mismatches at position 9 (Tyr – Phe) of HLA-A*02 alleles were between A*0206 and A*0201 in recipient–donor pairs.

Positional effect of amino acid substitution on OS and TRM

A multivariate Cox analysis showed that amino acid substitution at position 9 of the HLA-A molecule was an independent predictor for TRM risk (HR = 9.1, $P = 0.027$, Table 5) and shorter OS (HR = 9.7, $P < 0.001$, Table 6), with TBI (HR = 3.7, $P = 0020$). Amino acid substitution at position 156 (Leu-Arg) of the HLA-Cw molecule was also an independent risk factor for TRM (HR = 6.8, $P < 0.011$, Table 6), but not for OS. A Kaplan–Meier plot of OS in patients with or without a substitution at position 9 of the HLA-A locus is presented in Fig. 1, which shows that OS was shorter in recipients with a substitution at position 9 of the HLA-A molecule (33.3%) versus those without a substitution (82.8%; $P = 0.0002$).

Table 2. Frequency of substitutions in human leukocyte antigen (HLA) molecules at each amino acid position between donors and recipients ($n = 64$).

Amino acid position	Frequency of substitution
HLA-A locus	
Position 9	6
Position 99	2
Position 149, 152, 156	1
HLA-B locus	
Position 97	2
Position 99, 143, 147	1
HLA-C locus	
Position 9	20
Position 24	15
Position 66	5
Position 73	10
Position 77	12
Position 80	12
Position 97	12
Position 99	20
Position 114	12
Position 116	21
Position 147	3
Position 152	7
Position 156	11
Position 163	14
HLA-DR locus	
Position 28	1
Position 37	6
Position 57	7
Position 60	2
Position 67	3
Position 70	3
Position 71	3
Position 74	7
Position 86	8
HLA class I loci*	
Position 9	25
Position 97	13
Position 99	21
Position 147	4
Position 152	8
Position 156	11

*Positions with mismatch frequencies >2.

Molecular modeling and structural analysis of HLA-A position 9 and HLA-Cw position 156

The residues at position 9 of A*0201 and A*0206 are phenylalanine and tyrosine, respectively. These amino

Table 3. Logistic analysis of the association of amino acid positions with severe GVHD (≥grade 3).

	Univariate analysis	
Amino acid position	OR (95% CI)	*P* value
HLA-A locus		
Position 9 (Tyr-Phe)	**7.000 (1.174–41.740)**	**0.033**
Position 99 (Tyr-Cys)	5.667 (0.324–99.043)	0.235
HLA-B locus		
Position 97 (Ser-Thr)	0 (NA)	0.999
HLA-C locus		
Position 9 (Ser-Phe)	2.714 (0.681–10.819)	0.157
Position 24 (Ala-Ser)	1.597 (0.354–7.212)	0.543
Position 66 (Asn-Lys)	0 (NA)	0.999
Position 73 (Thr-Ala)	2.755 (0.573–13.237)	0.206
Position 77 (Asn-Ser)	2.048 (0.443–9.470)	0.359
Position 80 (Lys-Asn)	2.048 (0.443–9.470)	0.359
Position 97 (Arg-Trp)	1.194 (0.216–6.590)	0.838
Position 99 (Phe-Cys)	2.714 (0.681–10.819)	0.157
Position 114 (Asp-Asn)	2.048 (0.443–9.470)	0.359
Position 116 (Leu-Tyr)	1.373 (0.341–5.519)	0.656
Position 147 (Trp-Leu)	0 (NA)	0.999
Position 152 (Thr-Ala)	0 (NA)	0.999
Position 156 (Leu-Arg)	2.755 (0.573–13.237)	0.206
Position 163 (Thr-Leu)	1.800 (0.394–8.215)	0.448
HLA-DR locus		
Position 37 (Tyr-Ser)	3.000 (0.469–19.177)	0.246
Position 57 (Ser-Asp)	0 (NA)	0.999
Position 60 (His-Tyr)	0 (NA)	0.999
Position 67 (Ile-Phe)	0 (NA)	0.999
Position 70 (Arg-Gln)	0 (NA)	0.999
Position 71 (Arg-Lys)	**12.750 (1.033–157.438)**	**0.047**
Position 74 (Ala-Glu)	2.350 (0.387–14.260)	0.353
Position 86 (Gly-Val)	1.917 (0.327–11.226)	0.471
Positions with involvement in ≥2 HLA class I loci		
Position 9	**9.0 (1.715–47.221)**	**0.009**
Position 97	1.050 (0.193–5.725)	0.995
Position 99	4.071 (0.998–16.605)	0.050
Position 147	0 (NA)	0.999
Position 152	0.714 (0.078–6.538)	0.766
Position 156	2.755 (0.573–13.237)	0.206

NA, not available; HLA, human leukocyte antigen; GVHD, graft-versus-host disease. Bold values were used for statistical significant variables ($P < 0.05$).

Table 4. Multivariate analysis for the position effect of amino acid substitution on severe GVHD (≥grade 3).

	Multivariate analysis	
Characteristics	OR (95% CI)	*P* value
Residues (substitution versus no substitution)		
HLA-A position 9 (Tyr-Phe)	**7.000 (1.174–41.740)**	**0.033**
HLA-DR position 71 (Arg-Lys)	0.910 (0.087–9.544)	0.937
Conditioning regimens		
Bu or Cy – Flu – ATG (*n* = 19)	0.141 (0.008–2.484)	0.181
Bu – Cy (*n* = 13)	0.260 (0.014–5.005)	0.372
Cy – ATG (*n* = 1)	0 (NA)	0.999
Other combinations (*n* = 2)	Reference	

Cy, cyclosporine A; Bu, busulfan; Cy, cyclophosphamide; Flu, fludarabine; ATG, antithymocyte immunoglobulin; HLA, human leukocyte antigen; GVHD, graft-versus-host disease. Bold values were used for statistical significant variables ($P < 0.05$).

Table 5. Multivariate Cox regression analysis for the effect of amino acid substitution on transplant-related mortality.

	Multivariate analysis	
Characteristics	HR (95% CI)	*P* value
Recipient sex (female versus male)	0.340 (0.074–1.556)	0.165
Donor sex (female versus male)	**0.062 (0.009–0.450)**	**0.006**
Recipient age (*n* = 62, continuous)	**1.188 (1.014–1.392)**	**0.033**
Donor age (*n* = 60, continuous)	1.011 (0.949–1.077)	0.741
HLA-A locus		
Position 9 (Tyr-Phe)	**9.139 (1.288–64.856)**	**0.027**
Position 99 (Tyr-Cys)	5.283 (0.191–146.280)	0.326
HLA-C locus		
Position 156 (Leu-Arg)	**6.828 (1.548–30.117)**	**0.011**

Bold values were used for statistical significant variables ($P < 0.05$).

Table 6. Multivariate Cox regression analysis for the effect of amino acid substitution on overall survival.

	Multivariate analysis	
Characteristics	HR (95% CI)	*P* value
Recipient age (*n* = 62, continuous)	1.088 (0.958–1.236)	0.193
Donor age (*n* = 60, continuous)	0.987 (0.960–1.240)	0.662
HLA-A locus		
Position 9 (Tyr-Phe)	**9.733 (2.761–34.312)**	**<0.001**
Use of TBI (*n* = 62)		**0.020**
No (*n* = 19)	Reference	
Yes (*n* = 43)	3.728 (1.227–11.324)	

TBI, total body irradiation; HLA, human leukocyte antigen. Bold values were used for statistical significant variables ($P < 0.05$).

acids are structurally similar except for the presence of a hydroxyl group in the benzene ring of tyrosine. This hydroxyl group would collide with a leucine at position 2 (P2) of a bound peptide. As a result, the preference of A*0206 is shifted toward peptides with a P2 valine, which is shorter than leucine by one carbon, thus avoiding steric collision with Tyr-9 (Fig. 2).

Human leukocyte antigen-Cw molecules with arginine at residue 156 prefer peptides with aspartic acid at P3 because Arg156 can form a salt bridge with this negatively charged amino acid (Fig. 3a). As leucine is hydrophobic, substitution of this amino acid at residue 156 eliminates such polar interactions. Instead, leucine-substituted HLA-Cw favors peptides containing hydrophobic residues at P3 and P6 (Fig. 3b).

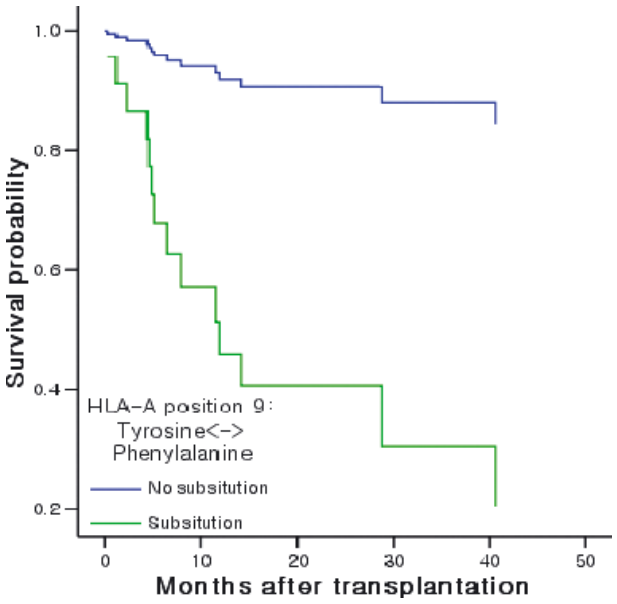

Figure 1 The effect of amino acid substitution (Tyr-Phe) at position 9 of the human leukocyte antigen (HLA)-A molecule on overall survival.

Discussion

Human leukocyte antigen allelic mismatch at the high-resolution level has been reported to play a major role in the development of severe acute GVHD following UHSCT [15,21]. The alloreactivity to mismatched HLA, which is the mechanism of acute GVHD, is thought to be due to HLA structural dissimilarity caused by a summation of amino acid substitutions in HLA molecules. However, it is unlikely that all differences have the same clinical impact on the development of acute GVHD because substituted amino acids might have different effects according to their type and position in the molecule. Therefore, identification of permissive and nonpermissive mismatches for each HLA allele is important in choosing one donor among several unrelated, but serologically matched, donors.

Kawase *et al.* [15] reported recently that high-resolution mismatches at HLA-A2, -A26, -B62, and -DR4 alleles were significantly associated with severe acute GVHD in Japanese UHSCT patients. They also reported that amino acid substitutions at positions 9, 99, 116, 156 of HLA-A and -C are high-risk mismatches and suggested that the identification of such nonpermissive mismatches would be beneficial for the selection of more suitable donors [15]. In a similar study, Ferrara *et al.* [12] suggested that substitution at position 116 of HLA class I increases the risk for acute GVHD in Italian patients undergoing UHSCT. However, nonpermissive mismatches might differ slightly according to ethnicity because the distribution of HLA alleles is ethnic-specific.

In this study, we evaluated the effect of substitutions at different amino acid positions of HLA molecules on acute GVHD in Korean pediatric patients. Using a multivariate Cox analysis, we, like Kawase *et al.*, found that mismatches involving residue 9 significantly increased the risk of acute GVHD. All mismatches at position 9 were between phenylalanine (HLA-A*0201) and tyrosine (HLA-A*0206). In HLA-A molecules, residue 9 is located in the β-sheet of the α2 domain and is involved in peptide-fragment binding [22]. Amino acid substitutions in these pockets can significantly alter peptide preference [23], which can provoke the alloreactivity of T cells. Our modeling results predict that the substitution of tyrosine in HLA-A*0206 for phenylalanine in HLA-A*0201 alters the preference of HLA-A molecule for the P2 residue of bound peptides to favor the smaller valine instead of leucine. This difference can cause changes in the interaction of T-cell receptors with the HLA-A2 molecule-peptide complex.

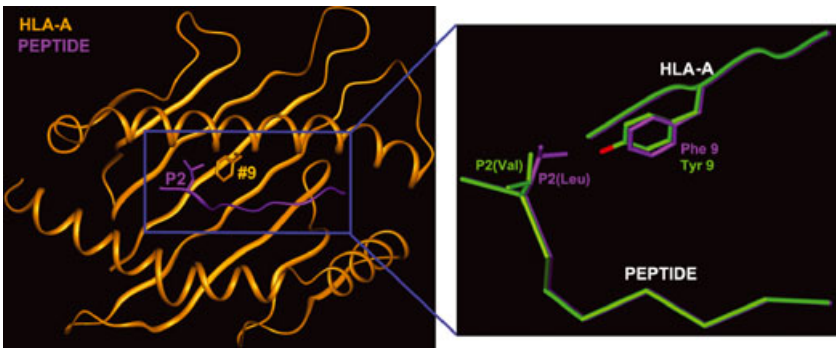

Figure 2 Modeling of structural alterations caused by mismatch of residue 9 in the human leukocyte antigen (HLA)-A molecule. The amino acids of residue 9 of the HLA-A molecule and P2 of a bound peptide are Phe and Leu, respectively. The close-up view shows that structural changes associated with the substitution of tyrosine for phenylalanine at position 9 alter the preference of the HLA-A molecule for the P2 residue of bound peptides.

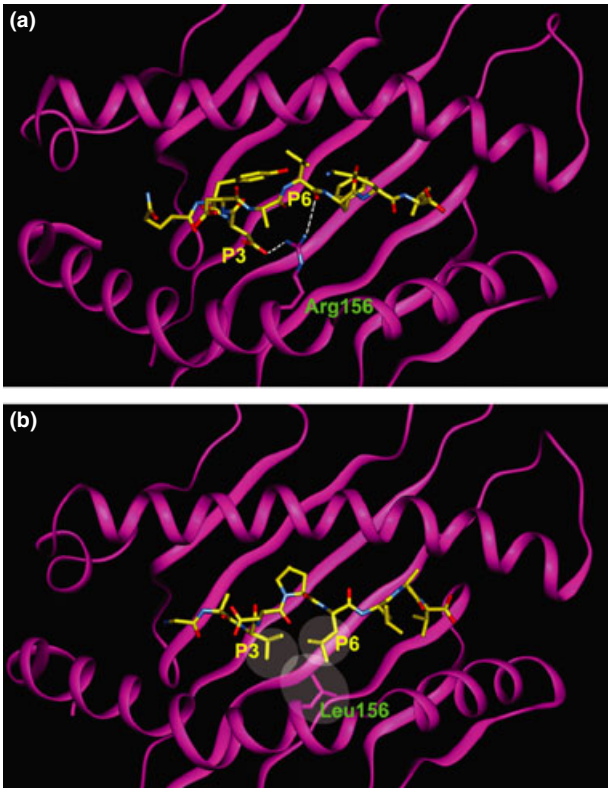

Figure 3 Modeling of structural alterations caused by mismatch of residue 156 in the human leukocyte antigen (HLA)-Cw molecule. (a) The crystal structure of HLA-Cw4 with a bound peptide (based on the coordinates of PDB ID 1IM9). The salt bridge between Arg156 and aspartic acid at P2 and the hydrogen bond between Arg156 and the backbone atom of P6 are depicted as dashed lines. (b) The crystal structure of HLA-Cw3 with a bound peptide (based on the coordinates of PDB ID 1EFX). The hydrophobic interactions involving Leu156 are depicted as overlapping ellipses.

Substitution of a leucine for arginine at position 156 of the HLA-Cw molecule was also an independent risk factor for TRM in this study. Residue 156 is located in the α2-helix and is involved in peptide binding. Leucine is a nonpolar aliphatic amino acid that favors hydrophobic interactions, whereas arginine is highly positively charged and favors ionic interactions with negatively charged peptide residues (e.g., aspartic acid), indicating that a mismatch at residue 156 would affect peptide binding. Position 156 of HLA-A [24] and HLA-B [9] molecules has been demonstrated to affect T-cell alloreactivity *in vitro*.

High-resolution typing of the A2 antigen has shown that most Korean individuals have A*0201 (15.7%), A*0206 (8.9%), and A*0207 (4.7%) [25]. This distribution is similar to that of the Japanese population studied by Kawase *et al.*, who found that multiple substitutions at position 9 were associated with severe GVHD. Our finding that only position 9 of HLA-A was associated with an increased risk of acute GVHD is possibly because of the small number of patients in our study. Nonetheless, the fact that the significance of residue 9 was replicated in this study reaffirms the importance of identifying nonpermissive mismatches. Moreover, Kawase *et al.* [15] showed that a change in amino acid at position 156 of the HLA molecule could increase the risk for acute GVHD in UHSCT. Although in the present study we were only able to demonstrate an association of position 156 of the HLA-Cw molecule with a significant risk for TRM (not acute GVHD), it is likely that amino acid substitution at position 156 of the HLA class I molecule, like substitution at position 9, increases the risk for severe acute GVHD as well as shorter OS in Korean pediatric recipients of UHSCT [15]. Support for this speculation will require further study of Korean patients using a larger number of UHSCT cases.

In conclusion, we found that a single amino acid substitution at position 9, primarily resulting from HLA-A*02 polymorphisms, was a major predictor of severe acute GVHD in Korean pediatric patients. We also present predictions for alloreactivity based on structural changes in the HLA molecule surrounding position 9. These findings indicate that reducing the risk of acute GVHD in Koreans will require that donor–recipient pairs be perfectly matched at high resolution at HLA-A, especially HLA-A*02.

Disclosure

No conflicts of interest.

Authorship

S-H Hwang: wrote the paper; S-W Cho, H-B Oh, C-L Chang and E-Y Lee: participated in the conception of this study; J-J Seo and J-H Lee: performed UHSCT; Y-S Heo: performed computational modeling; all authors checked and approved the final version of the paper.

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