の順に相関を示した。

３．ベトナム、インドネシア、フィリピン並びにAPBMT未参加国であるモンゴル、ミヤンマー、バングラデシュにおいて造血細胞移植を振興する目的で、新興国のための国際ワークショップをWHOとの共催でベトナム、ハノイで開催した。これら新興国の移植に対する熱意は強く、こうした国における移植振興のためのアルゴリズムとプログラムを策定中である。その中でも同種移植に必須のHLAタイピングシステムにつき、当研究班が主導的役割を果たして行くことが確認された。

D．考察

１．APBMT参加の各国/地域は人種、言語、宗教等において欧米とは比較にならないほど多様であるが、造血細胞移植というキーワードのもとに十分共同作業が可能であることが実証されたと考える。

２．移植法は、各国/地域とも国情に合わせて柔軟に行っていることが明らかになった。例えば、骨髄バンクを作る前に適正規模の臍帯血バンクを作るとか、更にその前に血縁者からのHLA不適合移植の可能性と限界を確立しておくとかのアプローチが、近い将来見られるようになろう。

３．いずれにせよHLAタイピングのシステム確立は、その国/地域で移植を始める上では必須であり、早急な技術供与が我が国からも必要であると思われる。

E.　結論

造血幹細胞移植は一過性には高額医療であるが、治癒率・社会復帰率が高く生涯医療費として眺めた場合、治療効率が良いので、むしろ新興国向けの医療である。ただ国力の問題で未だ実施に踏み切れない国もあるが、我々の経験を伝え、移植医療を速やかに振興するための経済効率も考慮に入れた施策をそれぞれの国が採用出来るようにすることは、ひいては我が国の本医療効率、医療経済にも益するものと考える。

F．健康危険情報

日本造血細胞移植学会との共同事業として行なわれている本事業を通じて得られたドナーに関わる健康危険情報は逐一同学会のホームページ上に開示される（一般からもアクセス可能）。又、非血縁ドナーに関しては骨髄移植推進財団の各種伝達機構により周知される。

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H. 知的財産権の出願・登録状況（予定を含む）

1．特許取得

なし

2．実用新案登録

なし

3．その他

なし

別紙４

研究成果の刊行に関する一覧表

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Acceptable HLA-mismatching in unrelated donor bone marrow transplantation for patients with acquired severe aplastic anemia

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We retrospectively analyzed the effect of HLA mismatching (HLA-A, -B, -C, -DRB1, -DQB1) with molecular typing on transplantation outcome for 301 patients with acquired severe aplastic anemia (SAA) who received an unrelated BM transplant through the Japan Marrow Donor Program. Additional effect of HLA-DPB1 mismatching was analyzed for 10 of 10 or 9 of 10 HLA allele-matched pairs (n = 169). Of the 301 recipient/donor pairs, 101 (33.6%) were completely matched at 10 of 10 alleles, 69 (23%) were mismatched at 1 allele, and 131 (43.5%) were mismatched at ≥ 2 alleles. Subjects were classified into 5 subgroups: complete match group (group I); single-allele mismatch group (groups II and III); multiple alleles restricted to HLA-C, -DRB1, and -DQB1 mismatch group (group IV); and others (group V). Multivariate analysis indicated that only HLA disparity of group V was a significant risk factor for poor survival and grade II-IV acute GVHD. HLA-DPB1 mismatching was not associated with any clinical outcome. We recommend the use of an HLA 10 of 10 allele-matched unrelated donor. However, if such a donor is not available, any single-allele or multiple-allele (HLA-C, -DRB1, -DQB1) mismatched donor is acceptable as an unrelated donor for patients with severe aplastic anemia. (*Blood.* 2011;118(11):3186-3190)

Introduction

BM transplantation from an unrelated donor (UBMT) is indicated as salvage therapy for patients with severe aplastic anemia (SAA) who fail to respond to immunosuppressive therapy. Early results of UBMT have not been encouraging because of a high incidence of graft failure and GVHD.[1-3] The Center for International Blood and Marrow Transplant Research (CIBMTR) reported the outcome of 232 patients with SAA who received an UBM transplant between 1988 and 1998.[3] The 5-year probabilities of overall survival (OS) were 39% and 36% after matched unrelated and mismatched unrelated donor transplantations, respectively. We previously reported the outcome of 154 patients with SAA who received an UBM transplant between 1993 and 2000 through the Japan Marrow Donor Program (JMDP).[4] The 5-year OS rate was 56% in that study.

In several recent studies, the effect of HLA high-resolution matching on outcome of patients who received an UBM transplant has been elucidated.[5-8] However, results have been derived primarily from an analysis of patients with hematologic malignancies. Major obstacles for UBMT are different between patients with hematologic malignancies and patients with SAA. Relapse is a main cause of death for patients with hematologic malignancies, and GVL effect may result in decrease of relapse rate. In contrast, graft failure is the main problem, and GVHD is the only negative effect for patients with SAA. Therefore, optimal HLA matching may be different between these 2 populations. Algorithms for donor selection derived from an analysis of patients with hematologic malignancies might not be useful for patients with SAA. However, a few studies have focused on the clinical significance of HLA-allele compatibility in patients with SAA.[2,4,9,10]

In a previous study, we analyzed the clinical significance of HLA allele mismatching in 142 patients with SAA, in whom data of high-resolution typing of HLA-A, -B, and -DRB1 were available.[4] Mismatching of HLA-A or -B alleles between donor and recipient was a strong risk factor for acute and chronic GVHD and OS, whereas mismatching of the HLA-DRB1 allele did not have a significant effect on patient outcomes. In the study from the National Marrow Donor Program, mismatching of HLA-DRB1 was the most crucial risk factor for OS.[2] These results indicate that better donor selection through high-resolution typing might result in improved outcome in patients with SAA who receive an UBM transplant. In fact, several recent studies showed a significantly improved outcome in patients with SAA who received and UBM transplant over time.[11,12] In particular, better HLA matching by high-resolution typing has been thought to contribute to these improvements.[4,9-11]

On the contrary, restricting BMT to donor-recipient pairs perfectly matched at high-resolution typing reduces the chance of undergoing UBMT for many patients. Therefore, strategies for selecting a partially HLA allele mismatched donor are required when a full matched donor cannot be identified. Here, we report a detailed analysis of outcome in 301 patients with SAA who were

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typed for HLA-A, -B, -C, -DRB1, -DQB1, and -DPB1 by a molecular technique and underwent UBMT through the JMDP.

Methods

Patients

From February 1993 to April 2005, 380 consecutive patients with acquired SAA received an UBM transplant through the JMDP. Patients with inherited AA, such as Fanconi anemia, and patients who received a BM transplant > 2 times were excluded. This study includes 301 patients in whom molecular analysis of HLA-A, -B, -C, DRB1, and -DQB1 were performed by DNA-based methods. HLA-DPB1 was analyzed in 299 of these patients. The previous study included 142 patients in whom molecular typing was performed only for HLA-A, -B, and -DRB1.

Characteristics of the 301 patients and donors are shown in Table 1. Briefly, patients (173 males and 128 females) were between birth and 64 years of age (median, 17 years of age). The median disease duration before BMT was 43 months (range, 4-436 months). All patients failed conventional immunosuppressive therapies and were considered candidates for UBMT. All patients or their guardians gave informed consent for transplantation and submission of the data to the JMDP.

Transplantation procedure

Characteristics of the transplantation procedures are also shown in Table 1. Patients underwent transplantations at individual centers following the local protocols for preconditioning regimens and GVHD prophylaxis. The various preconditioning regimens used by individual centers were classified into 5 categories: TBI or LFI + CY + ATG (n = 128), TBI or LFI + CY (n = 103), TBI or LFI + CY + Flu with or without ATG (n = 39), CY + Flu + ATG (n = 8), and others (n = 23). In 130 patients, CsA and MTX were used for prophylaxis against GVHD; 134 patients received FK instead of CsA. The remaining 35 patients received other GVHD prophylaxis. Ex vivo T-cell depletion was not used for any patient. The median number of infused nucleated marrow cells was 3.1×10^8/kg. One-half (n = 150) of the transplantations were performed before 2000, and 151 were done after 2001.

HLA typing and definition of mismatching

HLA matching between patients and donors was based on HLA serotyping according to the standard technique. Partial HLA-A and -B alleles and complete HLA-DRB1 alleles were identified as confirmatory HLA typing during the coordination process, and HLA-A, -B, -C, -DQB1, and -DPB1 alleles were retrospectively reconfirmed or identified after transplantation. Molecular typing of HLA-A, -B, -C, -DQB1, -DRB1, and -DPB1 alleles was performed by the Luminex microbead method (Luminex 100 system) adjusted for the JMDP and in part by the sequencing-based typing method. Mismatching was defined as the presence of donor antigens or alleles not shared by the recipient (rejection vector) or the presence of recipient antigens or alleles not shared by the donor (GVHD vector).

Definition of transplantation-related events

The day of engraftment was defined as the first day of 3 consecutive days on which neutrophil count exceeded 0.5×10^9/L. Patients who did not reach neutrophil counts > 0.5×10^9/L for 3 consecutive days after transplantation were considered to have primary graft failure. Patients with initial engraftment in whom absolute neutrophil counts declined to < 0.5×10^9/L subsequently were considered to have secondary graft failure. Acute GVHD was evaluated according to standard criteria in patients who achieved engraftment, and chronic GVHD was evaluated according to standard criteria in patients who achieved engraftment and survived > 100 days after transplantation.

Data collection and statistical analysis

Transplantation data were collected with the use of standardized forms provided by the JMDP. Patient baseline information and follow-up reports were submitted at 100 days and annually after transplantation. Analysis of patient outcome was performed with the date of last reported follow-up or date of death. Data were analyzed as of July 1, 2007.

Probability of OS and 95% confidence interval (95% CI) were estimated from the time of transplantation according to the Kaplan-Meier method. Cumulative incidence of neutrophil engraftment at day 42 was analyzed in the whole of patients by treating deaths until day 42 as a competing risk. Cumulative incidence of acute GVHD at day 100 was analyzed in patients who sustained engraftment by treating deaths until day 100 as a competing risk. Cumulative incidence of chronic GVHD at day 365 was analyzed in patients who sustained engraftment and survived longer than day 100 by treating deaths until day 365 as a competing risk. In univariate analysis, the log-rank test or Gray test was used to assess the significance of HLA allele mismatching on clinical outcomes. The Mann-Whitney *U* test was used to compare the median days of neutrophil engraftment. The chi-square test or Mann-Whitney *U* test was used to compare patient characteristics and transplantation procedures between the patient groups. All *P* values < .05 were considered statistically significant, whereas *P* values between .05 and .1 were considered as marginally significant.

Multivariate analyses were performed to assess the effect of HLA allele mismatching on the clinical outcome by Cox proportional hazard model (each mismatched group vs fully matched group; hazard risk = 1.0 as a reference group). Factors other than HLA mismatching included in the models were patient age, patient sex, donor age, donor sex, disease duration before BMT, infused cell dose, matching of ABO blood type, GVHD prophylaxis, and preconditioning regimens.

Results

HLA matching by DNA typing

Of the 301 recipient/donor pairs, 101 pairs (33%) were completely matched at HLA-A, -B, -C, -DRB1, and -DQB1 allele; 69 pairs (23%) were mismatched at 1 HLA allele; 59 pairs (20%) were mismatched at 2 HLA alleles; and 72 pairs (24%) were mismatched at ≥ 3 alleles (Table 2). The number and frequency of 1-allele and 2-allele mismatches in either GVHD or rejection vector or both vectors in each HLA allele were 55 (18.3%) and 7 (2.3%) in HLA-A allele, 32 (10.6%) and 2 (0.7%) in HLA-B allele, 130 (43.2%) and 10 (3.3%) in HLA-C allele, 68 (22.6%) and 5 (1.7%) in HLA-DRB1 allele, 80 (26.6%) and 13 (4.3%) in HLA-DQB1 allele, and 179 (59.5%) and 44 (14.6%) in HLA-DPB1 allele, respectively. Because the frequency of mismatching was too high at the DPB1 allele, analysis of DPB1 mismatching was separated from that of other alleles. In addition, because the number of single-allele mismatched pairs of HLA-A, -B, -C, -DRB1, and -DQB1 were too small for separate analyses, HLA-A and -B were grouped into the mismatch of the HLA-A or HLA-B allele (A/B) and HLA-DRB1 and -DQB1 into the mismatch of the HLA-DRB1 or HLA-DQB1 allele (DRB1/DQB1), respectively.

Survival

Of the 301 patients, 202 are alive at the time of analysis with an observation time from 3 to 128 months (median, 44 months) after transplantation. Five-year OS was 66.3% (95% CI, 60.7%-72.5%) in the whole population (supplemental Figure 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). Subgroup analyses were performed in 8 main subgroups (> 15 recipients) as follows: (1) complete match group (n = 101), (2) single locus (A/B) mismatch group (n = 20), (3) single (C) mismatch group (n = 42), (4) 2 loci (A/B + C) mismatch group (n = 20), (5) 2 loci (DRB1/DQB1) mismatch group (n = 19), (6) 3 loci (A/B + C) mismatch group (n = 15), (7) 3 loci (C + DRB1/DQB1) mismatch group (n = 29), and

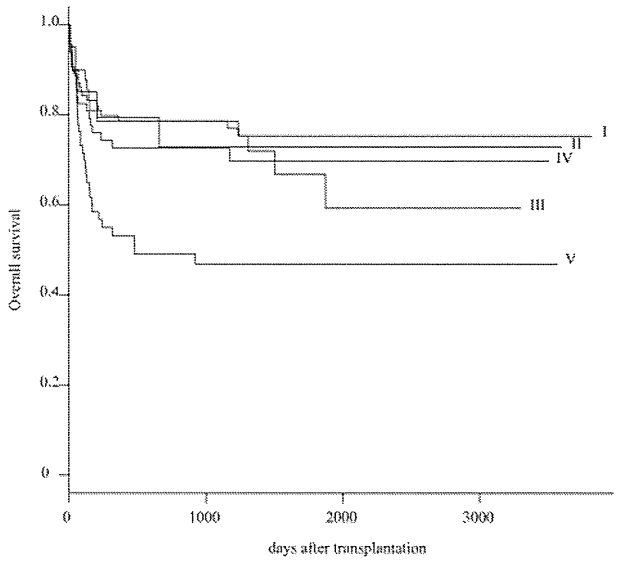

Figure 1. Kaplan-Meier estimates of OS in 5 HLA groups.

(8) 3 loci (A/B + C + DRB1/DQB1) mismatch group (n = 21). OS was significantly worse in the following groups than in the complete match group (75.2%): 2 loci (A/B + C) mismatch group (49.0%; $P = .022$), ≥ 3 loci (A/B + C) mismatch group (40.0%; $P = .002$), and A/B + C + DRB1/DQB1 mismatch group (56.1%; $P = .031$; supplemental Table 1).

On the basis of these primary results, 301 patients were reclassified into 5 subgroups: HLA complete match group (group I; n = 101), single-allele (A/B) mismatch group (group II; n = 20), single-allele (C or DRB1/DQB1) mismatch group (group III; n = 49), multiple-allele (restricted to C or DRB1/DQB1) mismatch group (group IV; n = 68), and others (group V; n = 63). The probability of OS at 5 years was 75.2% (95% CI, 84.8%-66.7%) in group I, 72.7% (95% CI, 96.7%-54.7%) in group II, 66.7% (95% CI, 85.1%-52.3%) in group III, 69.7% (95% CI, 82.6%-58.8%) in group IV, and 46.8% (95% CI, 61.7%-35.5%) in group V, respectively (Table 3; Figure 1). Survival rate was significantly inferior in group V than in group I ($P = .003$).

To avoid or minimize the effect of other HLA alleles mismatching, the effect of HLA-DPB1 mismatching was evaluated in group I (n = 101) and groups II + III (n = 69), independently. HLA-DPB1 was matched in 51 recipient/donor pairs (30%) and mismatched in 118 pairs (70%). Patient characteristics and transplantation procedures were not different between HLA-DPB1 matched and mismatched groups (supplemental Table 2). The probability of OS at 5 years in group I was equivalent between the HLA-DPB1 matched group (74.4%; 95% CI, 93.2%-59.4%) and the HLA-DPB1 mismatched group (75.7%; 95% CI, 87.2%-65.8%; $P = .894$; Table 4; Figure 2A). It was also equivalent in groups II + III (71.4%; 95% CI, 93.6%-54.5% in the HLA-DPB1 matched group and in the HLA-DPB1 mismatched group (67.1%; 95% CI, 85.6%-52.5%; $P = .826$; Table 4; Figure 2B). Multivariate analysis identified significant unfavorable variables as follows: recipient age (0-10 years: relative risk [RR] = 1.0; 11-20 years: RR = 4.092, $P = .002$; 21-40 years: RR = 3.970, $P = .004$; > 41 years: RR = 5.241, $P = .003$), conditioning regimen (Flu + CY + TBI/LFI ± ATG: RR = 1.0; CY + TBI/LFI: RR = 4.074, $P = .058$; others: RR = 6.895, $P = .013$), HLA mismatching (group I: RR = 1.0; group V: RR = 1.967, $P = .023$), donor sex (female: RR = 1.0; male: RR = 1.850, $P = .016$), and GVHD prophylaxis (FK + MTX: RR = 1.0; other: RR = 1.754, $P = .024$), blood type (ABO match or minor mismatch: RR = 1.0; major mismatch or bidirection: RR = 1.948, $P = .005$), and disease duration (< 7 years: RR = 1.0; > 7 years: RR = 1.540, $P = .084$; Table 5).

Engraftment

The cumulative incidence of neutrophil engraftment at day 42 was evaluated in 300 patients. It was 90.3% (95% CI, 93.7%-86.9%) in the whole population. Subgroup analyses showed that it was 93.0% (95% CI, 98.2%-87.8%) in group I, 90.0% (95% CI, 100%-74.6%) in group II, 89.8% (95% CI, 98.9%-80.7%) in group III, 92.6% (95% CI, 99.2%-86.0%) in group IV, and 84.1% (95% CI, 93.4%-74.8%) in group V ($P = .185$; Table 3). The median time to engraftment was 17 days in group I; 18 days in groups II, III, and IV; and 19 days in group V. Engraftment was marginally delayed in group V compared with group I ($P = .053$). Additional HLA-DPB1 mismatching did not affect the cumulative incidence of engraftment in the 10 of 10 and 9 of 10 matched groups, respectively (Table 4). In multivariate analysis, blood type (ABO match or

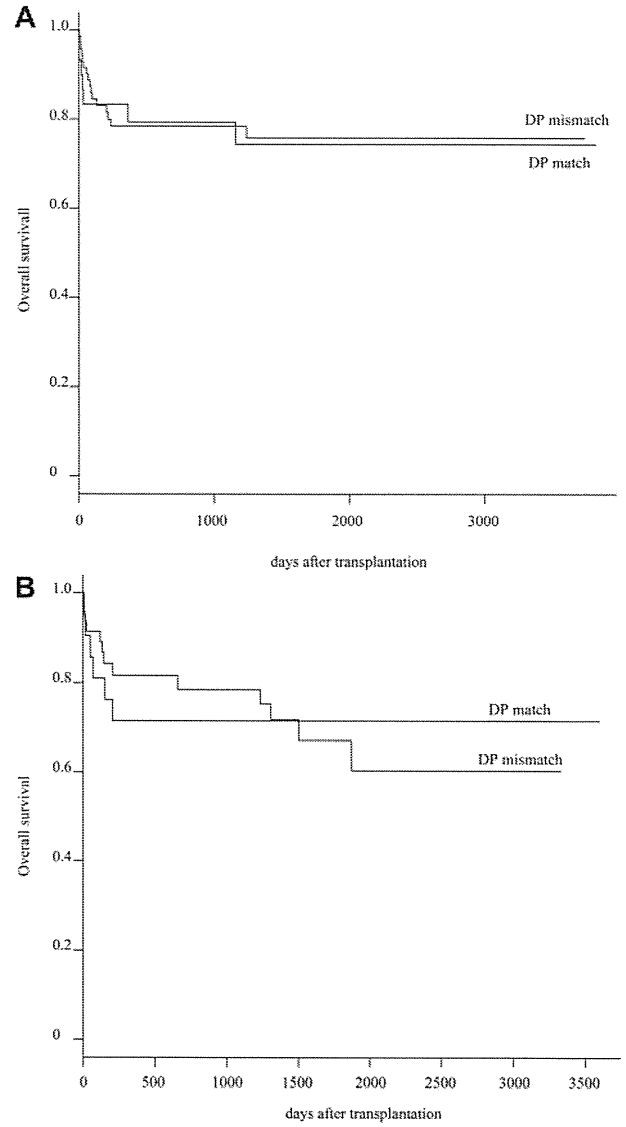

Figure 2. OS between HLA-DPB1 matched group and HLA-DPB1 mismatched group. (A) Difference of OS between HLA-DPB1 matched group and HLA-DPB1 mismatched group in 10 of 10 HLA allele matched pairs. (B) Difference of OS between HLA-DPB1 matched group and HLA-DPB1 mismatched group in 9 of 10 HLA allele matched pairs.

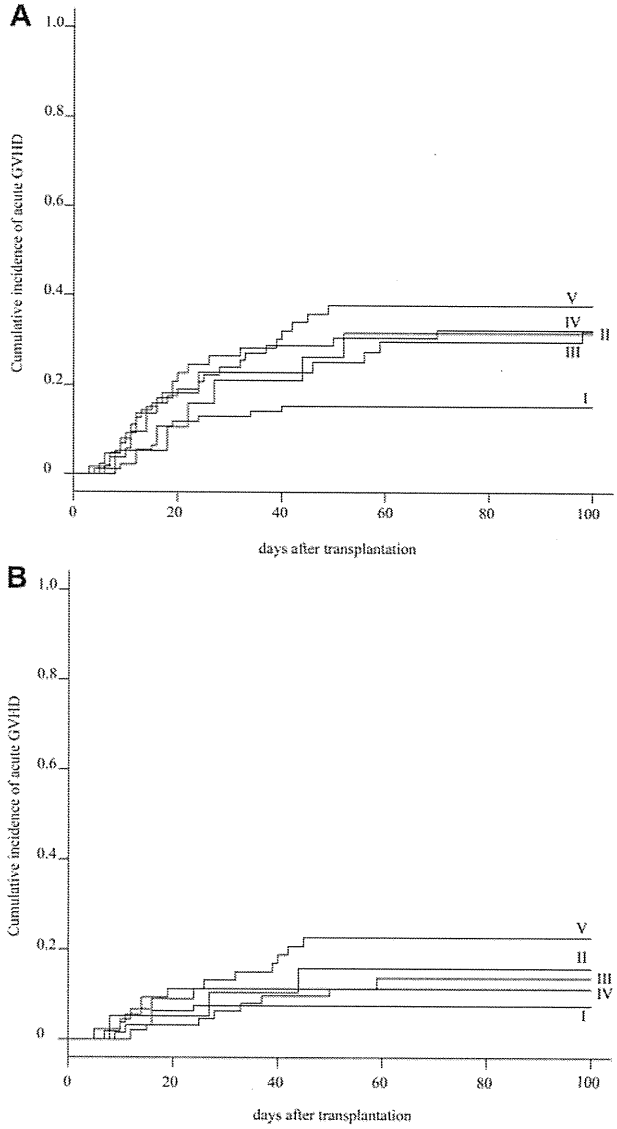

Figure 3. Cumulative incidence of acute GVHD. (A) Cumulative incidence of grade II-IV acute GVHD in 5 HLA groups. (B) Cumulative incidence of grade III-IV acute GVHD in 5 HLA groups.

minor mismatch: RR = 1.0; major mismatch or bidirection pair: RR = 5.102, P = .039) and HLA mismatching (group I: RR = 1.0; group V: RR = 4.906, P = .035) were significant risk factors for engraftment.

Acute GVHD

The cumulative incidence of acute GVHD at day 100 was evaluated in 272 patients. The cumulative incidence of grade II-IV and grade III-IV acute GVHD was 27.2% (95% CI, 32.5%-21.9%) and 12.9% (95% CI, 16.9%-8.9%) in the whole population, respectively (supplemental Figure 2). Subgroup analyses showed that the cumulative incidence of grades II-IV acute GVHD was statistically lower in group I (15.1%; 95% CI, 22.4%-7.8%) than in group V (37.7%; 95% CI, 50.9%-24.5%; P = .037), and marginally lower than in group III (31.8%; 95% CI, 45.8%-17.8%) and group IV (31.7%; 95% CI, 43.3%-20.1%; Table 3; Figure 3A). Whereas the cumulative incidence of grade III-IV acute GVHD was not significantly different among 5 groups: 7.5% (95% CI, 24.6%-0%) in group I, 15.8% (95% CI, 32.7%-0%) in group II, 13.6% (95% CI, 23.9%-3.3%) in group III, 11.1% (95% CI, 18.9%-3.3%) in group IV, and 22.6% (95% CI, 34.0%-11.2%) in group V (P = .139; Table 3; Figure 3B). Additional HLA-DPB1 mismatching evaluated in 155 patients did not affect the cumulative incidence of grade II-IV acute GVHD in the 10 of 10 and 9 of 10 matched groups, respectively (Table 4). Multivariate analysis showed that a significantly higher incidence of grade II-IV acute GVHD was associated with HLA mismatching (group I: RR = 1.0; group III: RR = 3.975, P = .002; group IV: RR = 3.334, P = .004; group V: RR = 3.665, P = .002). Other significant risk factors were the preconditioning regimen (Flu +CY + TBI/LFI ± ATG: RR = 1.0; TBI/LFI + CY: RR = 5.224, P = .003), and donor sex (female: RR = 1.0; male: RR = 1.844, P = .034; supplemental Table 3).

Chronic GVHD

The cumulative incidence of chronic GVHD at day 365 was evaluated in 232 patients. It was 24.5% (95% CI, 30.3%-18.7%) in the whole population. Subgroup analyses showed that it was comparable among the 5 HLA groups: 19.8% (95% CI, 28.8%-10.8%) in group I, 26.3% (95% CI, 49.3%-3.3%) in group II, 28.2% (95% CI, 43.3%-13.1%) in group III, 26.9% (95% CI, 39.2%-14.6%) in group IV, and 27.3% (95% CI, 42.1%-12.5%) in group V (P = .922; Table 3; supplemental Figure 3). HLA-DPB1 mismatching did not affect the cumulative incidence of chronic GVHD (Table 4).

Discussion

The survival rate in UBMT has increased substantially over the past 10 years in patients with SAA.[8-15] A 5-year survival rate of 90% has been reported in a small series of children.[16,17] A recent meta-analysis showed that detailed HLA-matching facilitated by DNA-based typing has contributed to the improved survival rate in patients with SAA who received an UBM transplant.[18] However, many patients with SAA who need hematopoietic stem cell transplantation do not have an HLA-complete matched donor. Our multivariate analysis indicated that among 4 HLA-mismatched groups, only HLA disparity of group V was a statistically significant unfavorable variable. We conclude that any type of HLA single-allele mismatch or multiple-allele mismatch within HLA-C and HLA class II (DRB1 or DQB1) is acceptable as an unrelated donor when an HLA complete match donor is unavailable.

We previously reported that HLA class I allele mismatching (HLA-A or -B) but not class II allele (HLA-DRB1) mismatching was a significant risk factor for survival when 6 alleles were analyzed.[4] HLA-A or -B mismatching pairs in the previous study were separated into 2 groups in the current study in which 10 alleles were analyzed. One group was a true single-allele mismatching pair of HLA-A or -B alleles (group II), and another was a multiple-allele mismatching pair of HLA-A or -B plus HLA-C and/or class II HLA alleles (group V). Because HLA-C and -DQB1 alleles were not typed, this type of multiple-allele mismatching might be mistaken as a single-allele mismatching pair, which was the reason for the inferior outcome of HLA-class I mismatching pairs in our previous study.

As the same in our previous study, mismatching of HLA-DRB1 did not provide a significant impact on clinical outcome. An HLA-DRB1 mismatching pair was also classified into a true single-allele mismatching of HLA-DRB1 (group III) and HLA-DRB1 plus HLA-C and/or HLA-DQB1 mismatching pairs (group IV). Interestingly, multiple mismatching of group IV was not

associated with increased mortality, which may explain why mismatching of HLA-DRB1 did not have a deleterious effect in the previous study.

The effect of HLA-DPB1 mismatching was also evaluated in HLA complete matched pairs (n = 101) and single-allele mismatched pairs (n = 69). The importance of DPB1 matching in the UBMT setting has been mainly discussed in patients with hematologic malignancies. Although results were controversial in early reports, recent studies support a significant effect of DPB1 mismatching on the incidence of acute GVHD, disease relapse, and OS.[19-22] In a large dataset of the International Histocompatibility Working Group, there was a statistically significant higher risk of both grade II-IV and grade III-IV acute GVHD.[19] The increased risk of acute GVHD was accompanied by a statistically significant decrease in disease relapse, probably because of the GVL effect, which offset the deleterious effect of acute GVHD. Survival rate was significantly better in DPB1-matched transplantations in patients with standard-risk leukemia but not in advanced leukemia. Conversely, in the HLA-mismatched group, there was a significant survival advantage in DPB1 mismatched pairs.

We expected that DPB1 matching might be beneficial for patients with AA who do not need the GVL effect. However, clinical outcomes, including incidence of acute GVHD, were not affected by DPB1 mismatching. HLA-DPB1 typing may not be essential to the donor selection algorithm for patients with SAA. Indeed, HLA-DPB1 mismatching was observed in 74% of recipient/donor pairs, and it may be practically difficult to find HLA 12 of 12 matched donors.

In conclusion, this retrospective study confirms the importance of HLA matching between recipients and donors to improve the outcome of UBMT for patients with SAA patients. However, this study showed that only 33% of patients received transplants from an HLA 10 of 10 matched donor. The availability of unrelated hematopoietic stem cell transplants can be increased through the judicious selection of donors with HLA mismatches that do not substantially lower survival.

Authorship

Contribution: H. Yagasaki analyzed the data and wrote the paper; S. Kojima designed the research and analyzed the data; and H. Yabe, K.K., H.K., H.S., M.T., S. Kato, T.K., Y.M., and Y.K performed and supervised the research.

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Predictions in the Face of Clinical Reality: *HistoCheck* versus High-Risk HLA Allele Mismatch Combinations Responsible for Severe Acute Graft-versus-Host Disease

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HLA polymorphism remains a major hurdle for hematopoietic stem cell transplantation (HSCT). In 2004, Elsner et al. proposed the *HistoCheck* Web-based tool to estimate the allogeneic potential between HLA-mismatched stem cell donor/recipient pairs expressed as a sequence similarity matching (SSM). SSM is based on the structure of HLA molecules and the functional similarity of amino acids. According to this algorithm, a high SSM score represents high dissimilarity between MHC molecules, resulting in a potentially more

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deleterious impact on stem cell transplant outcomes. We investigated the potential of SSM to predict high-risk HLA allele mismatch combinations responsible for severe acute graft-versus-host disease (aGVHD grades III and IV) published by Kawase et al., by comparing SSM in low- and high-risk combinations. SSM was calculated for allele mismatch combinations using the *HistoCheck* tool available on the Web (www.histocheck.org). We compared ranges and means of SSM among high-risk (15 combinations observed in 722 donor/recipient pairs) versus low-risk allele combinations (94 combinations in 3490 pairs). Simulation scenarios were created where the recipient's HLA allele was involved in multiple allele mismatch combinations with at least 1 high-risk and 1 low-risk mismatch combination. SSM values were then compared. The mean SSM for high- versus low-risk combinations were 2.39 and 2.90 at A, 1.06 and 2.53 at B, 16.60 and 14.99 at C, 4.02 and 3.81 at DRB1, and 7.47 and 6.94 at DPB1 loci, respectively. In simulation scenarios, no predictable SSM association with high- or low-risk combinations could be distinguished. No DQB1 combinations met the statistical criteria for our study. In conclusion, our analysis demonstrates that mean SSM scores were not significantly different, and SSM distributions were overlapping among high- and low-risk allele combinations within loci HLA-A, B, C, DRB1, and DPB1. This analysis does not support selecting donors for HSCT recipients based on low *HistoCheck* SSM scores.

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KEY WORDS: HLA, Mismatched, Unrelated donor, HSCT, *HistoCheck*

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) is the only curative therapy for many hematologic and nonhematologic disorders. The steady expansion of unrelated stem cell donor registries has facilitated finding a matched donor for many transplant candidates, particularly those with common human leukocyte antigens (HLA) alleles and haplotypes. However, the extensive polymorphism of HLA and the remarkable disparity in the distribution of alleles and haplotypes among individuals of different ethnic and racial backgrounds remain a major hurdle for access of many patients to HSCT. A number of studies have shown that donor/recipient matching for alleles at HLA-A, -B, -C, -DRB1, and -DQB1 loci lowers the risk of clinically severe acute graft-versus-host disease (aGVHD) [1-3]. Recently, HLA-DPB1 allele mismatches were also significantly associated with an increased incidence of GVHD [4-6]. When only HLA mismatched donors are available for a given patient, the challenge becomes determining which mismatch has a less deleterious impact on clinical outcomes. Bray and colleagues [7], in a comprehensive commentary, described the National Marrow Donor Program (NMDP) guidelines for unrelated HSC donor selection including the impact of mismatches at different loci on HSCT clinical outcomes. In 2004, Elsner and colleagues [8] proposed the *HistoCheck* Web-based tool to estimate the allogenicity of mismatches with a sequence similarity matching (SSM) concept. In this concept, an SSM score (ie, allogenicity index) is generated by rating the amino acid (AA) differences between HLA allelic products based on the position within the HLA molecule and the functional similarity of AA within proteins [9]. A high SSM score (also referred to as Dissimilarity Score [DSS]) represents high dissimilarity between HLA alleles resulting in a potentially greater deleterious impact on clinical outcomes. However, this algorithm has been challenged by 2 single-center analyses that could not associate higher SSM scores with aGVHD (in 26 patients) or in vitro T cell reactivity (in 74 patients) [10,11]. In the present study, we investigated the potential of SSM scores to predict high-risk HLA allele mismatch combinations responsible for severe aGVHD (grades III and IV) observed in a large cohort (5120 consecutive patients) of HSCT donor/recipient pairs. These allele combinations were observed in HSC transplants facilitated by the Japan Marrow Donor Program (JMDP) and published by Kawase et al. [12]. This investigation was conducted by comparing SSM scores in high-risk and low-risk allele combinations at HLA-A, -B, -C, -DRB1, and -DPB1 loci. No high-risk allele combinations at DQB1 locus met the predetermined level of statistical significance ($P < .005$) and thus SSM predictions were not evaluated in this study.

METHODS

Identification of High- and Low-Risk HLA Allele Mismatch Combinations

Significant high-risk HLA allele mismatch combinations were identified by retrospective analysis of 5210 consecutive registered patients who underwent transplantation through the JMDP. Patient characteristics, HLA matching and typing methods, and transplant procedures are described elsewhere [12]. Briefly, 15 mismatch allele combinations were identified as high-risk allele mismatch combinations (4 at HLA-A, 1 at HLA-B, 6 at HLA-C, 1 at HLA-DRB1, and 2 at HLA-DPB1 loci). Only 1-allele mismatched pairs in the same HLA locus were considered, and

adjusted by HLA locus matching in the other loci. These mismatch combinations were found to be associated with hish risk of severe aGVHD in a multivariable Cox regression model constructed with mismatch combinations and potential confounders. Confounders considered were sex, patient age, donor age, type of disease, risk of leukemia relapse, GVHD prophylaxis, and preconditioning. Each HLA mismatch combination was evaluated for each locus separately, for example, in the A*02:06-A*02:01 allele mismatch combination, the donor has HLA-A*02:06, recipient has HLA-A*02:01, and the other HLA-A allele of each donor and recipient was identical. This mismatch was compared with the HLA-A allele match. An allele mismatch combination was designated as a significant high-risk combination for severe aGVHD based on P values for hazard ratios (HR) for developing severe aGVHD of <.005. For example, the above mismatch combination was observed in 131 donor/recipient pairs and was associated with increased hazard of severe aGVHD (HR: 1.78, 95% confidence interval [CI]: 1.32-2.41, $P < .001$). Therefore, this was considered a high-risk mismatch allele combination. On the other hand, allele mismatch combinations with a 95% CI of the HR including 1.00 were considered low-risk combinations. For example, the combination A*24:02-A*24:20 was observed in 60 donor/recipient pairs and was not associated with increased risk of severe aGVHD (HR: 0.64, 95% CI: 0.32-1.30, $P = .225$). In addition, high-risk allele combinations observed in the context of 2 loci-linked mismatches such as (DRB1*14:03-DQB1*03:01) – (DRB1*14:01-DQB1*05:02) were excluded from this analysis because there are no explicit provisions regarding the utility of the *HistoCheck* algorithm in this setting.

SSM Score Calculation and Comparisons

SSM scores were calculated using the *HistoCheck* tool available online at http://www.histocheck.org/ according to the instructions posted on that Website. The averages and the distribution of SSM values were compared among high- and low-risk allele mismatch combinations in the same locus.

Simulation Scenarios

Hypothetical scenarios were created where the recipient's HLA allele was involved in multiple allele mismatch combinations with at least 1 high-risk mismatch and 1 low-risk mismatch. These scenarios were created to simulate clinical scenarios where multiple mismatched donors are considered for a given recipient in the absence of a matched donor. In these instances, SSM scores were compared among donors from both types of mismatch combinations. In addition, to investigate the impact of the direction of the allele mismatch, we assessed whether any of the identified 15 high-risk mismatch allele combinations associated with severe aGVHD were also a high-risk combination when considering the reverse direction of the mismatch between donor and recipient. For example, in the high-risk mismatch combination HLA-A*26:01/26:02, the recipient had the HLA-A*26:02 allele and the donor had the HLA-A*26:01. In this instance, we reviewed the list of the high- and low-risk mismatch allele combinations from the Kawase study to assess the risk associated with the reverse combination (ie, recipient is HLA-A*26:01 and donor is HLA-A*26:02). The same assessment was performed for all identified high-risk combinations.

Statistical Analysis

For the purpose of this analysis, low-risk mismatch allele combinations were used as a control group for SSM comparisons. SSM means and distributions were compared among aggregates of low-risk mismatch allele combinations and aggregates of high-risk combinations as well as individual high-risk combinations.

RESULTS

High- versus Low-Risk Allele Mismatch Combinations

In the HLA-A locus, the 4 high-risk allele mismatch combinations (observed 214 HSCT donor-recipient pairs) had a mean SSM of 2.39 (range: 1.04-4.30) compared to a mean SSM of 2.90 (range: 1.04-5.66) in 11 low-risk combinations (observed in 389 pairs). Individual high-risk combinations had the following SSM values: 1.04 (A*02:06-02:01), 2.87 (A*02:06-02:07), 1.36 (A*26:02-26:01), and 4.3 (A*26:03-26:01).

The 1 high-risk combination (B*15:01-15:07, observed in 19 pairs) at HLA-B locus had an SSM value of 1.06. The mean SSM for 4 B low-risk combinations was 2.53 (range: 1.74-2.81). In the HLA-C locus, the mean SSM for the 7 high-risk combinations (observed in 316 pairs) was 16.60 (range: 12.36-23.86) compared to a mean SSM of 14.99 (range 1.52-23.86) in 18 low-risk combinations (observed in 578 pairs). The 1 high-risk combination (DRB1*04:05-04:03, observed in 53 pairs) at HLA-DRB1 locus had an SSM value of 4.02. The mean SSM for 4 DRB1 low-risk combinations was 3.81 (range: 1.30-10.41). Finally, at the HLA-DPB1 locus, the mean SSM for the 2 high-risk combinations (observed in 120 pairs) was 7.47 (range: 6.98-7.95) compared to a mean SSM of 6.94 (range 1.21-12.87) in 36 low-risk combinations (observed in 1594 pairs).

A graphical representation of overall comparisons of SSM means and distributions among aggregates of low-risk mismatch allele combinations and aggregates of high-risk combinations as well as individual high-risk combinations across all loci is depicted in Figure 1. Without exception, in all these loci, the SSM

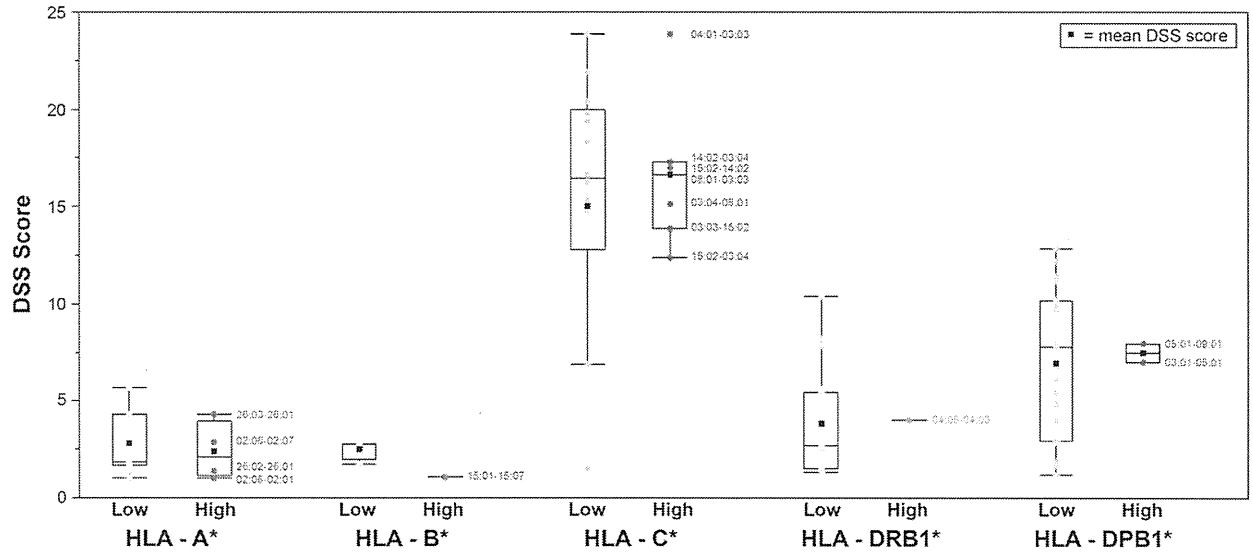

Figure 1. Comparison of SSM in high- versus low-risk mismatch allele combinations. SSM means are not significantly different, and SSM distributions are overlapping among high- and low-risk allele combinations within loci HLA-A, -B, -C, -DRB1, and -DPB1.

score for each high-risk combination fell within or below the range for the low-risk combinations. Two of 15 high-risk combinations had SSM scores that were lower than or equal to the lowest SSM score in any of the low-risk combinations in the corresponding locus. A detailed list of individual low-risk combinations is presented in the original publication by Kawase et al. [12].

Simulation Scenarios

To simulate clinical scenarios where multiple mismatched donors are considered for a given recipient, SSM values for high-risk and low-risk combinations including the same recipient allele were compared. The recipient allele in 13 of the 15 identified high-risk mismatch allele combinations (Loci HLA*A, C*, DRB1*, and DPB1*) was included in 1 or more low-risk allele mismatch combinations (Table 1). In 1 scenario, a recipient with an A*02:07 allele had 2 potential mismatched donors at this allele for either A*02:06 (high-risk mismatch allele combination) or A*02:01 (low-risk mismatch allele combination). SSM values for the high-risk and the low-risk combinations were 2.90 and 1.83, respectively, as one might expect. However, in another scenario, a recipient with an A*02:01 allele had 2 potential mismatched donors at this allele for either A*02:06 (high-risk mismatch allele combination) or A*02:07 (low-risk mismatch allele combination), the opposite was observed. SSM values for the high- and the low-risk combinations were 1.04 and 1.83, respectively. All simulation scenarios are summarized in Table 1.

Overall, no predictable SSM pattern of association with high- or low-risk mismatch allele combinations could be distinguished. In all but 2 instances (at HLA-A* and -DRB1* loci), at least 1 low-risk combination scored a higher SSM value than the high-risk mismatch combination including the same recipient allele.

Direction of the Allele Mismatch Combinations

None of the 15 high-risk combinations were high risk in both directions. In 10 of these combinations, when the direction of the allele mismatch was reversed between donor and recipient, there was no longer an association with severe aGVHD (Table 2). An example of such a unidirectional risk is the combination where the recipient has A*02:01 and the donor has A*02:06 (high risk) versus the recipient having A*02:06 and the donor having A*02:01(low risk). Both of these combinations have an SSM value of 1.04 according to *HistoCheck*.

DISCUSSION

Optimal effectiveness and safety of HSCT requires high degree of HLA allele matching between donors and recipients [7,13]. However, many patients who need HSCT do not have an HLA-matched donor, which lowers the probability of cure. Undoubtedly, a predictive algorithm for definition of "low-risk" HLA mismatches has the potential of broadening the use of mismatched donors and increasing the availability of unrelated donors. Several approaches have been proposed to identify such low-risk mismatches, with the simplest being a comparison between alleles based on the number of amino acid mismatches. However, prior reports have not found any evidence for selecting an allele with a lower number of AA substitutions of its allelic product [14,15]. Although hypothetically appealing, the notion that the lower the number of AA substitutions the more low-risk the mismatch is

Table 1. Simulation Scenarios for Comparison between SSM Scores among Multiple High- versus Low-Risk Mismatched Donors Potentially Available for a Patient with a Given HLA Allele

HLA Locus	MM Combination (Donor-Patient)	N	HR (95% CI)	*P*	Risk Group	SSM
A	02:06-02:01	131	1.78 (1.32-2.41)	<.001	High	1.04
	02:07-02:01	20	1.12 (0.42-3.02)	.81	Low	1.83
	02:06-02:07	27	3.45 (2.09-5.70)	<.001	High	2.90*
	02:01-02:07	28	0.83 (0.34-2.03)	.70	Low	1.83
C	04:01-03:03	42	2.81 (1.72-4.60)	.001	High	23.86
	08:01-03:03	80	2.32 (1.58-3.40)	.001	High	16.65
	07:02-03:03	18	2.16 (0.96-4.85)	.06	Low	21.90
	03:04-03:03	62	0.83 (0.41-1.68)	.61	Low	1.52
	03:04-08:01	69	2.34 (1.55-3.52)	.001	High	7.13
	03:03-08:01	76	1.07 (0.63-1.84)	.78	Low	16.65
	14:02-03:04	23	3.66 (2.00-6.68)	.001	High	17.30
	15:02-03:04	27	3.77 (2.20-6.47)	.001	High	12.36
	08:01-03:04	47	1.64 (0.98-2.76)	.06	Low	15.13
	01:02-03:04	12	1.85 (0.59-5.81)	.29	Low	18.30
	07:02-03:04	33	1.22 (0.58-2.59)	.59	Low	20.38
	03:03-03:04	83	1.08 (0.63-1.85)	.76	Low	1.52
	03:03-15:02	25	3.22 (1.75-5.89)	.001	High	13.88
	08:01-15:02	36	1.59 (0.79-3.21)	.19	Low	15.33
DRB1	04:05-04:03	53	2.13 (1.28-3.53)	.003	High	4.02*
	04:10-04:03	17	1.01 (0.32-3.21)	.98	Low	2.50
	04:06-04:03	30	0.99 (0.46-2.10)	.99	Low	1.45
DPB1	05:01-09:01	71	2.03 (1.30-3.16)	.002	High	7.95
	04:02-09:01	17	0.33 (0.04-2.36)	.27	Low	11.19
	02:01-09:01	47	1.37 (0.75-2.51)	.30	Low	9.98
	03:01-09:01	15	0.8 (0.19-3.22)	.75	Low	3.98
	04:01-09:01	11	0.9 (0.22-3.66)	.89	Low	12.87
	03:01-05:01	49	2.41 (1.49-3.89)	<.001	High	6.98
	06:01-05:01	13	2.5 (0.92-6.77)	.07	Low	7.95
	04:02-05:01	79	1.47 (0.90-2.40)	.12	Low	6.70
	02:02-05:01	41	0.43 (0.13-1.35)	.15	Low	6.18
	09:01-05:01	48	0.71 (0.29-1.73)	.46	Low	7.95
	04:01-05:01	29	0.73 (0.23-2.29)	.59	Low	5.46
	14:01-05:01	26	1.17 (0.48-2.84)	.73	Low	7.77

N indicates number of donor-patient pairs in whom the mismatch allele combination was observed; HR, hazard ratio of developing severe acutt graft-versus-host disease (aGVHD) compared to matched pairs as described in Kawase et al. [12]; SSM, sequence similarity matching; CI, confidence interval.

P values: for the corresponding estimated hazard ratio.

In all but 2 instances (marked with *), at least 1 low-risk combination scored a higher SSM value than the high-risk mismatch combination including the same recipient allele.

*The only 2 instances where SSM value was highest for the combination associated severe aGVHD.

challenged by 2 lines of evidence. First, a recent NMDP registry analysis suggested no difference in outcome when comparing antigen mismatch to allele level (high resolution) mismatch within the same antigen group [13]. Second, at least 2 reports have indicated that severe aGVHD can occur in the presence of only 1 AA mismatch between the donor and recipient allelic products [12,16].

In addition, at least 2 "epitope" based approaches have been proposed. One is based on serologically crossreactive groups (CREG) of antigens, and the other is based on comparisons of "functional epitope" structure through molecular viewing of the HLA structure and the determination of antibody-accessible polymorphic AAs (HLAMatchmaker) [17-19]. Neither of these 2 approaches have yielded predictions that were associated with a survival benefit in patients who underwent mismatched hematopoietic cell transplants from unrelated donors in registry analyses [20,21].

In the current analysis, we investigated whether *HistoCheck* SSM scores can predict 15 high-risk HLA allele mismatch combinations responsible for severe aGVHD observed in 5120 consecutive HSCT donor-recipient pairs facilitated through JMDP. It is noteworthy that the association between these 15 allele combinations and high risk for severe aGVHD has not yet been validated in an independent patient population; however, we believe that this association is reasonably robust because of the conservative approach and the rigorous statistical methods pursued in assigning these associations as described elsewhere [12]. SSM score comparisons were performed between high-risk and low-risk combinations at loci HLA-A, -B, -C, -DRB1, and -DPB1. Significant overlap exists between the high- and low-risk mismatches with respect to SSM scores. In all investigated loci, the SSM score for each high-risk combination fell within or below the range for the low-risk combinations, thus demonstrating the unreliability of SSM scores with respect to aGVHD risk. In addition, in 2 high-risk combinations (13%), SSM scores were less than or equal to the lowest SSM score in any of the low-risk combinations in the

Table 2. Mismatch Allele Combinations Where the Risk of aGVHD Depends on the Direction of the Mismatch

HLA Locus	MM Combination (Donor-Patient)	N	HR (95% CI)	P	Risk Group	SSM
A*	02:06-02:01	131	1.78 (1.32-2.41)	<.001	High	1.04
	02:01-02:06	138	1.23 (0.87-1.73)	.223	Low	
	02:06-02:07	27	3.45 (2.09-5.70)	<.001	High	2.87
	02:07-02:06	22	0.71 (0.23-2.24)	.571	Low	
	26:02-26:01	21	3.35 (1.89-5.91)	<.001	High	1.36
	26:01-26:02	24	0.64 (0.26-1.58)	.34	Low	
	26:03-26:01	35	2.17 (1.29-3.64)	.003	High	4.30
	26:01-26:03	34	1.37 (0.73-2.57)	.326	Low	
C*	08:01-03:03	80	2.32 (1.58-3.40)	.001	High	16.65
	03:03-08:01	76	1.07 (0.63-1.84)	.782	Low	
	03:04-08:01	69	2.34 (1.55-3.52)	.001	High	15.13
	08:01-03:04	47	1.64 (0.98-2.76)	.057	Low	
	04:01-03:03	42	2.81 (1.72-4.60)	.001	High	23.86
	03:03-04:01	31	1.73 (0.89-3.36)	.103	Low	
DRB1*	04:05-04:03	53	2.13 (1.28-3.53)	.003	High	4.02
	04:03-04:05	54	1.27 (0.74-2.20)	.379	Low	
DPB1*	05:01-09:01	71	2.03 (1.30-3.16)	.002	High	7.95
	09:01-05:01	48	0.71 (0.29-1.73)	.457	Low	
	03:01-05:01	49	2.41 (1.49-3.89)	<.001	High	6.98
	05:01-03:01	83	1.20 (0.75-1.94)	.434	Low	

N indicates number of donor-patient pairs in whom the mismatch allele combination was observed; HR, hazard ratio of developing severe acute graft-versus-host disease (aGVHD) compared to matched pairs as described in Kawase et al. [12]; SSM, sequence similarity matching; CI, confidence interval.
P values: for the corresponding estimated hazard ratio.
None of the 15 high-risk combinations were high risk in both directions. In 10 of these combinations, when the direction of the allele mismatch was reversed between donor and recipient, there was no longer an association with severe aGVHD.
*The only 2 instances where SSM value was highest for the combination associated with severe aGVHD.

corresponding locus. Furthermore, when simulation scenarios were constructed for recipients included in both high- and low-risk combinations, one would expect that high-risk mismatch combinations have higher SSM values compared to low-risk combinations of the same recipient allele. However, only 2 of the 15 high-risk combinations (13%) had higher SSM scores than alternative low-risk combinations potentially available for a patient with alleles included in these combinations. Interestingly, none of the identified 15 mismatch allele combinations were associated with high-risk for severe aGVHD in both directions. Ten of these combinations were no longer associated with severe aGVHD when the direction of the allele mismatch was reversed between the donor and the recipient. However, the SSM score remains unchanged because *HistoCheck* does not distinguish the direction of the allele mismatch between donor and recipient.

The lack of association between SSM and clinical data in our study may be attributed at least in part to the limitations acknowledged by the developers of the algorithm. Namely, underestimating the impact of single AA mismatches, not accounting for clinical data suggesting disproportionately larger impact of substitutions at given positions, particularly position 116, and assignment of positions based on crystallographic data of HLA-A2 for class I and of HLA-DR1 for class II alleles [8]. In addition, *HistoCheck* by design does not account for the direction of the allele mismatch, which has shown to be clinically significant [12].

Limitations of our study include relatively few numbers of high-risk allele mismatch combinations, not including mismatch combinations at the HLA-DQB1 locus, the potential for misclassification of some mismatch combinations as low risk because of relatively small number of subjects in subcategories, and inclusion of patients only from 1 ethnic background. Nevertheless, these results and previous reports do not support the utilization of *HistoCheck* predictions in unrelated donor selection [10,11].

In conclusion, safely maximizing access to mismatched HSCT unrelated donors requires a robust understanding of the rules that govern permissible HLA mismatching, and a better understanding of HLA-associated risks of GVHD. The lack of association between the *HistoCheck* predictions and all previously proposed prediction algorithms and the observed clinical outcomes strongly emphasizes the utmost importance of clinical validation of any prediction algorithm prior to its utilization in clinical patient care. Moving forward, it is prudent in evaluating any prediction algorithm to rely primarily on clinical correlations rather than simply putative biological plausibility.

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Frequent loss of HLA alleles associated with copy number-neutral 6pLOH in acquired aplastic anemia

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Idiopathic aplastic anemia (AA) is a common cause of acquired BM failure. Although autoimmunity to hematopoietic progenitors is thought to be responsible for its pathogenesis, little is known about the molecular basis of this autoimmunity. Here we show that a substantial proportion of AA patients harbor clonal hematopoiesis characterized by the presence of acquired copy number-neutral loss of heterozygosity (CNN-LOH) of the 6p arms (6pLOH). The 6pLOH commonly involved the HLA locus, leading to loss of one HLA haplotype. Loss of HLA-A expression from multiple lineages of leukocytes was confirmed by flow cytometry in all 6pLOH(+) cases. Surprisingly, the missing HLA-alleles in 6pLOH(+) clones were conspicuously biased to particular alleles, including HLA-A*02:01, A*02:06, A*31:01, and B*40:02. A large-scale epidemiologic study on the HLA alleles of patients with various hematologic diseases revealed that the 4 HLA alleles were over-represented in the germline of AA patients. These findings indicate that the 6pLOH(+) hematopoiesis found in AA represents "escapes" hematopoiesis from the autoimmunity, which is mediated by cytotoxic T cells that target the relevant autoantigens presented on hematopoietic progenitors through these class I HLAs. Our results provide a novel insight into the genetic basis of the pathogenesis of AA. (*Blood.* 2011;118(25):6601-6609)

Introduction

Acquired aplastic anemia (AA) is a rare condition associated with BM failure and pancytopenia.[1] A series of classic observations and experiments have unequivocally supported that the autoimmunity to hematopoietic stem/progenitor cells (HSPCs) critically underlies the pathogenesis of the BM failure in the majority of AA cases. According to the widely accepted model of immune-mediated BM failure, activated cytotoxic T cells (CTLs) that recognize an auto-antigen(s) presented on HSPCs through their class I HLA molecules have a major role in initiating the autoimmune reactions.[2-4] However, no definitive evidence exists that supports this model or the presence of such CTL repertoires. Moreover, little information is available about their target antigens or about the way by which they are recognized by effector T cells.

Another long-standing issue on AA is its close relationship with clonal hematopoiesis.[5,6] It was first suspected from an apparent overlap between AA and paroxysmal nocturnal hemoglobinuria (PNH)[7,8] and was also implicated by the frequent development of late clonal disorders in AA, such as myelodysplastic syndromes, PNH, or even acute myeloid leukemia (AML).[9-11] Clonal hematopoiesis can be explicitly demonstrated by conventional clonality assays at presentation in a substantial proportion of newly diagnosed typical AA cases.[12] Although it has been expected that the inciting autoimmune insult somehow confers selective pressures on the evolution of clonal hematopoiesis,[5] the exact mechanism for such immunologic selection or escape is still unclear.

The objectives of this study, therefore, were to characterize the clonal nature of the hematopoiesis that is maintained even under the severe autoimmune insult in AA, and to explore the genetic/immunologic mechanism that could underlie the pathogenesis of AA. To achieve these aims, we performed single nucleotide polymorphism (SNP) array-based analysis of genomic copy numbers and/or allelic imbalances in peripheral blood (PB) specimens obtained from 306 patients with AA. Initially, we found that AA patients frequently showed clonal/oligoclonal hematopoiesis that lost specific HLA alleles as a result of copy number-neutral loss of heterozygosity (CNN-LOH) of the 6p arms, which led us to further analyses of the contribution of 6pLOH(+) clones to residual hematopoiesis and a large-scale epidemiologic study on the HLA alleles that are over-represented in AA, involving a total of 6,613 transplants registered in the Japan Marrow Donor Program (JMDP).

Methods

Subjects

PB specimens from a total of 306 patients with AA were analyzed for the presence of genetic alterations using SNP arrays (see Figure 1). The clinical

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Table 1. Patient characteristics

	Newly diagnosed (n = 107)	Previously treated (n = 199)
Median age at diagnosis, mo (range)	64 (9-88)	24 (2-80)
Sex, male/female, no.	58/49	110/89
Severity of AA at onset, no. (%) of patients		
Severe	79 (74)	185 (93)
Nonsevere	28 (26)	14 (7)
History, mo, median (range)	19 (0.1-251)	51 (0.1-372)
Past treatment, no. (%) of patients		
ATG + CsA	—	39 (20)
CsA alone	—	51 (26)
Anabolic steroid alone	—	13 (7)
Unknown*	—	96 (48)

ATG indicates antithymocyte globulin; CsA, cyclosporine A; and —, not applicable.

*Information regarding previous therapies of 96 cases (from Japan Marrow Donor Program) was unavailable.

characteristics of these patients are summarized in Table 1 and supplemental Table 1 (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). Among the 306 patients, 107 were newly diagnosed and 199 were previously treated. Ninety-six patients received allogeneic BM transplantation from unrelated donors through the JMDP, and their HLA information was available from the JMDP. The other 210 were newly genotyped for HLA-A, -B, -C, -DRB1, -DQB1, and -DPB1 alleles as described elsewhere.[13] A total of 103 patients had been treated with anti–thymocyte globulin plus cyclosporine, cyclosporine alone, or anabolic steroids at the time of sampling. All patients and healthy persons provided their informed consent before sampling in accordance with the Declaration of Helsinki. The study protocol was approved by the ethics committee of the Graduate School of Medical Science, Kanazawa University and also by that of the Graduate School of Medicine, University of Tokyo.

Analysis of genomic copy numbers and detection of 6pLOH

Genomic copy numbers, as well as allele-specific copy numbers, were analyzed by using GeneChip 500K arrays (Affymetrix) as previously described.[14,15] Briefly, genomic DNA from AA patients and normal controls were analyzed on GeneChip 500K arrays separately. After adjusting several biases introduced during experiments, signal ratios of the corresponding probes between test (patient) and controls were calculated across the genome to obtain genome-wide copy numbers. Genetic lesions, including copy number gains and losses, as well as CNN-LOHs, were first detected using a hidden Markov model-based algorithm implemented in the CNAG software.[14,15] Known copy number variations were carefully excluded by referring to the Database of Genomic Variants (www.projects.tcag.ca/variation). CNN-LOH in 6p involving the HLA locus was more specifically and sensitively detected by statistically evaluating the mean differences in allele-specific copy numbers between heterozygous SNPs on 6p (N = ~ 1400) that were telomeric from the 5′-end of the HLA-A locus (rs1655927) and all non-6p heterozygous SNPs (N = ~ 105 000) using the Mann-Whitney *U* test with the R package (www.r-project.org). Possible false-positive findings arising from multiple testing involving the 306 samples were evaluated by maintaining the false discovery rate under 0.01 as previously described,[16] where the microarray data of 1000 JMDP donor specimens obtained from an ongoing whole genome association study (unpublished data) were used to calculate an empiric null distribution.[17,18]

Determination of the missing HLA alleles in 6pLOH(+) clones in patients with AA

The 500K SNP data of the 1800 JMDP donor-recipient pairs (JMDP dataset), together with their HLA genotyping information, was used to generate an HLA SNP haplotype table on the GeneChip 500K platform, which contains the consensus SNPs of the 3 major haplotypes (P1, P2, and P3) in Japanese subjects[18] and the SNP sequences of all observed HLA haplotypes complementary to P1 to P3 within the JMDP set (N = 1576; data not shown). To determine the missing HLA haplotype in each 6pLOH(+) patient, those "HLA" haplotypes were first selected from the aforementioned HLA haplotype table that were compatible with the observed HLA genotypes of that patient. Among these, a candidate haplotype was selected such that it contained the minimum number of SNPs that were incompatible with the patient's genotype. For each candidate haplotype, genomic copy numbers were inferred at the heterozygous SNPs along that haplotype using the circular binary segmentation algorithm,[19,20] which divided the haplotype into one or more discrete segments with different mean copy numbers. Finally, each copy number segment was thought to be "missing," when the alternative hypothesis ($H\alpha$: $S_i \neq \overline{S_i}$, for $^{\forall}{}_i$) was supported against the null hypothesis (H_0: $S_i = \overline{S_i}$, for $^{\forall}{}_i$) using the Wilcoxon signed rank test with a significance level of .05, where S_i represents the allele-specific copy number at the ith heterozygous SNP site within the segment of the candidate haplotype with $\overline{S_i}$ being the corresponding value for the complementary haplotype (supplemental Figure 1). Finally, for those HLA types that appeared more than 8 times among 6pLOH(+) cases, their contribution to the observed allelic loss of HLA haplotypes was evaluated by multivariate logistic regression analysis with stepwise backward selection.

Flow cytometry

Heparinized PB and BM were collected from the patients at diagnosis and/or after treatment. HLA-A expression on granulocytes, monocytes, B and T cells, and BM $CD34^+$ cells was analyzed by flow cytometry using a FACSCanto II instrument (BD Biosciences) with the FlowJo 7.6.1 program (TreeStar). The monoclonal antibodies used for this study are provided in supplemental Table 2.

Human androgen receptor assay

The human androgen receptor gene was amplified from genomic DNA of 23 female patients, including 3 6pLOH(+) patients, as described by Ishiyama et al[21] with some modifications. Clonality was assessed using an "S value" as a marker of skewing in granulocytes and T lymphocytes.

Association of HLA types with AA

A total of 6613 patients who had received allogeneic BM transplantation through the JMDP between 1992 and 2008 were investigated to see whether the HLA alleles frequently missing in CNN-LOH in 6p with the development of AA could represent risk alleles for the development of AA. Thus, the frequencies of patients with each of the candidate risk alleles (HLA-A*31:01, B*40:02, A*02:01, and A*02:06) and those having none of these alleles were compared between 407 patients with AA and those with other hematopoietic disorders (1827 with AML, 1606 with acute lymphocytic leukemia, 1014 with chronic myeloid leukemia, 825 with myelodysplastic syndrome, 566 with non-Hodgkin lymphoma, and 368 with other hematopoietic neoplasms; supplemental Table 3) by calculating the Fisher *P* values in the corresponding 2 × 2 contingency tables.

Results

Genetic lesions in AA detected by SNP array analysis

After excluding known or suspected copy number variations, a total of 50 genetic lesions were identified in 46 of the 306 (15%) PB specimens of our AA case series (Table 1; Figure 1). Among these by far, the most conspicuous was the recurrent CNN-LOH involving the 6p arm, which was detected in 28 cases as a significant dissociation of allele-specific copy number graphs in 6p regions using a hidden Markov model–based algorithm implemented in the CNAG software[2,14,15] (Figure 2A-2B). Of particular interest was that all CNN-LOH in 6p commonly affected the HLA locus, causing a haploid loss of HLA alleles and uniparental HLA expression. In some cases, the breakpoint of the 6pLOH was

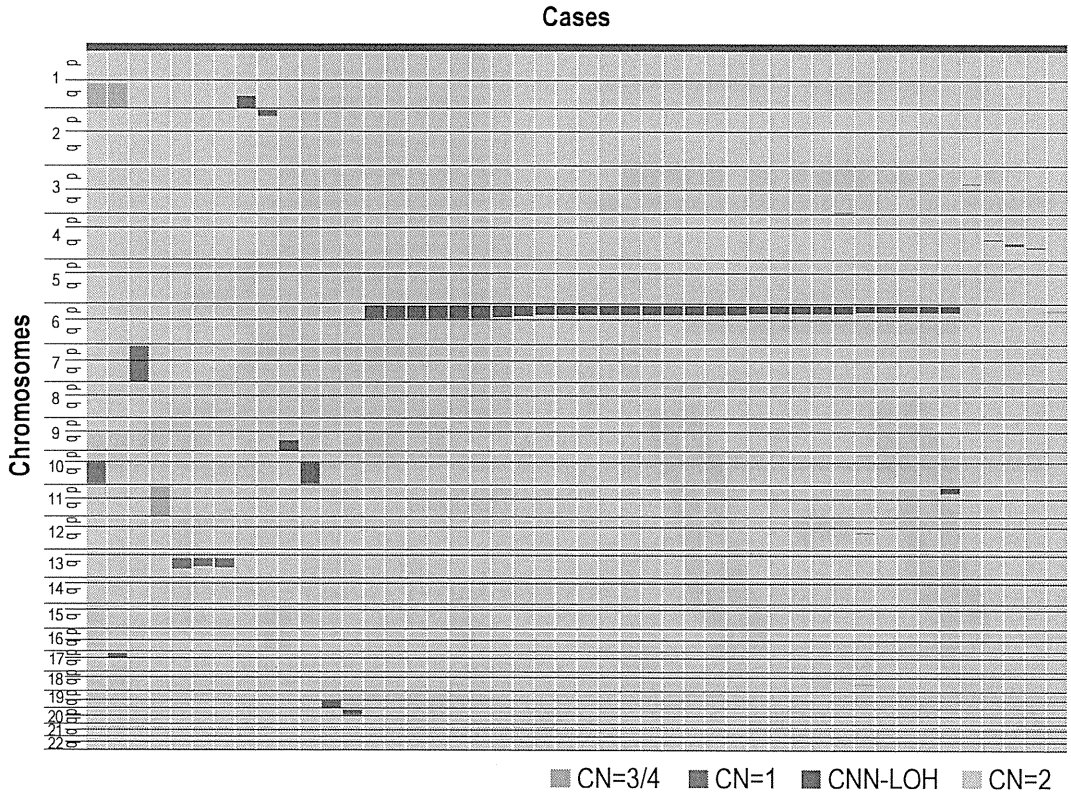

Figure 1. Copy number changes and allelic imbalances in 46 of the 306 AA cases. The copy number changes and allelic imbalances (or CNN-LOHs) in each case are summarized in the chromosomal order vertically for 46 AA cases with copy number abnormalities. Gains and losses, as well as CNN-LOHs, are shown in the indicated colors.

predicted to fall within the HLA locus (Figure 2B). These findings strongly indicated that the HLA locus was the genetic target of these 6pLOHs. Also supporting this was the finding that, in half of the cases, the dissociations in the allele-specific copy number graphs were gradually attenuated to the baseline over several mega base pair regions rather than showing a discrete breakpoint, indicating the presence of multiple 6pLOH(+) clones within a single case that had different breakpoints but still shared the same missing HLA alleles (Figure 2C). Moreover, the 6pUPDs existing only in a minor population were more sensitively detected by statistically evaluating the size of dissociation of allele-specific copy numbers in the 6p arm. With this improved statistical test, CNN-LOH in 6p was found in a total of 40 cases (13%; Figure 2D; supplemental Figure 2), where the false discovery rate was maintained at 0.01 to avoid too many false positive findings. In all 6pLOH(+) cases, substantial numbers of heterozygous SNP calls were retained within the affected regions, thus indicating that the CNN-LOHs in 6p were not constitutional but represented acquired genetic events only found in the affected subclones (Figure 1). Indeed, all 6pLOH(+) cases were shown to have "heterozygous" HLA alleles in high-resolution HLA typing of their PB (Table 2). Moreover, 6pLOH was not detected in the CD3-positive T cells in selected cases (cases 25 and 26, supplemental Figure 3). By quantitatively comparing the observed differences in allele-specific copy numbers in the 6pLOH segments with what were expected assuming 100% LOH(+) components, the 6pLOH(+) clones were estimated to account for 0.2% to 53.9% of the PB leukocytes (Table 2). The trend of the lower percentages of the 6pLOH(+) fraction in newly diagnosed patients compared with those in patients at remission was thought to reflect the fact that the former patients tended to have lower counts of granulocytes and monocytes, which were the predominant targets of 6pLOH (see supplemental Table 1).

The disease status of the 40 patients at the sampling was before treatment in 16 cases, during remission for 1 to 16 years after therapies in 15, and before BM transplantation for refractory disease in 9. All evaluable 6pLOH(+) AA cases responded to immunosuppressive therapy (IST) (23 of 23), whereas 101 of 126 evaluable cases with 6pLOH(−) responded ($P = .014$; Table 3).

Uniparental expression of HLA-A in multilineage hematopoietic cells

The genetic loss of one HLA haplotype in SNP array analysis was further confirmed by expression analysis of HLA-A in PB leukocytes using flow cytometry in 19 eligible cases with 6pLOH(+), in which the HLA-A alleles were heterozygous and fresh PB samples were available. Loss of expression of one HLA-A antigen was confirmed in all 19 6pLOH(+) cases (Figure 3A; supplemental Figure 4). The HLA-A missing cells in the PB were shown to have appeared shortly after the onset or before the initiation of treatments in 2 cases, and were confirmed to persist for 1 to 16 months (median, 6 months) in 14 patients (supplemental Table 1; supplemental Figure 5). The percentage of granulocytes lacking HLA-A antigens in the 2 patients who were responsive to IST remained almost the same during the convalescent period of 2 to 3 months (supplemental Figure 6). Importantly, uniparental expression of HLA-A alleles was detected in multiple cell lineages, including granulocytes, monocytes, B cells, and, to a lesser extent, in T cells. Moreover, uniparental HLA-A expression was demonstrated in BM $CD34^+$ cells in 5 patients whose BM samples were available for flow cytometry. All 5 patients possessed various proportions of BM $CD34^+$ cells (49.7%-71.3%), which had lost the expression of one