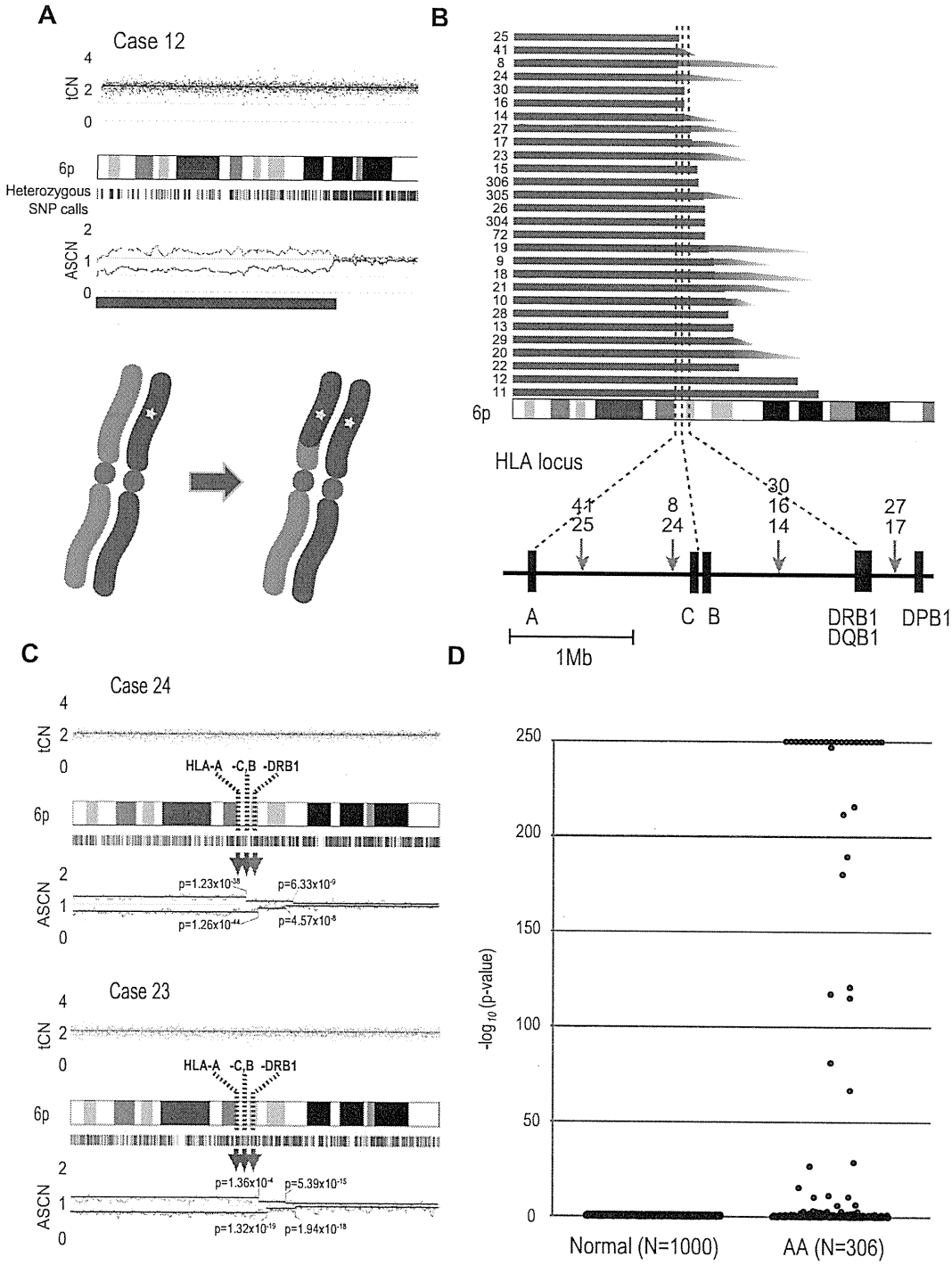


**Figure 2. Acquired 6pLOHs in AA patients that target the HLA locus.** (A) Typical CNAG outputs in SNP array analysis showing CNN-LOH (purple line) that appears as significant dissociation in allele-specific copy number graphs (red and green lines) from the baseline with normal total copy numbers (tCN; top panel). As a result of an allelic conversion, the affected segment causes LOH (* indicates 1; bottom panel). The "acquired" origin of these lesions is indicated by the retention of substantial numbers of heterozygous SNP calls (green bars below the chromatogram) that would otherwise mostly disappear. (B) The breakpoints of 6pLOHs found in a total of 28 AA cases, all involving the HLA locus in common. In more than half of cases (indicated by arrowheads in panel B), the exact location of the breakpoint was difficult to uniquely determine, where dissociation of the allele-specific copy number graphs continuously tapered along the 6p arm, indicating the presence of multiple 6pLOH(+) clones with common missing alleles (C). Indeed, the breakpoint containing regions are separated into multiple segments having significantly different copy numbers in the circular binary segmentation model, as indicated by solid lines with *P* values. Note that the most telomeric breakpoint is located within (case 24) or centromeric to (case 23) the HLA locus in each case. (D) A skewed distribution of the logarithm of *P* values in AA cases compared with normal persons. The *P* values were calculated in the Mann-Whitney *U* test, with which the difference in the mean allele-specific copy numbers between 6p and other chromosomal regions was evaluated (see "Analysis of genomic copy numbers and detection of 6pLOH"). A total of > 250 values are plotted as 250.

HLA-A antigen; and in each case, the missing HLA-A allele was identical to that in the PB leukocytes (Figure 3B). The uniparental expression of HLA-A in case 13 was also observed in the CD34$^+$ compartment of the archived BM specimen obtained 2 years before analysis (supplemental Figure 7). Together, these findings suggested that the 6pLOH involved early HSPCs and that the 6pLOH occurred at the level of long-term repopulating stem cells.

**Table 2. 6pLOH(+) AA cases and imputed allelic status of HLA alleles**

| UID | 6pUPD(+) fraction,* % | Missing alleles | | | | | | Retained alleles | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | A | B | C | DRB1 | DQB1 | DPB1 | A | B | C | DRB1 | DQB1 | DPB1 |
| 19 | 53.9 | 31:01††‡ | 40:02† | 03:04† | 12:01 | 03:01 | 05:01 | 24:02 | 52:01 | 12:02 | 15:02 | 06:01 | 05:01 |
| 12 | 51.8 | 02:01††‡ | 40:02† | 03:03 | 15:01 | 06:02 | 05:01 | 26:02 | 40:06 | 08:01 | 09:01 | 03:03 | 05:01 |
| 17 | 51.6 | 24:02 | 13:01 | 03:04† | 12:02 | 03:01 | 04:02 | 24:02 | 52:01 | 12:02 | 15:02 | 06:01 | 09:01 |
| 304 | 49.3 | 31:01††‡ | 55:02 | 01:02 | 12:02 | 03:01 | 41:01 | 24:02 | 07:02 | 07:02 | 01:01 | 05:01 | 04:02 |
| 11 | 48.0 | 02:06††‡ | 40:02† | 03:04† | 15:01 | 06:02 | ND‖ | 11:01 | 67:01 | 07:02 | 16:02 | 05:02 | ND‖ |
| 21 | 46.2 | 31:01‡§ | 51:01 | 14:02 | 14:05 | 05:03 | 03:01 | 24:02 | 07:02 | 07:02 | 01:01 | 05:01 | 04:02 |
| 24 | 44.9 | 31:01† | 40:02† | 03:04† | 11:01 | 03:01 | 02:01 | 24:02 | 40:06 | 08:01 | 09:01 | 03:03 | 05:01 |
| 26 | 44.3 | 31:01††§ | 40:01 | 03:04† | 04:05 | 04:01 | 03:01 | 26:03 | 52:01 | 12:02 | 15:02 | 06:01 | 09:01 |
| 27 | 43.5 | 02:06† | 40:02† | 03:04† | 04:10 | 04:02 | 02:01 | 11:01 | 52:01 | 12:02 | 15:02 | 06:01 | 09:01 |
| 10 | 42.1 | 31:01† | 40:02† | 03:04† | 08:03 | 06:01 | 02:01 | 24:02 | 51:01 | 14:02 | 09:01 | 03:03 | 02:01 |
| 8 | 40.8 | 02:06††‡ | 40:02† | 03:03 | 12:01 | 03:01 | 05:01 | 24:02 | 52:01 | 12:02 | 15:02 | 06:01 | 04:02 |
| 23 | 35.2 | 02:01† | 40:02† | 03:04† | 09:01 | 03:03 | 02:01 | 24:02 | 54:01 | 01:02 | 04:05 | 04:01 | 04:02 |
| 25 | 32.1 | 02:06††‡ | | | No LOH | | | 01:01 | | | No LOH | | |
| 9 | 23.5 | 02:06††‡ | 39:01 | 07:02 | 08:02 | 04:02 | 02:01 | 24:02 | 15:18 | 07:04 | 04:01 | 03:01 | 14:01 |
| 20 | 21.7 | 26:01‡ | 40:02† | 03:03 | 15:01 | 06:02 | 05:01 | 02:18 | 46:01 | 01:02 | 08:03 | 06:01 | 05:01 |
| 14 | 21.7 | 31:01††‡ | 51:01 | 14:02 | 09:01 | 03:03 | 05:01 | 24:02 | 52:01 | 12:02 | 15:02 | 06:01 | 09:01 |
| 22 | 20.6 | 02:01† | 39:01 | 07:02 | 08:03 | 06:01 | 05:01 | 24:02 | 52:01 | 12:02 | 15:02 | 06:01 | 09:01 |
| 18 | 17.6 | 02:01††‡ | 40:06 | 08:01 | 09:01 | 03:03 | 02:01 | 24:02 | 35:01 | 03:03 | 15:01 | 06:02 | 04:02 |
| 15 | 17.4 | 02:06† | 40:06 | 08:01 | 09:01 | 03:03 | 02:01 | 24:02 | 07:02 | 07:02 | 01:01 | 05:01 | 02:01 |
| 41 | 15.2† | 31:01††‡ | 35:01 | 03:03 | 09:01 | 03:03 | 03:01 | 26:01 | 39:01 | 07:02 | 08:03 | 06:01 | 05:01 |
| 28 | 12.8 | 24:02 | 54:01 | 01:02 | 01:01 | 05:01 | 04:02 | 24:02 | 52:01 | 12:02 | 15:02 | 06:01 | 09:01 |
| 29 | 11.7 | 31:01† | 40:02† | 03:04† | 15:01 | 06:02 | 02:01 | 24:02 | 54:01 | 01:02 | 04:05 | 04:01 | 05:01 |
| 305 | 10.3 | 02:06††‡ | 40:02† | 15:02 | 15:02 | 06:01 | 04:01 | 24:02 | 51:01 | 14:02 | 09:01 | 03:03 | 02:01 |
| 13 | 9.6 | 24:02‡ | 40:02† | 03:04† | 15:01 | 06:02 | 02:01 | 02:01‡ | 35:01 | 08:01 | 09:01 | 03:03 | 02:01 |
| 306 | 8.5 | 24:02‡ | 40:02† | 03:04† | 09:01 | 03:03 | 02:01 | 26:02 | 40:06 | 08:01 | 09:01 | 03:03 | 02:01 |
| 16 | 8.1 | 11:01 | 40:06 | 08:01 | | No LOH | | 24:02 | 46:01 | 01:02 | | No LOH | |
| 30 | 8.0 | 02:06† | 39:01 | 07:02 | | No LOH | | 24:02 | 40:06 | 08:01 | | No LOH | |
| 72 | 5.6 | 02:01† | 40:02† | 03:04† | 09:01 | 03:03 | 05:01 | 02:07 | 46:01 | 01:02 | 08:03 | 06:01 | 02:02 |
| 36 | 4.0 | 02:01††‡ | ND¶ | ND# | 15:02 | 06:01 | 09:01 | 24:02 | ND¶ | ND# | 15:02 | 06:01 | 09:01 |
| 124 | 3.5 | 24:02 | 40:02† | 03:04† | 12:01 | 03:01 | 02:01 | 24:02 | 52:01 | 12:02 | 15:02 | 06:01 | 09:01 |
| 223 | 2.8 | 31:01††‡ | 48:01 | 03:04† | 09:01 | 03:03 | 05:01 | 02:06† | 39:01 | 07:02 | 15:01 | 06:02 | 02:01 |
| 215 | 2.8 | 31:01† | 51:01 | 14:02 | 08:02 | 04:02 | 04:02 | 03:01 | 44:02 | 05:01 | 13:01 | 06:03 | 05:01 |
| 181 | 1.3 | 02:06† | 13:01 | 03:04† | 12:02 | 03:01 | 05:01 | 24:02 | 52:01 | 12:02 | 15:02 | 06:01 | 09:01 |
| 97 | 1.0 | 24:02 | 07:02 | 07:02 | 01:01 | 05:01 | 05:01 | 02:01† | 39:01 | 07:02 | 15:01 | 06:02 | 02:01 |
| 252 | 0.9 | ND** | 40:02† | 03:04† | 09:01 | 03:03 | 05:01 | ND** | 46:01 | 01:02 | 04:05 | 04:01 | 05:01 |
| 118 | 0.9 | 02:06‡§ | 40:02† | 03:04† | 08:02 | 03:02 | 05:01 | 24:02 | 52:01 | 12:02 | 15:02 | 06:01 | 09:01 |
| 298 | 0.8 | 24:02 | 40:02† | 03:04† | 15:01 | 06:02 | 05:01 | 24:02 | 52:01 | 12:02 | 15:02 | 06:01 | 09:01 |
| 188 | 0.7 | 24:02 | 52:01 | 12:02 | 15:02 | 06:01 | 09:01 | 02:01† | 52:01 | 12:02 | 11:01 | 03:01 | 05:01 |
| 291 | 0.7 | 31:01† | 51:01 | 14:02 | 15:01 | 06:02 | 02:01 | 24:02 | 40:01 | 03:04† | 11:01 | 03:01 | 05:01 |
| 196 | 0.2 | ND†† (A*02:06/24:02, B*35:01/51:01, C*03:03/15:02, DRB1*04:03/15:01, DQB1*03:02/06:02, DPB1*0:201/02:01) | | | | | | | | | | | |

UID indicates unique ID.
*The percentage of 6pUPD(+) fraction is derived from total peripheral blood leukocytes that include lymphoid as well as myeloid element.
†HLA types significantly deviated to missing alleles.
‡The allelic loss was confirmed by flow cytometry.
§The missing haplotype was determined by flow cytometry.
‖DPB1*04:02/05:01.
¶B*15:18/52:01.
#C*08:01/12:02.
**A*02:01/02:07.
††Missing allele was not determined because copy number changes in these segments were not statistically significant.

### Clonality of the HLA-missing granulocytes

The human androgen receptor-based clonality assays in granulocytes were performed in 3 6pLOH(+) and 20 6pLOH(−) patients, in which all 3 6pLOH(+) and 4 (20%) of the 6pLOH(−) patients showed evidence of clonality in granulocyte populations (supplemental Figure 8).

### Missing HLA alleles in 6pLOH

Given that the HLA is the genetic target of 6pLOH in AA, the missing HLA alleles in 6pLOH are of particular interest because in this context they are thought to be directly involved in the presentation of the target auto-antigens to CTLs and, therefore, to be critically important in the pathogenesis of AA. We determined the missing HLA alleles in each 6pLOH(+) AA patient by the haplotype imputation of HLA alleles based on the large data of HLA haplotypes observed in the JMDP set, followed by statistical evaluation of allele-specific copy numbers along the imputed haplotypes (Figure 4). The imputed haplotypes were confirmed in 4 cases by the family studies on the HLA. The allelic status was imputed at least partially in 39 of the 40 6pLOH(+) cases. The imputed results were consistent with the patterns of uniparental expression of HLA-A in flow cytometry in 18 cases with 6pLOH (Table 2; Figure 4), except for those in case 26, in which no valid SNP haplotype

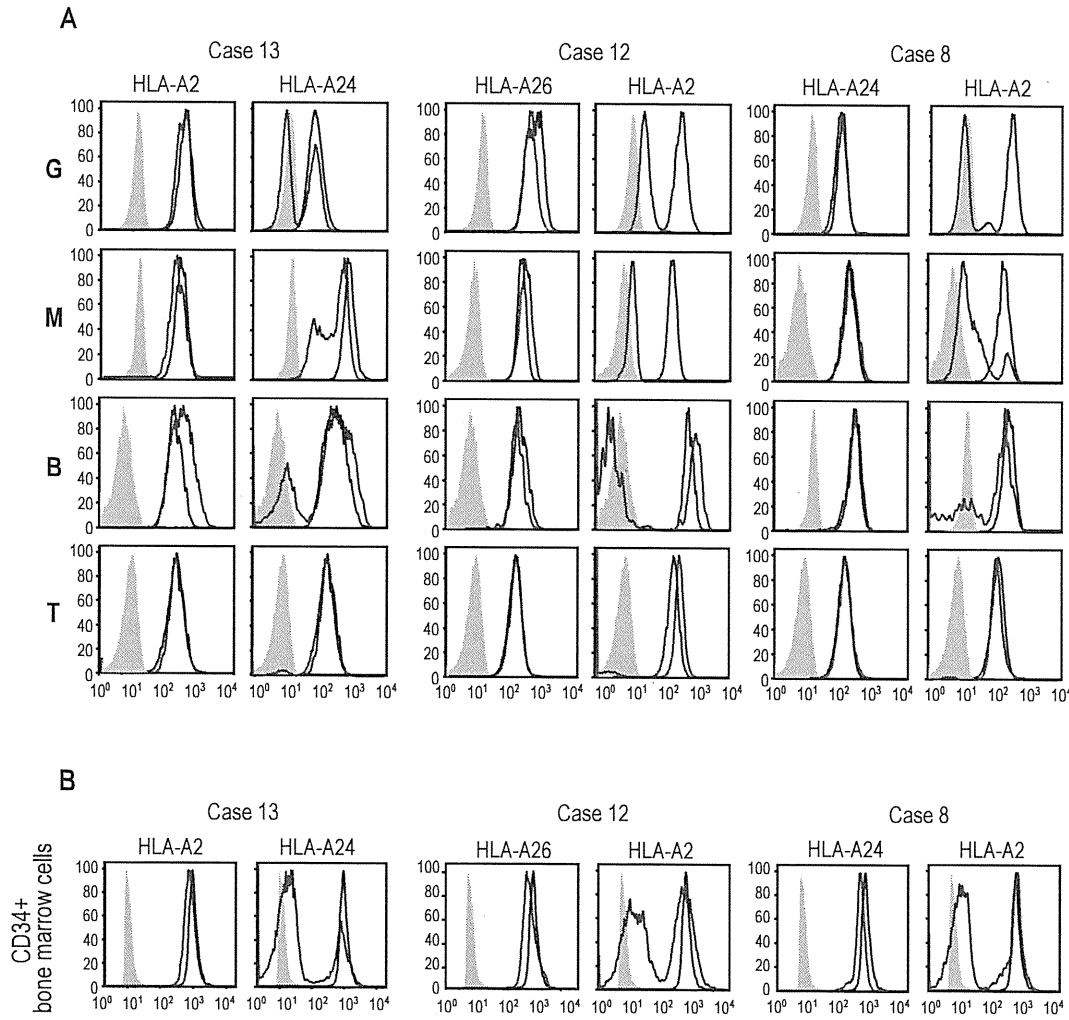


**Figure 3. Uniparental expression of HLA in AA cases with CNN-LOH in 6p.** Allele-specific expression of HLA-A antigens in AA specimens was examined by flow cytometry using monoclonal antibodies that specifically recognize the indicated HLA types (red lines), where leukocytes from healthy persons were used as a control (blue lines). (A-B) The uniparental expression of HLA-A antigens in PB leukocytes and BM $CD34^+$ cells obtained from 3 AA cases with CNN-LOH in 6p. Different leukocyte compartments were separately examined, including granulocytes (G), monocytes (M), B-lymphocytes (B), and T-lymphocytes (T).

around the HLA-A locus was identified and the status of HLA-A was determined by flow cytometry. The missing HLA alleles in 6pLOH(+) AA showed a conspicuous deviation to some selected HLA alleles, including HLA-A*31:01, B*40:02, C*03:04, and, to a lesser extent, HLA-A*02:01 and A*02:06. After the effects of linkage disequilibrium between individual HLA alleles were taken into consideration by multivariate analysis, 4 HLA alleles were shown to remain as the principal determinants of the missing haplotypes, HLA-A*31:01, B*40:02, A*02:01, and A*02:06 (supplemental Table 4).

### Over-representation of frequently missing HLAs in AA populations

Because these missing HLA alleles in 6pLOH could be involved in the pathogenesis of AA, we next tested whether these relevant HLA alleles are associated with the risk of the development of AA among the 6,613 JMDP registrants. As shown in Table 4, the 4 major missing HLA alleles, HLA-A*31:01, B*40:02, A*02:01, and A*02:06, were more frequently observed in AA cases compared with nonsignificant HLA alleles (ie, all HLA alleles other

**Table 3. Response rate (CR + PR) according to the Camitta criteria**

| | Newly diagnosed (n = 107) | | Previously treated (n = 103) | |
|---|---|---|---|---|
| | 6pLOH(−) (n = 91), no. (%) | 6pLOH(+) (n = 16), no. (%) | 6pLOH(−) (n = 88), no. (%) | 6pLOH(+) (n = 15), no. (%) |
| **Immunosuppressive therapies (all)** | 36/49 (73) | 11/11 (100) | 65/77 (84) | 12/12 (100) |
| ATG + CsA | 14/19 (74) | 7/7 (100) | 27/33 (82) | 5/5 (100) |
| CsA alone | 22/30 (73) | 4/4 (100) | 38/44 (86) | 7/7 (100) |
| Anabolic steroid alone | 0/0 (0) | 0/0 (0) | 7/11 (64) | 2/2 (100) |
| Unknown/not evaluable | 42 | 5 | 0 | 1 |

CR indicates complete remission; PR, partial remission; ATG, antithymocyte globulin; and CsA, cyclosporine A.

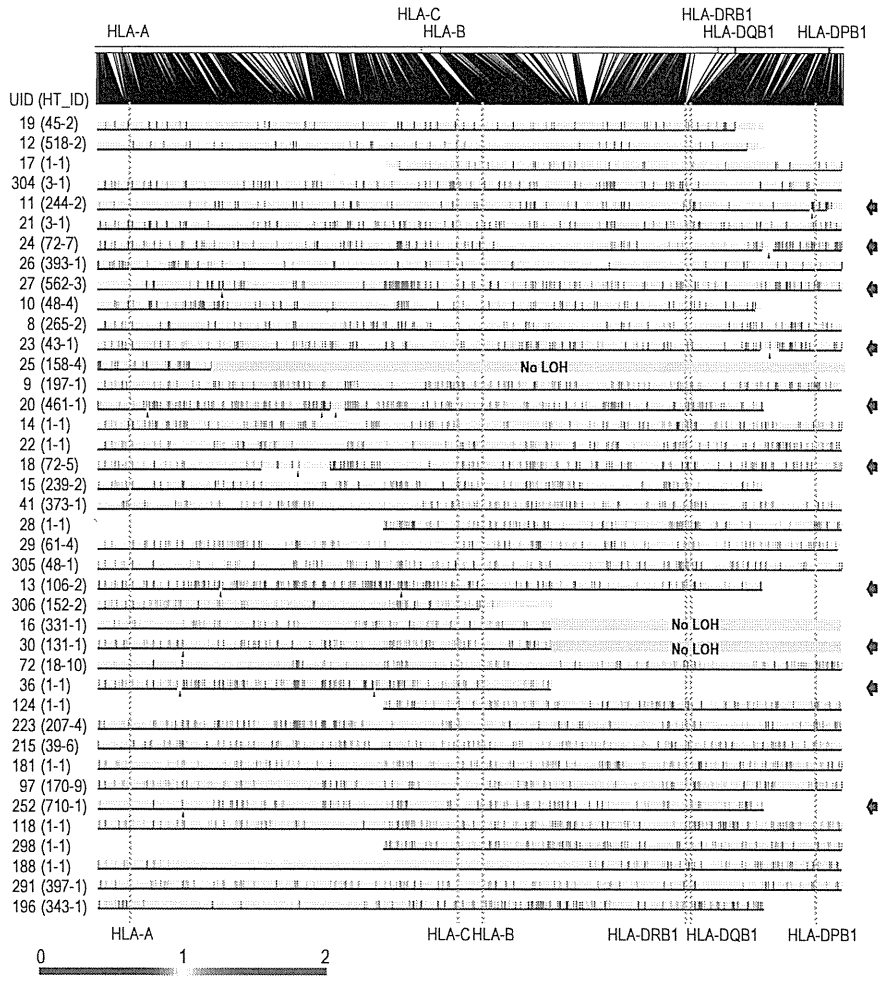


**Figure 4. Imputation of missing HLA haplotypes.** The observed allelic copy numbers at heterozygous SNP sites along each candidate SNP haplotype are color-coded as indicated at the bottom. Green bars showed the SNPs that are incompatible with the patient's genotype. Case IDs and haplotype ID (HT_ID) are indicated on the left. The locations of the 500K SNPs and HLA-A, C, B, DRB1, DQB1, and DPB1 are indicated in the figure. For each allele, genomic copy numbers were imputed using the circular binary segmentation algorithm. This divided each haplotype into one or more segments having discrete mean allelic copy numbers (blue arrows on the right). The positions of breakpoints are indicated by arrowheads. Finally, the mean allelic copy number of each segment was statistically compared with that of the corresponding segment on the other haplotype using the Wilcoxon signed rank test. Missing HLA haplotypes were determined based on the result of the statistic tests. Purple and blue lines indicated the retained and missing segments, respectively, whereas the allelic status was not determined statistically for those segments shown by green lines.

than these 4 alleles), where the odds ratios for the risk of the development of AA between each of these alleles and nonsignificant alleles were 1.87 (95% confidence interval [CI], 1.43-2.43) for A*02:01, 2.22 (95% CI, 1.70-2.90) for A*02:06, 1.37 (95% CI, 1.00-1.88) for A*31:01, and 1.95 (1.48-2.58) for B*40:02 (Table 4). The combined relative risk for all these alleles was 1.75 (1.42-2.17; $P = 1.3 \times 10^{-7}$).

## Discussion

The origin of clonal hematopoiesis in AA is a focus of long-standing disputes, in which a profoundly reduced hematopoietic stem cell pool and/or escape from the autoimmune insults have been implicated in the evolution of the clonal hematopoiesis in AA.[5,22,23] Our findings on 6pLOH in AA provide an intriguing insight not only into the underlying mechanism of the clonal hematopoiesis in AA but also into the origin of the autoimmunity that is responsible for the pathogenesis of AA. A recent study from the United States also reported 3 cases with 6pLOH.[24] With a sensitive detection algorithm, the presence of the 6pLOH(+) components was demonstrated in as many as 13% of typical cases with AA, and the evidence from the subsequent studies strongly indicated that the HLA genes are the genetic targets of 6pLOH in AA patients. First, the HLA locus was commonly and critically involved in all 6pLOHs found in AA. Second, some AA patients carried multiple 6pLOH(+) subclones with different breakpoints, but in all cases, the 6pLOH involved the HLA locus and occurred in a manner that targeted the same parental HLA allele. Moreover, particular class I HLA alleles were over-represented among 6pLOH(+) cases and consistently found in the missing haplotypes. Finally, many of these HLA alleles were shown to be tightly

**Table 4. Association of missing HLA alleles with AA in Japanese patients**

| Risk allele | AA (N = 407) | Other diseases (N = 6206) | Total (N = 6613) | $P$ ($\chi^2$ test) | Odds ratio (95% CI) (vs no risk alleles) |
|---|---|---|---|---|---|
| A*02:01 | 103 | 1173 | 1276 | $2.5 \times 10^{-6}$ | 1.87 (1.43–2.43) |
| A*02:06 | 100 | 957 | 1057 | $< 1.0 \times 10^{-7}$ | 2.22 (1.70–2.90) |
| A*31:01 | 58 | 899 | 957 | 0.048 | 1.37 (1.00–1.88) |
| B*40:02 | 86 | 938 | 1024 | $1.8 \times 10^{-6}$ | 1.95 (1.48–2.58) |
| All risk alleles | 268 | 3250 | 3518 | $1.3 \times 10^{-7}$ | 1.75 (1.42–2.17) |
| No risk alleles | 139 | 2956 | 3095 | — | — |

— indicates not applicable.

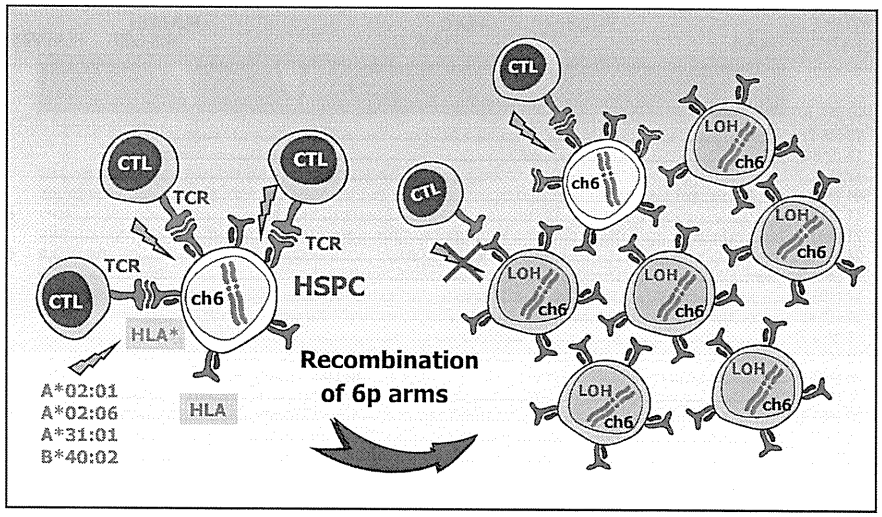


**Figure 5. A proposed mechanism for escape hematopoiesis in 6pLOH(+) AA.** In AA, the targets of CTLs are the HSPCs that present some auto-antigen through particular class I HLA molecules, including HLA-A*02:01, A*02:06, A*31:01, and B*40:02. In the presence of these autoimmune insults, the HSPCs that lose their expression of the antigen-presenting HLA molecule as a result of CNN-LOH in 6p would acquire a growth advantage over other HSPCs expressing the relevant HLA, leading to clonal outgrowth of the 6pLOH(+) progenies.

associated with the development of AA in Japanese patients in case-control studies using the large JMDP registry.

The conspicuous bias of the missing HLA alleles in 6pLOH to particular HLA types and the significant association of AA with those HLA types strongly suggest that the recurrent 6pLOH in AA is a phenomenon tightly related to the pathogenesis of AA rather than mere secondary event during the course of AA. Based on these observations, it is well reasoned that, in 6pLOH(+) AA cases, the autoimmunity to HSPCs is mediated by the CTLs that target the antigens presented via specific class I HLA molecules and that the 6pLOH(+) cells found in AA could be explained as escape hematopoiesis that survives the autoimmune insult by genetically deleting the relevant HLA species that are required for antigen presentation (Figure 5). These scenarios are further supported by the recent reports showing that the CNN-LOH in 6p provides a common mechanism of leukemic relapse after HLA haploidentical stem cell transplantations, in which leukemic cells that lost the mismatched HLA haplotype through CNN-LOH in 6p are thought to escape the immunologic surveillance of the engrafted donor T cells.[25,26] Importantly, it was experimentally demonstrated by immunologic assays that the 6pLOH(+) leukemic cells actually escaped GVL by CTLs, whereas 6pLOH(−) leukemic cells were effectively killed by the same CTLs. Although the immunologic targets of CTLs are different between relapse after haploidentical transplants (mismatched HLAs themselves) and AA (still unknown autoantigens presented on missing HLAs), the prominent similarities found in both cases further support that CNN-LOH in 6p confers an escape mechanism from autoreactive CTLs in AA.

In light of the above considerations, the chronologic behavior of the 6pLOH(+) components in PB is also interesting and worth discussing. Despite the assumption that 6pLOH is an effective escape mechanism from CTLs, the 6pLOH(+) stem cells were unable to repopulate the BM to cure AA, unless effective IST was applied (supplemental Figure 6). This is most probably explained by the presence of inflammatory cytokines, such as IFN-γ and TNF-α, which have also been shown to play an important role in the BM failure in AA and are thought to be responsible for the continued prevention of the 6pLOH(+) stem cells from fully expanding and reconstituting the BM (supplemental Figure 9A-B).[27,28]

When the autoimmune insults are removed after IST, no further injury of normal stem cells would occur. However, this does not necessarily mean the surviving normal stem cells can eventually outnumber the 6pLOH(+) stem cells over time. Note that, once the autoimmune insults disappear, nothing could biologically or immunologically discriminate a 6pLOH(+) stem cell from a 6pLOH(−) stem cell (supplemental Figure 9A). In particular, a 6pLOH(+) stem cell and a 6pLOH(−) stem cell will produce the same number of progeny on average and feed the same number of mature blood cells. As a consequence, once established, the predominance of 6pLOH(+) stem cells over 6pLOH(−) stem cells should be maintained, after the severely reduced hematopoietic stem cell pool has been re-expanded with removal of the inciting autoimmunity. It is also of note that the recovery of myeloid components after IST, which are affected more strongly by 6pLOH than lymphoid cells, contributes to an apparent increase in 6pLOH components in the SNP array analysis in PB (supplemental Figure 6A).

One of the most significant findings in the current study is the identification of the HLA alleles that are over-represented in the Japanese AA populations, including HLA-A*31:01, B*40:02, A*02:01, and A*02:06. All of these HLA alleles belong to class I MHCs and thus are thought to be involved in the antigen presentation to CTLs. This provides another prominent example, in which specific HLA types play a critical role in the development of a human disease, and the information about these particular HLA types provides a solid basis on which we can ultimately isolate the relevant antigens responsible for the development of AA. Of particular note, there was a previous report indicating that HLA-B*40:02 and A*02:06 were over-represented in PNH as well as AA, although the study size was much smaller than the current study.[29] Combined with our study, these findings support the hypothesis that AA and PNH are the different outcomes of the same immunologic insult[5,30] and may also provide the genetic basis of the high prevalence of AA and PNH in East Asia.[31,32]

In some AA cases, hematopoiesis could be maintained over years by the progenitors that escaped and survived the inciting autoimmune insult by deleting the target HLA through CNN-LOH in 6p. Given that the 6pLOH was detected in only 13% of our series, it is probable that other escape mechanisms may also operate to maintain hematopoiesis in AA. Indeed, clonality was clearly demonstrated in 20% of the 6pLOH(−) cases in the human androgen receptor assay study (supplemental Figure 8). In addition, our SNP array analysis also revealed a variety of clonal abnormalities in AA cases (Figure 1), although it is still open to question

whether these abnormalities actually represent the mechanism of escape hematopoiesis or were related to some neoplastic process. Further studies on the genetic basis of the escape mechanisms would contribute to our understanding of the molecular pathogenesis of AA.

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## Authorship

Contribution: S. Ohtake, S. Ogawa, and S.N. developed the concept of the study and supervised the project; T.K., S. Ohtake, and S.N. designed the experiments; T.K., A.S.-O., Y. Sato, Y. Mori, M.K., M.S., K.H., and Y. Sasaki performed the experiments and analyzed the data; K.K. performed high-resolution HLA typing; S.M. and Y. Morishima provided the information of JMDP donor-recipient pairs (JMDP dataset); T.K., A.S.-O., S. Ogawa, and S.N. wrote the paper; and all authors approved the final version of the manuscript.

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# A Genetic Variant in the IL-17 Promoter Is Functionally Associated with Acute Graft-Versus-Host Disease after Unrelated Bone Marrow Transplantation

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## Abstract

Interleukin IL-17 is a proinflammatory cytokine that has been implicated in the pathogenesis of various autoimmune diseases. The single nucleotide polymorphism (SNP), rs2275913, in the promoter region of the IL-17 gene is associated with susceptibility to ulcerative colitis. When we examined the impact of rs2275913 in a cohort consisting of 438 pairs of patients and their unrelated donors transplanted through the Japan Marrow Donor Program, the donor IL-17 197A allele was found to be associated with a higher risk of acute graft-versus-host disease (GVHD; hazard ratio [HR], 1.46; 95% confidence interval [CI], 1.00 to 2.13; $P=0.05$). Next, we investigated the functional relevance of the rs2275913 SNP. *In vitro* stimulated T cells from healthy individuals possessing the 197A allele produced significantly more IL-17 than those without the 197A allele. In a gene reporter assay, the 197A allele construct induced higher luciferase activity than the 197G allele, and the difference was higher in the presence of T cell receptor activation and was abrogated by cyclosporine treatment. Moreover, the 197A allele displayed a higher affinity for the nuclear factor activated T cells (NFAT), a critical transcription factor involved in IL-17 regulation. These findings substantiate the functional relevance of the rs2275913 polymorphism and indicate that the higher IL-17 secretion by individuals with the 197A allele likely accounts for their increased risk for acute GVHD and certain autoimmune diseases.

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## Introduction

Interleukin 17 (IL-17), also known as IL-17A, plays an important role in tissue inflammation, and is involved in the pathophysiology of autoimmune diseases and organ allograft rejection [1,2,3,4,5,6,7,8,9,10,11]. Moreover, several reports have shown that Th17 cells and IL-17 have a significant impact on the development of acute graft-versus-host disease (GVHD) in mouse models [12,13,14,15,16,17]. The 197A allele, which is the result of a single nucleotide polymorphism (SNP) rs2275913 (G197A) in the promoter region of the IL-17 gene, has been reported to be associated with the susceptibility to rheumatoid arthritis [18] and ulcerative colitis [19]. In our previous study, we demonstrated that the 197A allele was also implicated in the development of acute GVHD in patients who underwent unrelated myeloablative bone marrow transplantation (BMT) [20]. In the present study, we extended this investigation to a validation cohort of patients who received an unrelated BMT, including patients who underwent reduced intensity transplantation.

Interestingly, the rs2275913 SNP is located within a binding motif for the nuclear factor activated T cells (NFAT), which is a critical regulator of the IL-17 promoter [21]. Therefore, it is conceivable that the rs2275913 SNP exerts an effect on the transcriptional regulation of IL-17. The present study shows that allele 197A correlates with more efficient IL-17 secretion, and that this resulted from its higher affinity for NFAT.

## Materials and Methods

### Patients

IL-17 genotyping was performed on 438 recipients with hematological malignancies and their unrelated donors who underwent BMT through the Japan Marrow Donor Program (JMDP) with T-cell-replete marrow from HLA-A, -B, -C, -DRB1,

-DQB1, and -DPB1 allele-matched donors between January 1993 and December 2007. The HLA genotypes of patients and donors were determined by the Luminex microbead method as described previously (Luminex 100 System; Luminex, Austin, TX) [22,23]. Although the Luminex microbead method does not provide unambiguous HLA 4-digit typing for all genotypes, the JMDP has confirmed that this method can identify all HLA alleles with >0.1% frequency among the Japanese population [24].

None of the present patients had a history of any prior transplantation. The final clinical survey of these patients was completed by November 1, 2008. The diagnoses were acute myeloid leukemia (AML) in 149 (34%), acute lymphoblastic leukemia (ALL) in 109 (25%), myelodysplastic syndrome (MDS) in 78 (18%), malignant lymphoma (ML) in 55 (15%), chronic myeloid leukemia in 42 (10%), and multiple myeloma (MM) in 5 (1%; **Tables 1** and **2**). The recipients were defined as having standard risk disease if they had AML or ALL in the first complete remission, ML in any complete remission, CML in any chronic phase, or MDS. All others were designated as having high-risk disease. The myeloid malignancies include AML, MDS and CML, and the lymphoid malignancies included ALL, ML and MM. Cyclosporine- or tacrolimus-based regimens were used in all patients for GVHD prophylaxis, and anti-T cell therapy, such as anti-thymocyte globulin and *ex vivo* T cell depletion were not in any of the patients. All patients and donors gave their written informed consent at the time of transplantation to participate in molecular studies of this nature according to the declaration of Helsinki. This project was approved by the Institutional Review Board of Kanazawa University Graduate School of Medicine and the JMDP.

**Table 1.** The donor and recipient characteristics (first part).

| Variable | No. | Ratio |
|---|---|---|
| No. of cases | 438 | |
| Recipient age, years | | |
| Median | 39 | |
| Range | 1–70 | |
| Donor age, years | | |
| Median | 35 | |
| Range | 20–57 | |
| Year of transplant | | |
| Median | 2003 | |
| Range | 1993–2007 | |
| Recipient IL-17 genotype | | |
| G/G | 180 | 41% |
| A/G | 200 | 46% |
| A/A | 58 | 13% |
| Donor IL-17 genotype | | |
| G/G | 166 | 38% |
| A/G | 200 | 46% |
| A/A | 66 | 15% |
| Recipient sex | | |
| Male | 281 | 64% |
| Female | 157 | 36% |
| Donor sex | | |
| Male | 296 | 68% |
| Female | 142 | 32% |
| Donor/recipient sex | | |
| Sex matched | 299 | 68% |
| Female/male | 62 | 14% |
| Male/female | 77 | 18% |

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## IL-17 genotyping

Genotyping of IL-17 was performed using the TaqMan-Allelic discrimination method with the Assay ID C__15879983_10 (Applied Biosystems) as described in a previous report [20].

## Cells and reagents

Primers and oligonucleotides were obtained from Hokkaido Science Systems (Sapporo, Japan). The GST-NFATc1 construct [25] was a generous gift from Dr Shoichiro Miyatake. An NFATc binding consensus oligonucleotide (sc-2577) was purchased from Santa Cruz Biotechnology (Santa Cruz, California).

**Table 2.** Donor and recipient characteristics (second part).

| Variable | No. | Ratio |
|---|---|---|
| Disease | | |
| Acute myeloid leukemia | 149 | 34% |
| Acute lymphoblastic leukemia | 109 | 25% |
| Myelodysplastic syndrome | 78 | 18% |
| Malignant lymphoma | 55 | 13% |
| Chronic myeloid leukemia | 42 | 10% |
| Multiple myeloma | 5 | 1% |
| Disease stage | | |
| Standard risk | 178 | 41% |
| High risk | 260 | 59% |
| ABO matching | | |
| Major or/and minor mismatch | 160 | 37% |
| Major mismatch | 91 | 21% |
| Minor mismatch | 86 | 20% |
| Bidirectional | 17 | 4% |
| Missing | 8 | 2% |
| Conditioning regimen | | |
| Myeloablative | 325 | 74% |
| Reduced intensity | 113 | 26% |
| With total body irradiation | 333 | 76% |
| Pretransplant CMV serostatus | | |
| CMV positive recipient | 324 | 74% |
| Missing | 40 | 9% |
| GVHD prophylaxis | | |
| With cyclosporine | 190 | 43% |
| With tacrolimus | 248 | 57% |
| TNC, $\times 10^8$ per kg | | |
| Median | 4.6 | |
| Range | 0.1–316.8 | |

Abbreviations: TNC: total nucleated cell count harvested.

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## Cell preparation, cell culture and measurement of IL-17

Heparinized blood samples were collected from 54 healthy Japanese volunteers. The ages of the subjects (30 males and 24 females) ranged from 20 and 56 years (median, 32 years). Peripheral blood mononuclear cells (PBMCs) were isolated using a Ficoll-Hypaque gradient (Pharmacia Biotech, Uppsala, Sweden) and were induced to secrete IL-17 by culturing the PBMCs ($10^6$/well) in 24 well plates for 48 hours in RPMI 1640 supplemented with 10% fetal bovine serum in the presence or absence of 5 μg/ml phytohemagglutinin (PHA; Sigma) at 37°C in 5% $CO_2$. In some experiments, PBMCs ($10^6$ cells/well) were seeded in 48 well plates coated with anti-CD3 (2 μg/ml) and anti-CD28 (1 μg/ml) monoclonal antibodies (Miltenyi Biotec, Gladbach, Germany) to activate T cells selectively, and then were cultured for 48 hrs. The concentrations of IL-17 in collected supernatants were measured by an enzyme-linked immunosorbent assay (ELISA; Mabtech, Nacka Strand, Sweden). For some functional assays, PBMCs ($6\times10^6$ cells/well) were cultured in six well plates for 72 hrs in the presence of 5 μg /ml of PHA and 100 U/ml of IL-2, and are hereafter designated as PHA-PBMCs.

## Quantitative RT- PCR

RNA was extracted from resting or PHA-activated PBMCs using the high pure RNA isolation kit (Roche). Reverse transcription was carried out with the PrimeScript RT reagent/gDNA eraser kit (Catalog RR047A, Takara). Quantitative real time PCR was performed in a StepOne Plus PCR system (Applies Biosystems) using the SYBR premix ExTaq perfect Real Time (Catalog RR041A, Takara) with the IL-17 primers described previously [26] and a set of primers for human GAPDH (Takara). The relative IL-17 mRNA levels normalized to GAPDH were calculated by the ΔΔCT method using the relative expression function included in the StepOne v2.2 software program. The specificity of the PCR products was monitored by a melting curves analysis.

## Luciferase assay

The promoter region of the IL-17 gene was amplified from the genomic DNA of individuals homozygous for the rs2275913 SNP (A197A or G197G) by polymerase chain reaction (PCR) with forward 5′-ACGCGTGGATCTCAGGACAAACAGGTTC-3′ and reverse 5′-AAGCTTGACTCACCACCAATGAGGT-CTT-3′ primers as described previously [21]. The resultant fragments IL-17/197A or IL-17/197G were subcloned into the pGL3-enhancer vector at the MluI and HindIII sites (Promega, Madison, WI) to generate pGL3-197A-enhancer or pGL3-197G-enhancer constructs. The fragments were inserted with the same orientation, and their nucleotide sequences were confirmed by DNA sequencing. Equimolar amounts of the following reporter plasmids: pGL3-enhancer, pGL3-197A-enhancer and pGL3-197G enhancer designated thereafter as pGL3-Luc, IL-17/A-Luc IL-17/G-Luc respectively, were transfected into PHA-PBMCs using the Exfect transfection reagent following the manufacturer instructions (Takara Bio, Japan). To control for differences in the transfection efficiency, cells were cotransfected with a renilla reporter plasmid, pRL-TK. In some experiments, the cells were treated with anti-CD3 and anti-CD28 mAbs or with Cyclosporine A (CsA) 24 hours after the transfection, and were cultured for other 24 hours. The activity of both luciferase and renilla in the transfected cells was measured with the Dual Luciferase Reporter Assay System (Promega).

## Electrophoresis motility shift assay (EMSA)

Double stranded IL-17 probes, including those harboring G197A, were generated by annealing the following oligonucleotides to their complementary oligonucleotides: CAT TTT CCT TCA GAA G**A**A GAG ATT CTT CTA (197A allele) and CAT TTT CCT TCA GAA G**G**A GAG ATT CTT CTA (197G allele). These oligomers encompass nucleotides −180 to −210 upstream of the transcriptional start site, based on data in the human genomic DNA Gene bank accession number AY460616.1. Before annealing, both complementary oligonucleotides were separately biotin-labeled at their 3′ ends, using the 3′ end DNA labeling kit (Thermo Fisher Scientific, Suwanee, USA) following the manufacturer's recommendations. Nuclear extracts from PHA-PBMCs were prepared using a nuclear extraction kit (Thermo Fisher Scientific). The DNA/protein binding assay was performed with 10 μg of nuclear extracts using the Light Shift Chemiluminescent EMSA kit (Thermo Fisher Scientific) according to the manufacturer's recommendations with minor modifications as follows: In the DNA/NFAT recombinant protein assay 0.5% bovine serum albumin was included in the binding reaction and purified GST-NFAT-recombinant proteins were desalted using Zeba spin desalting columns (Pierce). The DNA/protein complexes were detected by streptavidin peroxidase and visualized in a Luminescent Image Analyzer LAS-4000 (Fujifilm, Tokyo, Japan).

## *Data management and statistical analysis*

The data were collected by the JMDP using a standardized report form. Follow-up reports were submitted at 100 days, 1 year and annually after transplantation. The pre-transplant cytomegalovirus (CMV) serostatus was routinely tested for only patients, but not the donors. Engraftment was confirmed by an absolute neutrophil count of more than $0.5\times10^9$/L for at least 3 consecutive days. After collecting the data, acute and chronic GVHD were diagnosed and graded based on the classically defined criteria [27,28], namely, acute GVHD develops within the first 100 days post-transplant while the manifestation of GVHD occurring after day 100 is classified as chronic GVHD. Data using the updated criteria for assessment of GVHD [29,30] were not available in our cohort. The overall survival (OS) was defined as the number of days from transplantation to death from any cause. Disease relapse was defined as the number of days from transplantation to disease relapse. Transplant-related mortality (TRM) was defined as death without relapse. Any patients who were alive at the last-follow-up date were censored. The data about the causative microbes of infections and postmortem changes in the cause of death, as well as the data on supportive care, including prophylaxis for infections and therapy for GVHD, which were given on an institutional basis, were not available for this cohort.

The analysis was performed using the Excel 2007 software program (Microsoft Corp, Redmond, WA, USA) and modified R (The R Foundation for Statistical Computing, Perugia, Italy) software programs [31,32], as described in a previous report [33,34]. The probability of OS was calculated using the Kaplan-Meier method and compared using the log-rank test. The probabilities of TRM, disease relapse, acute GVHD, chronic GVHD, and engraftment were compared using the Grey test [35] and analyzed using a cumulative incidence analysis [31], while considering relapse, death without disease relapse, death without acute GVHD, death without chronic GVHD, and death without engraftment as respective competing risks. The variables included the recipient age at the time of transplantation, sex, pretransplant CMV serostatus, disease characteristics (disease type, disease lineage and disease risk at transplantation), donor characteristics (age, sex, sex compatibility, and ABO compatibility), transplant characteristics (conventional or reduced-intensity conditioning [36], total body irradiation-containing regimen, tacrolimus versus

**Table 3.** The results of the univariate analysis of the association of IL-17 polymorphisms with the clinical outcomes after transplantation.

| Variable | No. | 5-year OS | *P* | 5-year TRM | *P* | 5-year relapse | *P* | II–IV acute GVHD | *P* | Chronic GVHD | *P* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Recipient IL-17 genotype | | | | | | | | | | | |
| G/G | 180 | 41% | | 29% | | 37% | | 35% | | 41% | |
| A/G or A/A | 258 | 50% | 0.59 | 28% | 0.48 | 30% | 0.10 | 30% | 0.21 | 40% | 0.78 |
| Donor IL-17 genotype | | | | | | | | | | | |
| G/G | 166 | 50% | | 29% | | 31% | | 27% | | 37% | |
| A/G or A/A | 272 | 43% | 0.22 | 28% | 0.71 | 33% | 0.77 | 38% | 0.03 | 42% | 0.18 |

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cyclosporine, and total nucleated cell count harvested per recipient weight [TNC]), and the year of transplantation. The median was used as the cutoff point for continuous variables. The chi-square test and the Mann-Whitney U test were used to compare the two groups. The Hardy-Weinberg equilibrium for the IL-17 gene polymorphism was determined using the Haploview software program [37].

Multivariate Cox models were used to evaluate the hazard ratio associated with the IL-17 polymorphism. The covariates found to be P≤0.10 according to univariate analyses were used to adjust the hazard ratio.

For both the univariate and multivariate analyses, the P values were two sided, and the outcomes were considered to be significant for P≤0.05.

## Results

### Transplant outcome according to the IL-17 genotype

The genotype frequencies of 197G/G, 197A/G and 197A/A were 41%, 46% and 13% in recipients, and 38%, 46% and 15% in donors. These were similar to previous reports [18,38], and were in accord with the Hardy-Weinberg equilibrium ($P = 0.88$).

The transplant outcomes according to the IL-17 genotype are summarized in **Table 3**. The presence of the 197A allele in the 197A/G or 197A/A genotype in the donor was associated with a significantly higher incidence of grades II to IV acute GVHD (38% vs. 27%, $P = 0.03$; **Fig. 1**), while no significant differences between the 197A/G genotype and the 197A/A genotype in the recipient were seen in the incidence of grades II to IV acute GVHD (38% vs. 36%, $P = 0.78$). The acute GVHD-related mortality did not differ between the donor 197A/G or 197A/A genotypes and the donor 197G/G genotype (2% vs. 2%, $P = 0.83$).

All of the factors found to be significant in the univariate analyses were included in the model. The 197A/G or 197A/A genotype in donors remained statistically significant in the multivariate analyses for the development of grades II to IV acute GVHD (**Table 4**). The 197A/G or 197A/A genotype in the donor resulted in a higher incidence of grades II to IV acute GVHD (hazard ratio [HR], 1.46; 95% confidence interval [CI], 1.00 to 2.13; $P = 0.05$) even when adjusted for the other factors in the models. The IL-17 genotype showed no significant effects on the OS, TRM or relapse (**Table 5**).

### The impact of the rs2275913 SNP on the secretion of IL-17

To substantiate the biological significance of the rs2275913 SNP, we first examined whether the different genotypes correlated with IL-17 secretion. PBMCs from a total of 54 healthy individuals (197G/G in 24, 197A/G in 24 and 197A/A in 6) were stimulated *in vitro* with PHA and the levels of secreted IL-17 were determined by ELISA. As shown in **Fig. 2A**, the 197A allele positive (197A/G or 197A/A genotype) PBMCs secreted significantly higher levels of IL-17 than the 197A allele negative cells (197G/G genotype). Similar results were obtained when T cells were selectively stimulated with anti-CD3 and anti-CD28 mAbs (data not shown). The quantitative RT-PCR analysis showed that PHA-stimulated PBMCs from donors harboring the 197A allele had a significantly higher IL-17 mRNA level than those from 197A allele negative donors (**Fig. 2B**). Of note, the IL-17 mRNA levels in unstimulated cells were very low, irrespective of 197A allele positivity, and resulted in no differences between the two groups (data not shown). Together, these results suggested that the sequence variant rs2275913 influences the response of the IL-17 gene promoter to factors released in response to T cell activation, thus leading to a differential IL-17 production.

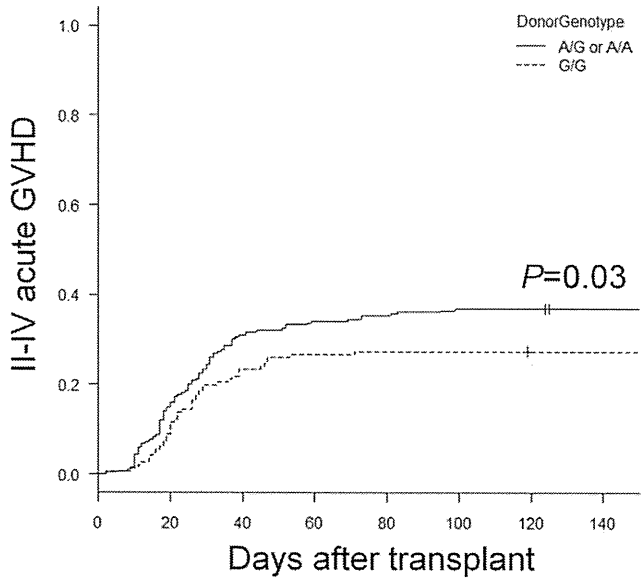


**Figure 1. The estimated cumulative incidence curve of grades II–IV acute GVHD according to the donor IL-17 genotype.** The solid line represents the donor 197A/G or A/A genotype, and the dashed line represents the donor 197G/G genotype.
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**Table 4.** The results of the multivariate analysis of the association of IL-17 polymorphisms with the GVHD after transplantation.

| | II–IV acute GVHD | | | Chronic GVHD | | |
|---|---|---|---|---|---|---|
| **Variable** | **Adjusted HR** | **95% CI** | **P** | **Adjusted HR** | **95% CI** | **P** |
| Recipient IL-17 genotype, A/G or A/A | 0.80 | 0.56–1.13 | 0.20 | 1.32 | 0.86–1.03 | 0.21 |
| Donor IL-17 genotype, A/G or A/A | 1.46 | 1.00–2.13 | 0.05 | 1.08 | 0.70–1.67 | 0.72 |

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### Functional relevance of the rs227513 SNP in the IL-17 gene promoter region

To address the functional significance of the rs2275913 SNP, reporter gene constructs containing the 197A and 197G alleles were prepared and used to transfect PHA-PBMCs. The results revealed that the insertion of the IL-17 promoter fragment consistently resulted in an augmentation of the luciferase activity compared with the construct without the fragments, however, the cells transfected with the IL-17/A-Luc construct had significantly higher luciferase activity than cells transfected with the IL-17/G-Luc construct (**Fig. 3A**). The differences in luciferase expression induced by these two constructs were more evident when the transfected cells were treated with anti-CD3 and anti-CD28 mAbs (**Fig. 3B**). Notably, treatment of the transfected cells with CsA abrogated the differences in luciferase activity induced by the two alleles, thus suggesting that the effects of the rs2275913 SNP on the regulation of the IL-17 promoter function are dependent on T cell activation.

### The 197A allele has a stronger interaction with NFAT than the 197G allele

To substantiate the functional relevance of the rs2275913 SNP, an EMSA assay was performed. Oligomers containing the 197A or 197G variants were biotin-labeled and allowed to interact with nuclear extracts derived from PHA-PBMCs. Despite the fact that the probes differed in just one nucleotide (A/G), the shift band corresponding to 197A probe-protein complexes was significantly more intense than that corresponding to 197G probe-protein complexes (**Fig. 4B**), thus suggesting that the two alleles have different affinities for some transcription factor in the nuclear extracts. A 50-fold excess of unlabeled IL-17 probes abrogated the formation of DNA-protein complexes, confirming the specificity of these interactions. Since NFAT has been demonstrated to play a crucial role in the regulation of IL-17 production [21] and the rs2275913 SNP maps to within the NFAT binding motif (**Fig. 4A**), DNA-protein interactions were subsequently carried out using recombinant NFAT instead of the nuclear extracts. The 197A probe-NFAT complexes displayed more a intense band than 197G probe-NFAT complexes (**Figs. 4C, D**), which were both completely eliminated by adding a competitor with a 50-fold excess of unlabeled IL-17 probes or an oligonucleotide containing a known NFAT target consensus in the binding reaction, thus suggesting that NFAT is the transcription factor which binds with differential affinities to the IL-17 probes.

## Discussion

The present study showed that the 197A allele of the IL-17 gene in the donors was associated with a higher risk of acute GVHD after unrelated fully HLA-matched BMT through the JMDP. The reason that this association did not significantly influence the TRM and OS might have resulted from the low incidence of acute GVHD-related mortality, regardless of the donor IL-17 genotype in the present cohort. Of note we have found that 197A allele positive PBMCs can produce IL-17 more efficiently than 197A allele negative PBMCs, which has not been reported so far, thus implying that the high inducibility of IL-17 might be correlated with the development of acute GVHD.

The role of IL-17 in the pathogenesis of acute GVHD remains unclear. In several mouse model experiments transfer of IL-17 producing cells induced acute GVHD [15,16,17], while in contrast there is a report [13] showing that donor IL-17 producing cells ameliorated acute GVHD. Host dendritic cells (DCs) are critical in the initiation of acute GVHD [39,40,41], thus leading to a hypothesis that IL-17 producing cells could modify the function of host DCs through unknown mechanisms. Direct interaction between IL-17 and host DCs may be supported by the fact that DCs expressed IL-17 receptors [1].

The IL-17 197A allele, which was associated with the higher production of IL-17 in comparison with the 197G allele, exhibited a higher promoter activity, as well as a higher affinity to transcriptional factor NFAT. The functional relevance of rs2275913 SNP was supported by the findings in our gene reporter assay showing that the higher promoter activity induced by 197A allele was stronger in the presence of T cell receptor activation by anti-CD3 and anti-CD28 treatment which is an upstream event in NFAT induction whereas in conditions leading to NFAT inactivation, namely CsA treatment, the differences in promoter activity induced by the 197A and 197G constructs were completely abrogated. Consistent with these observations, EMSA assay using recombinant NFAT proteins directly demonstrated a higher in affinity of 197A

**Table 5.** The results of the multivariate analysis of the association of IL-17 polymorphisms with the clinical outcomes after transplantation.

| | OS | | | TRM | | | Relapse | | |
|---|---|---|---|---|---|---|---|---|---|
| **Variable** | **Adjusted HR** | **95% CI** | **P** | **Adjusted HR** | **95% CI** | **P** | **Adjusted HR** | **95% CI** | **P** |
| Recipient IL-17 genotype, A/G or A/A | 1.01 | 0.71–1.42 | 0.97 | 1.43 | 0.84–2.41 | 0.87 | 0.75 | 0.49–1.16 | 0.19 |
| Donor IL-17 genotype, A/G or A/A | 1.29 | 0.90–1.84 | 0.16 | 1.31 | 0.75–2.31 | 0.34 | 1.24 | 0.79–1.93 | 0.35 |

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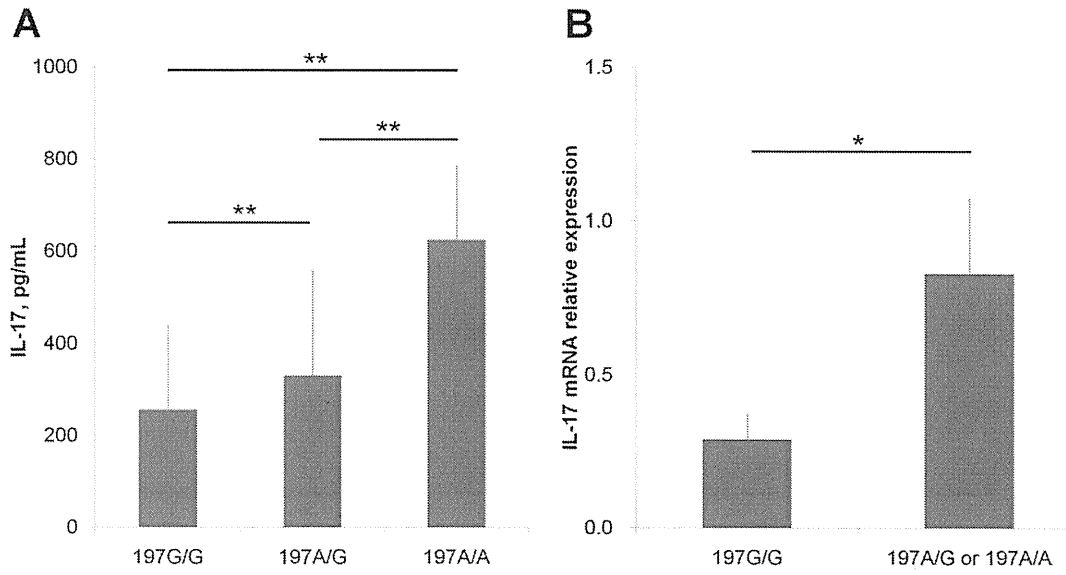


**Figure 2. The influence of the IL-17 SNP on IL-17 secretion by *in vitro* stimulated PBMCs.** (A) PBMCs from healthy individuals were stimulated for 48 hours in the presence of PHA or in 48 well plates coated with anti-CD3 and anti-CD28 mAbs. The values are expressed as the means +/− SD. (B) PBMCs from healthy donors (197A allele positive, n = 12 and 197A allele negative, n = 10) were cultured for 24 hrs in the presence of PHA. Total RNA was extracted, and the IL-17 mRNA levels were determined by quantitative RT-PCR and normalized to GAPDH. The data are the means +/−SD of triplicate measurements in each donor. *indicates $P<0.05$ and **$P<0.01$.
doi:10.1371/journal.pone.0026229.g002

allele. NFAT is a transcription factor crucial for the regulation of T cell-mediated IL-17 gene transcription [21], and the rs2275913 SNP is located in the promoter adjacent to the NFAT binding region (**Fig. 4A**). These findings suggest that the rs2275913 SNP plays a functional role in the promoter activity of the IL-17 gene through influencing the transcriptional activity of NFAT, affecting the production of IL-17 from T cells.

Previous studies have reported an association between the G197A SNP in the IL-17 promoter region and the susceptibility of the Japanese population to ulcerative colitis [19], as well as to rheumatoid arthritis in the Caucasian population [18]. The present study demonstrated that the 197A genotype is related to high IL-17 production, and the results of a previous Japanese study [19] showed that the 197A genotype was a risk factor for the

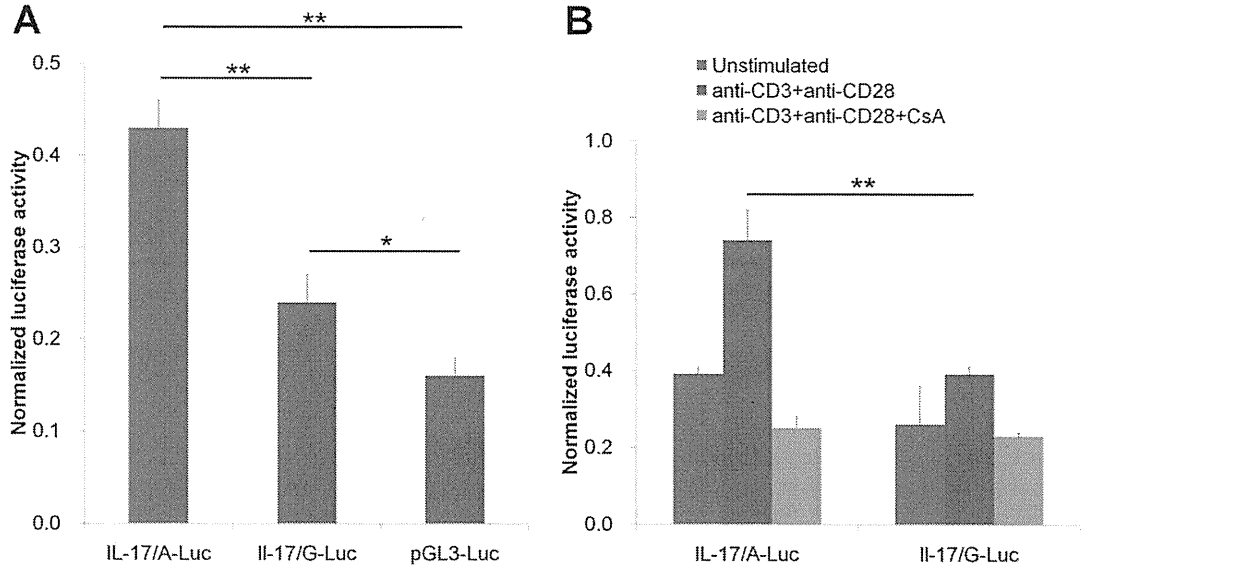


**Figure 3. The modulation of the reporter gene expression by the rs2275913 SNP.** (A) PHA-PBMCs were transfected with a luciferase expression vector alone (pGL3-Luc) or with a luciferase expression vector containing fragments of the IL-17 promoter with the 197A or 197G alleles (IL-17/A-Luc and IL-17/G-Luc). The transfected cells were cultured for 48 hr, and firefly luciferase activities were measured and normalized to Renilla luciferase. (B) The PHA-PBMCs were transfected as described above. Twenty four hours after transfection, the cells were treated with anti-CD3 and anti-CD28 mAbs or with CsA, and cultured for other 24 hr. The firefly luciferase activities were measured and normalized to Renilla luciferase. The values represent the normalized levels +/− S.E.M. from five independent experiments. *indicates $P<0.05$ and **$P<0.01$.
doi:10.1371/journal.pone.0026229.g003

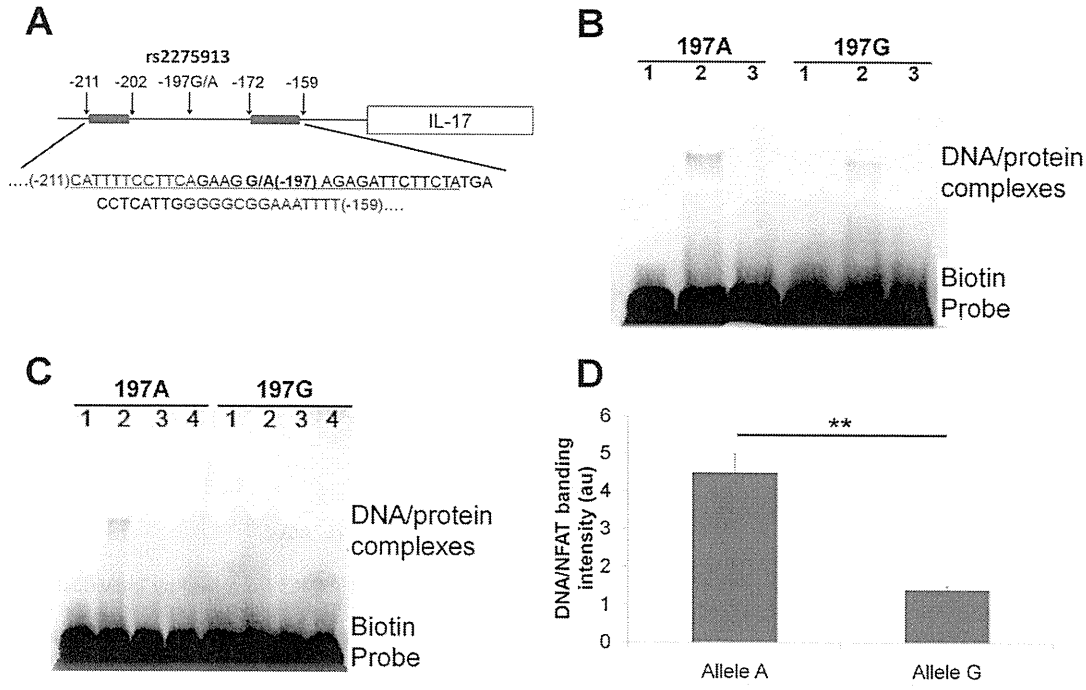


**Figure 4. The rs2275913 SNP results in differential binding of NFAT.** (A) The location of the rs2275913 SNP within the promoter region of the IL-17 gene. The underlined sequence corresponds to the oligomers used in EMSA assay. The NFAT binding sites [21] are indicated in red and by red boxes. (B) IL-17 probes were allowed to interact with nuclear extracts from PHA-PBMCs in an EMSA assay. Lane 1, free probes; lane 2, biotin-labeled probes plus nuclear extracts; lane 3, biotin-labeled probes plus nuclear extracts plus a 50-fold molar excess of unlabeled probes. The figure shown is representative of five independent experiments. (C) IL-17 probes were allowed to interact with recombinant NFAT proteins in an EMSA assay. Lane 1, free probes; lane 2, biotin-labeled probes plus GST-NFAT; lane 3, biotin-labeled probes plus GST-NFAT plus a 50-fold molar excess of unlabeled probes; lane 4, biotin-labeled probes plus GST-NFAT plus a 50-fold molar excess of unlabeled oligomers containing a NFAT consensus site. The figure shown is representative of five independent experiments. (D) The intensity of the bands corresponding to the DNA/protein interaction (lane 2 in **Fig. 4C**) were evaluated by densitometry to compare the binding affinity of the 197A allele and 197G allele for recombinant NFAT. The values are represented as arbitrary units (au). **indicates $P<0.01$.
doi:10.1371/journal.pone.0026229.g004

development of ulcerative colitis. Together, these results may explain the previous observations of increased expression of IL-17 in patients with inflammatory bowel disease such as ulcerative colitis, which promotes the recruitment of inflammatory cells into the intestinal mucosa through an increase in chemoattractants and the expression of adhesion molecules [4,42,43,44]. However, another study from Norway [18] suggested an association between the 197A genotype and resistance to developing rheumatoid arthritis Since many studies have demonstrated higher levels of IL-17 in patients with rheumatoid arthritis and the essential roles of IL-17 in mediating joint damage [45,46,47], the G197A SNP might affect the initiation of rheumatoid arthritis, but not disease progression and severity. This issue should thus be clarified using larger cohort studies in the future.

Our earlier report [34] showed an association with the IL-17 197A genotype in the recipient, but not the donor, as in the present study, with a higher incidence of acute graft-versus-host disease. However, unlike in the previous study [34], the current cohort mainly consisted of patients receiving relatively recent transplants, including reduced-intensity transplantation. The reason for these discrepancies is unclear, because the year of the transplant and conditioning intensity were considered as co-factors in the multivariate analysis. This issue should be clarified by further investigations in patients at higher risk for acute GVHD, including those receiving peripheral blood stem cell or HLA-mismatched transplants.

In conclusion, we have reported that the G197A SNP in the IL-17 promoter predicts the development of acute GVHD and plays a functionally important role in the regulation of IL-17 production. Given that the 197A allele is significantly associated with the higher production of IL-17, G197A genotyping may be used to predict the susceptibility and severity of other IL-17-related diseases and complications including rheumatoid arthritis, periodontal disease, multiple sclerosis, allergic rhinitis, psoriasis, inflammatory bowel disease, and organ allograft rejection [11]. Furthermore, a better understanding of the molecular mechanism by which this promoter SNP controls the production of IL-17 may therefore offer some novel therapeutic insights into the mechanisms of such diseases.

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## Author Contributions

Conceived and designed the experiments: JLE AT. Performed the experiments: JLE KN. Analyzed the data: AT. Contributed reagents/materials/analysis tools: AT MO HA KM YM TF YK SN TK. Wrote the paper: AT JLE.

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# Genetic Variants of Human Granzyme B Predict Transplant Outcomes after HLA Matched Unrelated Bone Marrow Transplantation for Myeloid Malignancies

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## Abstract

Serine protease granzyme B plays important roles in infections, autoimmunity, transplant rejection, and antitumor immunity. A triple-mutated granzyme B variant that encodes three amino substitutions (Q48R, P88A, and Y245H) has been reported to have altered biological functions. In the polymorphism rs8192917 (2364A>G), the A and G alleles represent wild type QPY and RAH mutant variants, respectively. In this study, we analyzed the impact of granzyme B polymorphisms on transplant outcomes in recipients undergoing unrelated HLA-fully matched T-cell-replete bone marrow transplantation (BMT) through the Japan Donor Marrow Program. The granzyme B genotypes were retrospectively analyzed in a cohort of 613 pairs of recipients with hematological malignancies and their unrelated donors. In patients with myeloid malignancies consisting of acute myeloid leukemia and myelodysplastic syndrome, the donor G/G or A/G genotype was associated with improved overall survival (OS; adjusted hazard ratio [HR], 0.60; 95% confidence interval [CI], 0.41–0.89; $P=0.01$) as well as transplant related mortality (TRM; adjusted HR, 0.48; 95% CI, 0.27–0.86, $P=0.01$). The recipient G/G or A/G genotype was associated with a better OS (adjusted HR, 0.68; 95% CI, 0.47–0.99; $P=0.05$) and a trend toward a reduced TRM (adjusted HR, 0.61; 95% CI, 0.35–1.06; $P=0.08$). Granzyme B polymorphism did not have any effect on the transplant outcomes in patients with lymphoid malignancies consisting of acute lymphoid leukemia and malignant lymphoma. These data suggest that there is an association between the granzyme B genotype and better clinical outcomes in patients with myeloid malignancies after unrelated BMT.

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## Introduction

Hematopoietic stem cell transplantation (HSCT) represents the only potentially curative option for many malignant conditions. Although substantial improvements in the supportive care of transplanted patients have been achieved in recent years, the profound compromise in the immune system associated with HSCT constitutes a significant risk for life threatening complications including GVHD, severe infections and disease relapse.[1] HLA matching represents the major genetic determinant in clinical outcomes after allogeneic HSCT, however, several studies have suggested that non-HLA genes associated with immune functions are also involved in determining the clinical outcome.[2] Single nucleotide polymorphisms (SNPs) in genes involved in the immune response to infections and inflammatory reactions have been identified as additional predictive markers of clinical outcomes in HSCT.[3,4,5,6,7,8,9,10,11,12,13,14]

Following HSCT, cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells, mainly derived from the donor, constitute the most important effector cells that eliminate allogeneic cells, including malignant cells, virus-infected cells and healthy cells. The destruction of the target cells occurs by at least one of the three defined mechanisms: TNF-α release, the Fas/Fas ligand interaction, and the granzyme/perforin pathway.[15] The later has been postulated as being the predominant mechanism for immune-mediated apoptosis of allogeneic cells.[16,17]

Granzyme B, the most abundant serine protease stored in secretory granules of CTLs and NK cells, is released upon target cell recognition, then specifically enters into the target cell cytoplasm via perforin, finally leading to target cell lysis.[15] Although the induction of target cell death by its pro-apoptotic properties has been considered the central function of granzyme B, growing evidence indicates that this protease also possesses additional non-death-related functions. These non-classical or extracellular functions are perforin-independent mechanisms and include immunosuppression, receptor cleavage, and cytokine-like effects.[15,18,19] Initially believed to be expressed exclusively by NK cells and CTLs, recent reports have shown that granzyme B can be expressed by various additional cell types, such as mast cells, neutrophils, dendritic cells (DCs), B cells, keratinocytes, chondrocytes, and vascular smooth muscle cells.[20,21,22,23,24]

Granzyme B is involved in the pathophysiology of viral and bacterial infections, solid organ rejection, autoimmune diseases, and antitumor immunity.[25,26,27,28,29,30] In the granzyme B gene, a triple-mutated allele (Q48R, P88A, and Y245H) in strong linkage disequilibrium is found in European, African, and Asian populations, including the Japanese population, at an allelic frequency of 25–30%.[31,32] The biological and functional relevance of the RAH mutant granzyme B, however, still remains controversial. Although it was reported that the RAH variant was incapable of inducing apoptosis,[31] and γδT cells derived from donors possessing the RAH variant had impaired cytotoxicity against target cells;[33] other studies have reported that RAH mutant granzyme B displays normal proteolytic and cytotoxic properties[34] and the cytotoxicic activity of T lymphocytes did not differ among donors with QPY or RAH genotypes.[32]

In this study, we hypothesized that a defect of inducing apoptosis in mutant granzyme B could influence the clinical outcomes of HSCT. To test this hypothesis, we investigated the influence of the QPY/RAH variants on the clinical outcomes after HSCT. Because these variants are in clear linkage disequilibrium, the study was focused on genotyping the polymorphism rs8192917 (2364A>G) in the granzyme B gene, which results in Q48R variants, and analyzed its impact on the clinical outcomes of patients undergoing allogeneic bone marrow transplantation (BMT) using an HLA allele-matched unrelated donor. The data herein show that the donor G/G or A/G allele, which represents mutant granzyme B, is associated with a significantly improved overall survival (OS) and reduced transplant-related mortality (TRM) in patients with myeloid malignancies.

## Methods

### Patients

Granzyme B genotyping was performed on 613 recipients with hematological malignancies and their unrelated donors who underwent BMT through the Japan Marrow Donor Program (JMDP) with T-cell-replete marrow from HLA-A, -B, -C, -DRB1, -DQB1, and -DPB1 allele-matched donors between January 1993 and December 2007. The HLA genotypes of patients and donors were determined by the Luminex microbead method, as described previously (Luminex 100 System; Luminex, Austin, TX).[35,36] Although the Luminex microbead method does not provide unambiguous HLA 4-digit typing for all genotypes, the JMDP has confirmed that this method can identify all HLA alleles with >0.1% frequency among the Japanese population.[37]

None of the present patients had a history of any prior transplantation. The final clinical survey of these patients was completed by November 1, 2008. The diagnoses were acute myeloid leukemia (AML) in 240 (39%), acute lymphoblastic leukemia (ALL) in 170 (28%), myelodysplastic syndrome (MDS) in 113 (18%), and malignant lymphoma (ML) in 90 (15%; **Tables 1 and 2**). The recipients were defined as having standard risk disease if they had AML or ALL in the first complete remission, ML in any complete remission, or MDS. All others were designated as having high-risk disease. The myeloid malignancies include AML and MDS, and the lymphoid malignancies included ALL and ML. Cyclosporine- or tacrolimus-based regimens were used in all patients for GVHD prophylaxis, and anti-T cell therapy, such as anti-thymocyte globulin and *ex vivo* T cell depletion were not in any of the patients. All patients and donors gave their written informed consent at the time of transplantation to participate in molecular studies of this nature according to the declaration of Helsinki. This project was approved by the Institutional Review Board of Kanazawa University Graduate School of Medicine and the JMDP.

### Granzyme B genotyping

Genotyping of granzyme B was performed using the TaqMan-Allelic discrimination method in a StepOne Plus Real Time PCR system (Applied Biosystems, Foster City, CA, USA), and the results

**Table 1.** Donor and recipient characteristics (first part).

| Variable | No. | Ratio |
|---|---|---|
| No. of cases | 613 | |
| Recipient age, years | | |
| Median | 36 | |
| Range | 1–70 | |
| Donor age, years | | |
| Median | 34 | |
| Range | 20–57 | |
| Year of transplant | | |
| Median | 2002 | |
| Range | 1993–2007 | |
| Recipient Granzyme B genotype | | |
| G/G | 30 | 5% |
| A/G | 202 | 33% |
| A/A | 381 | 62% |
| Donor Granzyme B genotype | | |
| G/G | 27 | 4% |
| A/G | 194 | 32% |
| A/A | 392 | 64% |
| Recipient sex | | |
| Male | 383 | 62% |
| Female | 230 | 38% |
| Donor sex | | |
| Male | 402 | 66% |
| Female | 210 | 34% |
| Missing | 1 | 0% |
| Donor/recipient sex | | |
| Sex matched | 409 | 67% |
| Female/male | 92 | 15% |
| Male/female | 111 | 18% |
| Missing | 1 | 0% |

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**Table 2.** Donor and recipient characteristics (second part).

| Variable | No. | Ratio |
|---|---|---|
| Disease | | |
| Acute myeloid leukemia | 240 | 39% |
| Myelodysplastic syndrome | 113 | 18% |
| Acute lymphoblastic leukemia | 170 | 28% |
| Malignant lymphoma | 90 | 15% |
| Disease stage | | |
| Standard risk | 357 | 58% |
| High risk | 256 | 42% |
| ABO matching | | |
| Major or/and minor mismatch | 246 | 40% |
| Major mismatch | 136 | 22% |
| Minor mismatch | 126 | 21% |
| Bidirectional | 18 | 3% |
| Missing | 7 | 1% |
| Conditioning regimen | | |
| Myeloablative | 499 | 81% |
| Reduced intensity | 114 | 19% |
| With total body irradiation | 472 | 77% |
| Pretransplant CMV serostatus | | |
| CMV positive recipient | 440 | 72% |
| Missing | 70 | 11% |
| GVHD prophylaxis | | |
| With cyclosporine | 296 | 48% |
| With tacrolimus | 314 | 51% |
| Missing | 3 | 0% |
| TNC, $\times 10^8$ per kg | | |
| Median | 4.9 | |
| Range | 0.1–79.1 | |

Abbreviations: TNC: total nucleated cell count harvested.
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were analyzed using the Allelic Discrimination software program (Applied Biosystems). The genotyping assay was conducted in 96-well PCR plates. The amplification reaction contained template DNA, TaqMan universal master mix and the specific probe designed for SNP rs8192917 (2364A>G) of granzyme B (product No. C_2815152_20 ; Applied Biosystems).

### Data management and statistical analysis

The data were collected by the JMDP using a standardized report form. Follow-up reports were submitted at 100 days, 1 year and annually after transplantation. The pre-transplant cytomegalovirus (CMV) serostatus was routinely tested for only patients, but not the donors. Engraftment was confirmed by an absolute neutrophil count of more than $0.5\times10^9$/L for at least 3 consecutive days. On collecting data, acute and chronic GVHD were diagnosed and graded using the previous criteria[38,39] and data using updated criteria for assessment of GVHD[40,41] were not available in our cohort. The OS was defined as the number of days from transplantation to death from any cause. Disease relapse was defined as the number of days from transplantation to disease relapse. TRM was defined as death without relapse. Any patients who were alive at the last-follow-up date were censored. The data about causative microbes of infections and postmortem changes in cause of death, as well as the data on supportive care, including prophylaxis for infections and therapy for GVHD, which were given on an institutional basis, were not available for this cohort.

The analysis was performed using the Excel 2007 (Microsoft Corp, Redmond, WA, USA) and modified R (The R Foundation for Statistical Computing, Perugia, Italy) software programs.[42,43] The probability of overall survival (OS) was calculated using the Kaplan-Meier method and compared using the log-rank test. The probabilities of TRM, disease relapse, acute GVHD, chronic GVHD, and engraftment were compared using the Grey test[44] and analyzed using the cumulative incidence analysis,[42] considering relapse, death without disease relapse, death without acute GVHD, death without chronic GVHD, and death without engraftment as respective competing risks. The variables were recipient age at time of transplantation, sex, pretransplant CMV serostatus, disease characteristics (disease type, disease lineage and disease risk at transplantation), donor characteristics (age, sex, sex compatibility, and ABO compatibility), transplant characteristics (conventional or reduced-intensity conditioning,[45] total body irradiation-containing regimen, tacrolimus versus cyclosporine, and total nucleated cell count harvested per recipient weight [TNC]), and the year of transplantation. The median was used as the cutoff point for continuous variables. The chi-square test and Mann-Whitney U test were used to compare two groups. The Hardy-Weinberg equilibrium for the granzyme B gene polymorphism was tested using the Haploview software program.[6]

Multivariate Cox models were used to evaluate the hazard ratio associated with the granzyme B polymorphism. Covariates found to be $P\leq0.10$ in the univariate analyses were used to adjust the hazard ratio. The covariates were selected according to myeloid and lympoid malignancies.

For both the univariate and multivariate analyses, P values were two sided, and outcomes were considered to be significant for $P\leq0.05$.

## Results

### The frequencies of the granzyme B genotypes

Granzyme B gene polymorphism was analyzed in 613 unrelated bone marrow donor-transplant recipient pairs (**Tables 1 and 2**). The genotype frequencies of G/G, A/G and A/A were 5%, 33% and 62% in recipients and 4%, 32% and 64% in donors. These were similar to HapMap data in the Japanese (9%, 29% and 62%, respectively) and European (5%, 35% and 60%, respectively) populations, and thus were in accord with the Hardy-Weinberg equilibrium ($P=0.79$).

### Transplant outcome according to the granzyme B genotype

The median follow-up duration in the cohort was 55 months among the survivors (range 4 to 168 months), and 191 recipients (31%) had relapsed or progressed, and 309 (50%) had died. Eighteen patients (3%) died before engraftment. The donor and recipient granzyme B genotype did not significantly influence the cumulative incidence of engraftment (data not shown).

The transplant outcomes according to the granzyme B genotype are summarized in **Table 3**. Patients with myeloid malignancies, which included AML and MDS, who received transplants from donors with the G/G or A/G genotype had a significantly better 5-year OS (58% vs. 42%, $P=0.01$; **Fig. 1A**) and a trend toward lower 5-year relapse rate (27% vs. 36%, $P=0.09$) than those receiving transplants from donors with the A/A genotype. No

**Table 3.** The results of the univariate analysis of the association of the Granzyme B genotype with clinical outcomes after transplantation.

| Variable | No. | 5-year OS | *P* | 5-year TRM | *P* | 5-year relapse | *P* | II-IV acute GVHD | *P* | Chronic GVHD | *P* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Myeloid malignancy | 353 | | | | | | | | | | |
| Recipient Granzyme B genotype | | | | | | | | | | | |
| A/G or G/G | 139 | 52% | 0.13 | 21% | 0.17 | 33% | 0.71 | 25% | 0.23 | 40% | 0.14 |
| A/A | 214 | 46% | | 26% | | 33% | | 31% | | 49% | |
| Donor Granzyme B genotype | | | | | | | | | | | |
| A/G or G/G | 141 | 58% | 0.01 | 20% | 0.21 | 27% | 0.10 | 28% | 0.72 | 47% | 0.79 |
| A/A | 212 | 42% | | 26% | | 37% | | 30% | | 45% | |
| Lymphoid malignancy | 260 | | | | | | | | | | |
| Recipient Granzyme B genotype | | | | | | | | | | | |
| A/G or G/G | 93 | 48% | 0.14 | 24% | 0.26 | 35% | 0.97 | 35% | 0.66 | 31% | 0.44 |
| A/A | 167 | 43% | | 26% | | 34% | | 33% | | 36% | |
| Donor Granzyme B genotype | | | | | | | | | | | |
| A/G or G/G | 80 | 43% | 0.93 | 29% | 0.60 | 33% | 0.78 | 34% | 0.88 | 32% | 0.49 |
| A/A | 180 | 46% | | 24% | | 35% | | 34% | | 36% | |

Abbreviations: OS, overall survival; TRM, Transplant-related mortality.

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difference was noted in the TRM, II-IV acute GVHD, or chronic GVHD in relation to the donors' polymorphism status. A comparison between the donor G/G and A/G genotypes showed no significant difference in OS (71% vs. 56%, $P=0.36$), TRM (6% vs. 23%, $P=0.36$), or the relapse rate (30% vs. 26%, $P=0.65$). When patients with AML and MDS were separately analyzed, the donor G/G or A/G genotypes remained statistically significant for a better OS in AML patients (58% vs. 45%; **Fig. 1B**), and had a tendency to be related to a better OS in MDS patients (58% vs. 37%; **Fig. 1C**). In patients with lymphoid malignancies consisting of ALL and ML, the donor granzyme B genotype had no significant effects on transplant outcomes (**Table 3**). This was true even when ALL and ML were separately analyzed (data not shown).

### Multivariate analysis

All of the factors found to be significant in the univariate analyses were included in the model. In patients with myeloid malignancies, the G/G or A/G genotype in donors were statistically significant in the multivariate analyses for better OS (adjusted hazard ratio [HR], 0.60; 95% confidence interval [CI], 0.41–0.89; $P=0.01$; **Table 4**) and TRM (adjusted HR, 0.45; 95% CI, 0.25–0.80; $P=0.01$) when adjusted for the other factors in the models. Despite not evident in the univariate analysis, the multivariate analysis revealed the donor granzyme B G/G or AG genotype was associated with lower incidence of chronic GVHD (adjusted HR, 0.61; 95% CI, 0.37–0.99; $P=0.05$; **Table 5**). In the independent analyses for AML patients and MDS patients, beneficial effects on OS by the donor G/G or A/G genotype were also found, which was close to being significant in both the AML patients (adjusted HR, 0.68; 95% CI, 0.42–1.09; $P=0.10$) and the MDS patients (adjusted HR, 0.61; 95% CI, 0.35–1.08; $P=0.09$). In addition, the recipient G/G or A/G genotype was associated with a significantly better OS (adjusted HR, 0.68; 95% CI, 0.47–0.99; $P=0.05$) and a trend toward a reduced TRM (adjusted HR, 0.61; 95% CI, 0.35–1.06; $P=0.08$). The difference between the donor G/G and A/G genotype did not reach statistical significance in relation to transplant outcomes (data not shown). The granzyme B genotype did not significantly influence the transplant outcomes in patients with lymphoid malignancies.

## Discussion

The current study showed that the granzyme B G/G or A/G genotype at rs8192917 (2364A>G) in the donor side representing the triple variant RAH granzyme B was associated with a significantly better OS and TRM compared to the granzyme A/A genotype, corresponding to wild type QPY granzyme B, for patients with myeloid malignancies receiving HLA-matched unrelated BMT through the JMDP. The G/G or A/G genotypes in the recipient also significantly improved the OS, as well the TRM, although to a lesser extent. This is the first report to show that the granzyme B polymorphism affects transplant outcomes.

The beneficial effects of the G/G or A/G genotype were absent in patients with lymphoid malignancies, irrespective of whether it was ALL or ML. A possible explanation for this may be that ALL and ML cells express the apoptosis inhibitor Bcl-2[46] and the endogenous inhibitor of granzyme B, proteinase inhibitor 9 (PI-9).[47,48,49,50] The expression of these two factors by malignant lymphoid cells may protect them from granzyme B-induced apoptosis and proteolysis[46,48] and might thus negate the differential effects of the different granzyme B genotypes.

Based on the traditional view that the triple-mutated granzyme B has an impaired pro-apoptotic function, it was expected that the presence of the RAH variant would predict an adverse clinical outcomes after HSCT, namely poor survival or an increased relapse rate. The results presented here, however, do not support that assumption. The mechanisms by which the mutant granzyme B genotype improved transplant outcomes remain unclear. This may be due, in part, because the reports on the biochemical and physiological properties of the triple variant RAH granzyme B are still controversial.

Although an initial study[31] reported that RAH granzyme B was unable to induce apoptosis in tumor cell lines, it was later

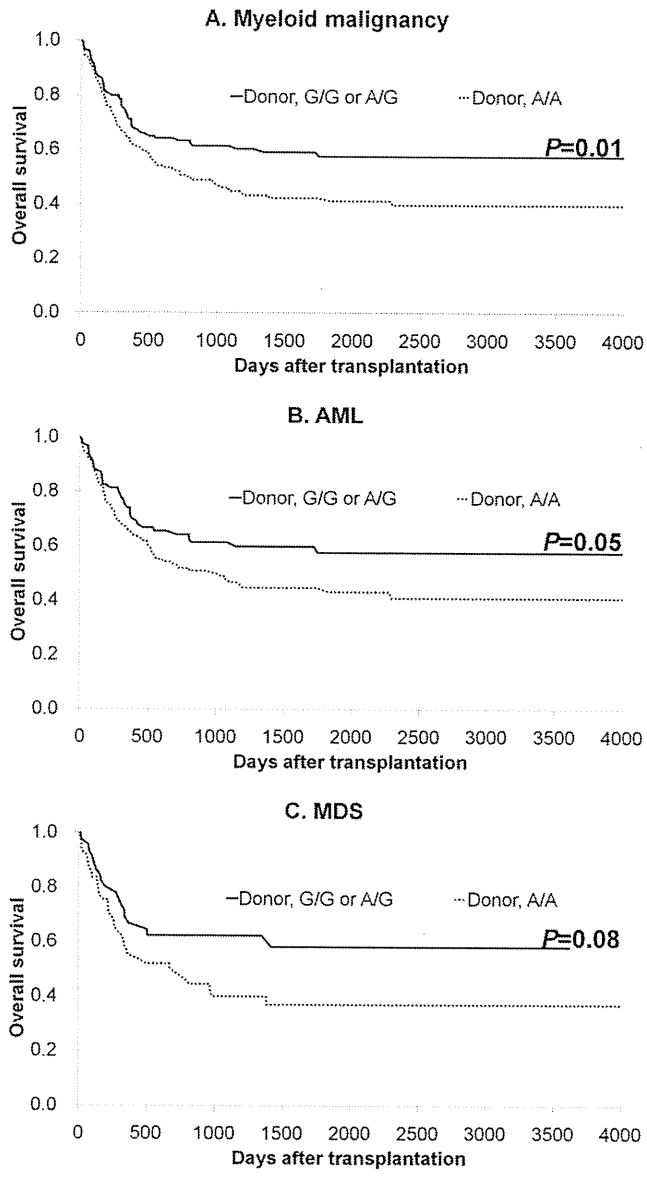


**Figure 1. The Kaplan-Meier analysis of OS after BMT according to the donor granzyme B genotype in patients with myeloid malignancies (A), AML (B), and MDS (C).**
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reported by others that RAH granzyme B retains its pro-apoptotic activity.[34] In addition to the classical role of granzyme B in mediating apoptosis within target cells by NK cells and CTLs, increasing evidence shows that extracellular granzyme B also has alternative functions, including extracellular matrix remodeling, immunosuppressive and cytokine-like effects.[18,19,24,51,52] A recent report[19] showed that human DCs abundantly secrete granzyme B, which can suppress T-cell expansion. Another report revealed a pivotal function of granzyme B in immunosuppression directed by regulatory T cells, leading to promotion of tumor escape.[52] In addition, extracellular granzyme B potentially induces apoptosis in various organs and tissues, thus leading to chronic inflammatory, autoimmune, and degenerative diseases.[18,24] In line with these observations, it is plausible that in patients receiving HSCT, extracellular granzyme B could contribute to significant effects, such as modulation of T-cell functions and organ damage, because high serum levels of extracellular granzyme B have been reported in HSCT recipients.[29]

Based on the results presented herein, it may therefore be reasonable to hypothesize that the granzyme B variants have differential biochemical properties whose biological consequences are more relevant on the non-classical functions exerted by the extracellular granzyme B. The analysis of patient serum may offer useful information on this issue, although these samples were not available for the present study. The fact that functional granzyme B is also secreted by nonhematopoietic cells, including keratinocytes, chondrocytes, and smooth muscle cells[18,53] may explain the findings that granzyme B variants in the recipient side, in addition to that in the donor side, had an impact on the transplant outcomes. Furthermore, this finding supports the view that the presence of the triple-mutated Granzyme B is indeed responsible for the beneficial effect in HSCT for myeloid malignancies.

The effects of the granzyme B G/G or A/G genotype on the reduced TRM in patients with myeloid malignancies might be a consequence of increased resistance to infections in these recipients. This hypothesis, although attractive, is highly speculative and is not supported by the present study because of the unavailability of data on the causes of infections in this cohort. Further studies will be needed to clarify whether the granzyme B genotypes can differentially affect the responses of patients against infections.

Two recent reports have described a significant correlation between disease susceptibility and the RAH/QPY polymorphism in the granzyme B gene. The wild type QPY genotype was associated with an increased incidence of Epstein-Barr-virus-associated

**Table 4.** The results of a multivariate analysis of the association of the Granzyme B genotype with the clinical outcomes after transplantation.

| Variable | OS | | | TRM | | | Relapse | | |
|---|---|---|---|---|---|---|---|---|---|
| | Adjusted HR | 95% CI | *P* | Adjusted HR | 95% CI | *P* | Adjusted HR | 95% CI | *P* |
| Myeloid malignancy | | | | | | | | | |
| Recipient Granzyme B genotype, G/G or A/G | 0.68 | 0.47–0.99 | 0.05 | 0.61 | 0.35–1.06 | 0.08 | 0.99 | 0.65–1.51 | 0.97 |
| Donor Granzyme B genotype, G/G or A/G | 0.60 | 0.41–0.89 | 0.01 | 0.45 | 0.25–0.80 | 0.01 | 0.75 | 0.48–1.15 | 0.19 |
| Lymphoid malignancy | | | | | | | | | |
| Recipient Granzyme B genotype, G/G or A/G | 0.99 | 0.60–1.57 | 0.96 | 0.93 | 0.44–1.96 | 0.84 | 1.40 | 0.84–2.34 | 0.20 |
| Donor Granzyme B genotype, G/G or A/G | 0.72 | 0.43–1.28 | 0.23 | 0.84 | 0.32–2.22 | 0.72 | 0.87 | 0.49–1.56 | 0.65 |

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**Table 5.** The results of a multivariate analysis of the association of the Granzyme B genotype with GVHD after transplantation.

| | II-IV acute GVHD | | | Chronic GVHD | | |
|---|---|---|---|---|---|---|
| Variable | Adjusted HR | 95% CI | *P* | Adjusted HR | 95% CI | *P* |
| Myeloid malignancy | | | | | | |
| Recipient Granzyme B genotype, G/G or A/G | 0.78 | 0.51–1.19 | 0.24 | 0.83 | 0.53–1.31 | 0.42 |
| Donor Granzyme B genotype, G/G or A/G | 0.94 | 0.62–1.43 | 0.76 | 0.61 | 0.37–0.99 | 0.05 |
| Lymphoid malignancy | | | | | | |
| Recipient Granzyme B genotype, G/G or A/G | 0.90 | 0.55–1.45 | 0.69 | 0.90 | 0.54–1.50 | 0.69 |
| Donor Granzyme B genotype, G/G or A/G | 1.07 | 0.65–1.76 | 0.79 | 1.13 | 0.68–1.89 | 0.64 |

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hemophagocytic lymphohistiocytosis (HLH) in children.[32] Conversely, a subsequent study reported an association of the mutant RAH genotype with the incidence of breast cancer.[33] However, to link the genetic susceptibility of granzyme B to disease based on the presented data is difficult, because no patient developed HLH or breast cancer following HSCT in the current cohort.

In conclusion, the present data suggest that the granzyme B polymorphism may affect the prognosis after BMT from an unrelated donor, and therefore, the granzyme B genotyping in transplant donors and recipients may provide opportunities to choose an ideal donor. However, care should be made in drawing conclusions, because the number of patients evaluated in the present study is limited. Experimental evidence is also required to substantiate the effects of extracellular granzyme B according to the polymorphism on organ and tissue damage. Further studies are warranted to ascertain whether the findings of this study can be extended to other disease groups, other stem cell sources, or HLA-mismatched transplantation, as well as to validate the present data.

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## Author Contributions

Conceived and designed the experiments: LJE AT. Performed the experiments: LJE KY. Analyzed the data: AT KN. Contributed reagents/materials/analysis tools: AT MO TK HS HA KM SO MI TF YM YK. Wrote the paper: AT LJE. Conducted the study: SN.

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