Table 3
Univariate Analysis of Associations between *PTPN22* Variations and Clinical Outcomes after BMT

Variable	Number	5-Year OS, %	P	5-Year TRM, %	P	5-Year Relapse, %	P	Grade II-IV aGVHD, %	P	Grade III-IV aGVHD, %	P	Chronic GVHD, %	P
Recipient *PTPN22* genotype													
G/G	228	48		25		28		33		11		43	
G/C	331	50	.73	28	.67	27	.75	35	.69	15	.26	47	.36
C/C	104	48	.64	19	.43	40	.06	**18**	**.009**	6	.18	42	.79
Donor *PTPN22* genotype													
G/G	219	48		22		34		32		13		42	
G/C	324	48	.59	30	.08	**27**	**.04**	31	.73	11	.57	45	.42
C/C	120	53	.38	21	.62	29	.35	33	.85	14	.79	49	.24

Significant values ($P \leq .05$) are in bold.

genotypes, leading to an estimated statistical power of 57% to detect the difference between the recipient C/C genotype and recipient G/C or G/G genotype in both subcohort analyses. The association between recipient C/C genotype and a lower incidence of grade II-IV aGVHD remained positive in the analyses of subcohort 1 ($P = .04$) and subcohort 2 ($P = .03$) (Supplemental Figure 1).

In addition, the recipient C/C genotype was associated with a higher incidence of relapse (40%) compared with that seen in the recipient G/G (28%; $P = .06$) and G/C (27%; $P = .02$) genotypes (Figure 1B). This difference had no significant influence on OS or TRM, however.

In a comparison of the impact of the *PTPN22* genotype in recipients with standard-risk disease and those with high-risk disease to investigate the significant effect of recipient genotype on relapse rate, the effect of recipient genotype on the incidence of grade II-IV aGVHD appeared unchanged. In patients with high-risk disease, the incidence of grade II-IV aGVHD was 33% in those with the recipient G/G genotype, 38% in those with the G/C genotype, and 17% in those with the C/C genotype ($P = .10$). In patients with standard risk disease, these values were 33%, 34%, and 18% ($P = .09$), respectively. In patients with high-risk disease, the 5-year cumulative incidence of relapse associated with the recipient C/C genotype was as high as 50%, which was not significantly different from that in those with the recipient G/G (39%; $P = .28$) and G/C (35%; $P = .14$) genotypes; however, this likely contributed to a significantly lower 5-year OS rate associated with the recipient C/C genotype (20%) compared with the recipient G/C (37%; $P = .02$) and G/G genotypes (32%; $P = .05$) (Figure 2A). In patients with standard-risk disease, the 5-year cumulative incidence of relapse was 32% in those with the recipient C/C genotype, 22% in those with the G/G phenotype ($P = .23$), and 32% in those with the G/C genotype ($P = .17$), and there were no significant differences in OS rate (Figure 2B).

After adjusting for clinical factors in the multivariate model, recipient C/C genotype remained statistically significant compared with the recipient G/G genotype with respect to the development of grade II-IV aGVHD (HR, 0.50; 95% confidence interval [CI], 0.29-0.85; $P = .01$; Table 4) and relapse (HR, 1.78; 95% CI, 1.10-2.90; $P = .02$; Table 5). Although analysis of the entire cohort revealed no considerable effects of the *PTPN22* genotype on OS rates (Table 5), compared with recipient G/G genotype. recipient C/C genotype was associated with significantly lower OS in patients with high-risk disease (HR, 1.60; 95% CI, 1.02-2.51; $P = .04$; Table 6) and with a significantly higher incidence of relapse in patients with standard-risk disease (HR, 2.02; 95% CI, 1.02-4.00; $P = .04$). No effects of recipient C/C genotype on OS rates were seen in patients with standard-risk disease.

The increased risk of relapse associated with recipient C/C genotype could be outweighed by the decreased risk of grade II-IV aGVHD, given that the absence of grade II-IV aGVHD was closely linked to the higher incidence of relapse (31% versus 19% at 5 years; $P = .01$) in the landmark analysis completed at day 60, in agreement with a previous report [18]. Consequently, we analyzed the impact of recipient *PTPN22* genotype on relapse according to the development of grade II-IV aGVHD. The landmark time for aGVHD analysis was chosen as day 60 post-BMT, as in a previous study [18], because more than 90% of patients who develop grade II-IV aGVHD do so within 60 days after transplantation [19]. In patients who developed grade II-IV aGVHD before day 60, the cumulative incidence of relapse was higher in those with the recipient C/C genotype (47% at 5 years) compared with

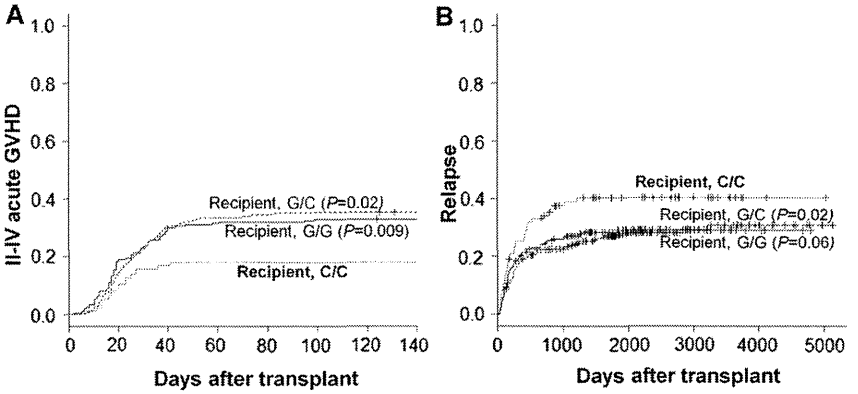

Figure 1. Estimated cumulative incidence curves of grade II-IV aGVHD (A) and relapse (B) according to recipient *PTPN22* genotype. Solid lines represent the recipient G/G genotype; dashed lines, the recipient G/C genotype; and dotted lines, the recipient C/C genotype.

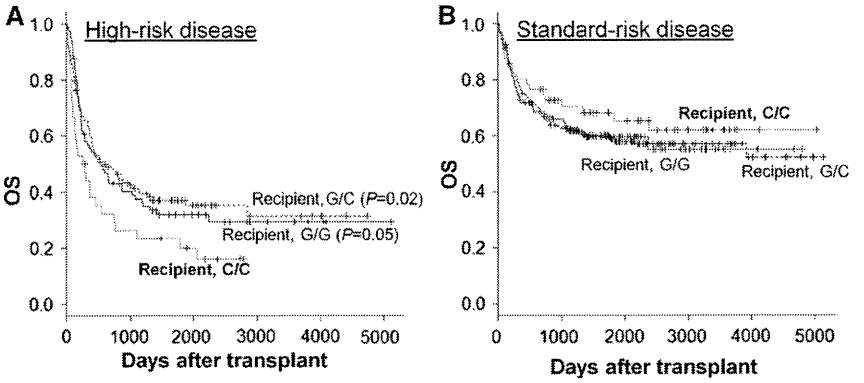

Figure 2. Kaplan-Meier analysis of OS after BMT according to the recipient *PTPN22* genotype in patients with high-risk disease (A) and those with standard-risk disease (B). Solid lines represent the recipient G/G genotype; dashed lines, the recipient G/C genotype; and dotted lines, the recipient C/C genotype.

those with the G/G (22%; $P = .04$) or G/C (20%; $P = .03$) genotype. The increased incidence of relapse associated with the recipient C/C genotype was confirmed on multivariate analysis, with an HR for relapse for the recipient C/C genotype versus G/G genotype as high as 4.5 (95% CI, 1.56-12.78; $P = .005$). In patients who survived more than 60 days without developing grade II-IV aGVHD, the 5-year cumulative incidence of relapse was higher in those with the recipient C/C genotype (39%) than in those with the recipient G/G (30%; $P = .22$), G/C (28%; $P = .24$), and G/G or G/C genotypes (30%; $P = .21$). After adjustment of covariates using the multivariate model, the increased incidence of relapse associated with the recipient C/C genotype was close to being significant compared with recipient G/G (HR, 1.79; 95% CI, 0.98-3.26; $P = .06$) and G/G or G/C (HR, 1.64; 95% CI, 0.99-2.71; $P = .06$) genotypes. Accordingly, the effects of recipient C/C genotype in increasing the incidence of relapse are considered independently significant irrespective of the development of grade II-IV aGVHD.

Effects of Donor PTPN22 Genotype on Transplantation Outcomes

Compared with donor G/G genotype, donor G/C genotype was correlated with a significantly lower incidence of relapse (27% versus 34%; $P = .04$) and with a trend toward increased TRM (30% versus 22%; $P = .08$). The effects of the lower relapse rate associated with the donor G/C genotype were also evident in the multivariate analysis (HR, 0.58; 95% CI, 0.40-0.85; $P = .005$; Table 5). The effects of donor G/C genotype on relapse and TRM had no significant impact on OS; this also held true in the analysis performed according to disease risk (data not shown).

DISCUSSION

In our study cohort, the recipient C/C genotype at the rs2488457 (−1123G>C) variant of the *PTPN22* promoter gene was associated with a lower incidence of grade II-IV aGVHD and a higher incidence of relapse after unrelated HLA-matched BMT performed through the JMDP. The recipient C/C genotype negatively affected OS in patients with high-risk disease, but not in those with standard-risk disease. In addition, the donor G/C genotype predicted a lower incidence of relapse, but had no significant impact on OS irrespective of disease risk.

Previous studies have identified 4 variations in the *PTPN22* gene associated with susceptibility to autoimmune diseases. The +1858C>T variation (rs2476601) is in near-perfect disequilibrium with rs6679677 [20] and is closely linked to the −1123G>C variation (rs2488457) analyzed in the present study [2,5,21-23]. The +1858C>T variation was first identified as associated with type 1 diabetes using a candidate gene approach [24]. Subsequent studies have confirmed this finding, as well as the variation's association with other autoimmune diseases, including Crohn's disease, ulcerative colitis, rheumatoid arthritis, Graves disease, autoimmune thyroid disease, vitiligo, alopecia, systemic lupus erythematosus, and acute allograft rejection [25]. The +1858C>T variation is not polymorphic in the Asian population [5]; instead, the −1123G>C variation is associated with type 1 diabetes and rheumatoid arthritis [2]. In addition, the −1123G>C variation is more closely associated with type 1 diabetes than the +1858C>T variation in the European population [5]. The remaining variation, +788G>A (Lyp-R263Q, rs33996649), is associated with ulcerative colitis, rheumatoid arthritis, and systemic lupus erythematosus [26].

Table 4
Multivariate Analysis of the Association between *PTPN22* Variations and GVHD after BMT

Variable	Grade II-IV aGVHD			Grade III-IV aGVHD			cGVHD		
	Adjusted HR	95% CI	*P*	Adjusted HR	95% CI	*P*	Adjusted HR	95% CI	*P*
Recipient *PTPN22* genotype, G/C (331) versus G/G (n = 228)	1.64	0.79-1.44	.68	1.32	0.80-2.18	.28	1.08	0.81-1.44	.59
Recipient *PTPN22* genotype, C/C (104) versus G/G (n = 228)	**0.50**	**0.29-0.85**	**.01**	0.54	0.22-1.34	.18	0.89	0.58-1.34	.59
Donor *PTPN22* genotype, G/C (324) versus G/G (n = 219)	0.95	0.70-1.30	.76	0.81	0.48-1.36	.42	1.13	0.84-1.53	.42
Donor *PTPN22* genotype, C/C (120) versus G/G (n = 219)	1.08	0.72-1.61	.72	1.10	0.59-2.07	.76	1.33	0.93-1.90	.11
Recipient age ≥34 years							**1.31**	**1.00-1.72**	**.05**
Total body irradiation–containing conditioning regimen							**1.44**	**1.01-2.06**	**.05**
High-risk disease							**0.75**	**0.56-0.99**	**.05**
Year of BMT 2001 or later				0.69	0.42-1.11	**.12**			

Covariates identified as significant in the univariate analyses ($P \leq .10$) were used to adjust the HR for the *PTPN22* genotype. Significant results ($P \leq .05$) are in bold.

Table 5
Multivariate Analysis of the Association between *PTPN22* Variations and Prognostic Outcomes after Transplantation

Variable	OS			TRM			Relapse		
	Adjusted HR	95% CI	*P*	Adjusted HR	95% CI	*P*	Adjusted HR	95% CI	*P*
Recipient *PTPN22* genotype, G/C (331) versus G/G (n = 228)	0.94	0.71-1.25	.69	0.84	0.55-1.28	.84	1.08	0.73-1.64	.71
Recipient *PTPN22* genotype, C/C (104) versus G/G (n = 228)	1.03	0.68-1.56	.87	0.67	0.33-1.35	.27	**1.78**	**1.10-2.90**	**.02**
Donor *PTPN22* genotype, G/C (324) versus G/G (n = 219)	0.91	0.68-1.21	.51	1.24	0.78-1.97	.37	**0.58**	**0.40-0.85**	**.005**
Donor *PTPN22* genotype, C/C (120) versus G/G (n = 219)	0.78	0.53-1.15	.21	1.08	0.60-1.97	.79	0.64	0.40-1.04	.07
Minor ABO incompatibility				**1.74**	**1.10-2.77**	**.002**			
Recipient age ≥34 years	**1.61**	**1.23-2.10**	**.001**	**2.21**	**1.45-3.37**	**<.001**			
CMV-positive recipient				**2.15**	**1.13-4.08**	**.002**	1.49	0.95-2.34	.08
Conventional conditioning regimen				1.33	0.64-2.78	.45			
Total body irradiation–containing conditioning regimen				0.95	0.60-1.52	.84			
High-risk disease	2.08	**1.60-2.69**	**<.001**	**1.75**	**1.14-2.70**	**.01**	**1.76**	**1.22-2.53**	**.003**
Female donor/male recipient							0.67	0.40-1.11	.12
TNC ≥ 5.0×10^8/kg				0.92	0.63-1.36	.69			
Year of BMT 2001 or later	0.98	0.74-1.31	.90						

Covariates identified as significant in the univariate analyses ($P \leq .10$) were used to adjust the HR for the *PTPN22* genotype. Significant results ($P \leq .05$) are in bold.

Recent genome-wide association studies and meta-analyses have validated the association of these variations with type 1 diabetes, inflammatory bowel disease, Graves disease, rheumatoid arthritis, and systemic lupus erythematosus [27].

Experimental evidence has demonstrated that +1858C>T (Lyp-R620W, rs2476601) and +788G>A (Lyp-R263Q, rs33996649) are functional [28,29]. Lyp-Trp620 (+1858T) is associated mainly with an increased risk of autoimmune diseases and impaired constitutive biding of Lyp with c-Src tyrosine kinase (Csk) [30]. The inability of Lyp-Trp620 to bind Csk results in a less efficient inhibition of TCR signaling, because Lyp and Csk concertedly down-regulate TCR signaling [28]. Previous studies in cell lines and primary human cells have shown conflicting results, however [28]. Lyp-Trp620–positive primary human T cells were found to produce less IL-2 on TCR signaling, and Lyp-Trp620 more potently reduced TCR signaling in a dose-dependent manner, suggesting a gain-of-function mutation [30]. Conversely, the

Table 6
Impact of Recipient *PTPN22* Genotype on OS and TRM According to Disease Risk in the Multivariate Analysis

Variable	OS			TRM			Relapse		
	Adjusted HR	95% CI	*P*	Adjusted HR	95% CI	*P*	Adjusted HR	95% CI	*P*
Patients with high-risk disease									
Recipient *PTPN22* genotype, G/C (128) versus G/G (n = 89)	0.95	0.57-1.35	.78	0.89	0.47-1.69	.73	1.05	0.60-1.84	.87
Recipient *PTPN22* genotype, C/C (40) versus G/G (n = 89)	**1.60**	**1.02-2.51**	**.04**	0.92	0.36-2.34	.85	1.51	0.75-3.05	.25
Donor *PTPN22* genotype, G/C (186) versus G/G (n = 142)	0.90	0.63-1.28	.55	1.29	0.60-2.80	.51	**0.53**	**0.31-0.91**	**.02**
Donor *PTPN22* genotype, C/C (69) versus G/G (n = 142)	0.81	0.51-1.29	.37	1.54	0.64-3.75	.34	0.56	0.29-1.11	.10
Minor ABO incompatibility				**2.32**	**1.14-4.73**	**.02**			
Recipient age ≥34 years	**1.76**	**1.28-2.43**	**.001**	**2.43**	**1.28-4.59**	**.006**			
CMV-positive recipient				1.33	0.58-3.06	.50	1.26	0.66-2.41	.49
Conventional conditioning regimen				1.33	0.35-5.14	.68			
Total body irradiation–containing conditioning regimen				1.80	0.53-6.15	.35			
Female donor/male recipient							0.85	0.41-1.78	.67
TNC ≥ 5.0×10^8/kg				1.11	0.61-2.03	.74			
Year of BMT 2001 or later	0.93	0.67-1.29	.67						
Patients with standard-risk disease									
Recipient *PTPN22* genotype, G/C (199) versus G/G (n = 138)	0.96	0.67-1.37	.81	0.78	0.46-1.34	.37	1.12	0.63-2.00	.70
Recipient *PTPN22* genotype, C/C (60) versus G/G (n = 138)	0.84	0.49-1.43	.52	0.51	0.18-1.41	.19	**2.02**	**1.02-4.00**	**.04**
Donor *PTPN22* genotype, G/C (186) versus G/G (n = 142)	1.17	0.82-1.69	.39	1.23	0.67-2.24	.51	0.65	0.39-1.10	.11
Donor *PTPN22* genotype, C/C (69) versus G/G (n = 142)	0.83	0.50-1.38	.48	0.81	0.35-1.86	.62	0.74	0.38-1.45	.39
Minor ABO incompatibility				1.39	0.72-2.71	.33			
Recipient age ≥34 years	**1.68**	**1.20-2.36**	**.003**	**2.04**	**1.16-3.59**	**.01**			
CMV-positive recipient				**3.45**	**1.19-9.96**	**.02**	1.74	0.90-3.39	.10
Conventional conditioning regimen				1.10	0.46-2.64	.83			
Total body irradiation–containing conditioning regimen				0.79	0.45-1.36	.39			
Female donor/male recipient							0.51	0.25-1.07	.08
TNC ≥ 5.0×10^8/kg				0.84	0.50-1.39	.50			
Year of BMT 2001 or later	1.24	0.88-1.74	.23						

Covariates identified as significant in the univariate analyses ($P \leq .10$) were used to adjust the HR for the *PTPN22* genotype. Significant values ($P \leq .05$) are in bold.

Lyp-Gln263 mutation, which is associated with a reduced risk of autoimmune diseases, reportedly results in loss of function [29].

The mechanisms through which the recipient −1123C allele of the *PTPN22* gene affects the incidence of aGVHD and disease relapse remain unclear. Previous reports of the number of regulatory T cells (Tregs) increasing inversely with the level of *PTPN22* in the thymus [31] and of thymus-derived Tregs operating to prevent aGVHD and promote disease relapse [32] suggest the hypothesis that in transplant recipients, the *PTPN22* −1123G>C variant influences the production of Tregs from the thymus. This hypothesis may be supported by the fact that the *PTPN22* gene has a functional variant, +1858C>T, that is closely linked to the −1123G>C variant [2,5,21-23], and that the minor +1858T allele functionally inhibits TCR signaling more potently than the major +1858C allele [30]. Hyporesponsive TCR signaling might lead to increased Treg production by the thymus, given that decreased TCR signaling can promote the development of intrathymic Tregs [33]. Thus, an increased number of Tregs in relation to the recipient −1123C/C genotype might prevent aGVHD at the expense of decreased graft-versus-tumor effects. These hypotheses must be considered speculative, however, given the lack of functional data on the −1123G>C variant. Elucidating the role of the *PTPN22* −1123G>C variant in Treg production will provide useful information in this regard.

A second possible mechanism includes the involvement of host DCs, which are critical for the initiation of aGVHD [34]. This possibility may be supported by a recent report indicating that the *PTPN22*+1858C>T variant plays key roles in antigen receptor signaling of DCs [28].

Why the *PTPN22* −1123G>C genotype displays different behaviors in the donor and recipient genotypes is obscure. Of note, the donor heterozygous −1123G/C genotype was associated with a reduced incidence of relapse, which could be attributed to increased graft-versus-tumor effects owing to donor G/C genotype. The effects of the heterozygous −1123G/C genotype on autoimmunity may be related to the association between this genotype and increased risk of developing autoimmune diseases, including type 1 diabetes and rheumatoid arthritis, in Asian populations [2,3,5]. However the present study showed no gene dose responses, and whether this phenomenon reflects a molecular heterosis is unclear [3,5,35].

The lack of considerable survival advantage in relation to donor *PTPN22* genotype may suggest that the beneficial effects of *PTPN22* genotyping are limited. However, determination of the recipient *PTPN22* genotype before transplantation might provide a recipient harboring the *PTPN22* G/C or G/G genotype an opportunity to avoid the risk of aGVHD by favoring a bone marrow or cord blood HLA-matched graft over a peripheral blood stem cell (PBSC) or HLA-mismatched graft. Conversely, a PBSC or HLA-mismatched graft, along with minimal aGVHD prophylaxis, could be acceptable for a recipient harboring the *PTPN22* C/C genotype. In addition, a recipient with the −1123G/G or G/C genotype may require a bone marrow or cord blood graft to avoid aGVHD. This may apply especially to recipients with a benign disease, such as severe aplastic anemia or primary immunodeficiency, in whom relapse does not matter.

A previous study investigated the impact of the *PTPN22* +1858C>T variant on transplantation outcomes in a cohort of European patients who underwent hematopoietic stem cell transplantation for hematologic malignancies [36]. Although a relatively small number of patients were included in that analysis, the authors found that the donor +1858C/C genotype was consistently linked with severe bacterial infections [36]. Another study [37] showed that recipient–donor pairs carrying 2 or more PTPN22 −1858T alleles were at increased risk for grade III-IV aGVHD, but not for grade II-IV aGVHD. Although determining whether such associations are also present in Japanese patients is not possible, because the +1858C>T variant is not polymorphic in Asian populations [2,3,5], these results might support involvement of the *PTPN22* gene in the pathophysiology of aGVHD, as suggested in the present study.

In conclusion, our data suggest that the specific *PTPN22* variant affects prognosis after unrelated donor BMT. Thus, *PTPN22* genotyping in transplant donors and recipients can be a useful tool for evaluating pretransplantation risk and, in combination with other known risk factors, can form the basis for tailoring individual treatment strategies. Nonetheless, care should be taken when drawing conclusions from our data; experimental evidence is needed to verify the effects of *PTPN22* variations. Moreover, the present study did not include adjustment for multiple testing, because the analyses were conducted in an exploratory context, and thus the interpretation of analyses in the subgroups should be taken into account. Finally, transplantation outcomes, including aGVHD and relapse are multifactorial, and single polymorphisms in one cytokine gene are unlikely to determine the majority of outcomes. Further studies are needed to ascertain whether the findings of this study can be extended to other stem cell sources or to HLA-mismatched transplantation, and to validate these data in other ethnic groups.

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Authorship statement: J. Luis Espinoza and Akiyoshi Takami designed research and wrote the manuscript. J. Luis Espinoza performed experiments. Akiyoshi Takami analyzed data and performed statistical analysis. All authors contributed to data and sample collection and reviewed the manuscript.

SUPPLEMENTARY DATA

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.bbmt.2012.09.014.

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

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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 (Ha: $S_i \neq \overline{S_i}$, 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

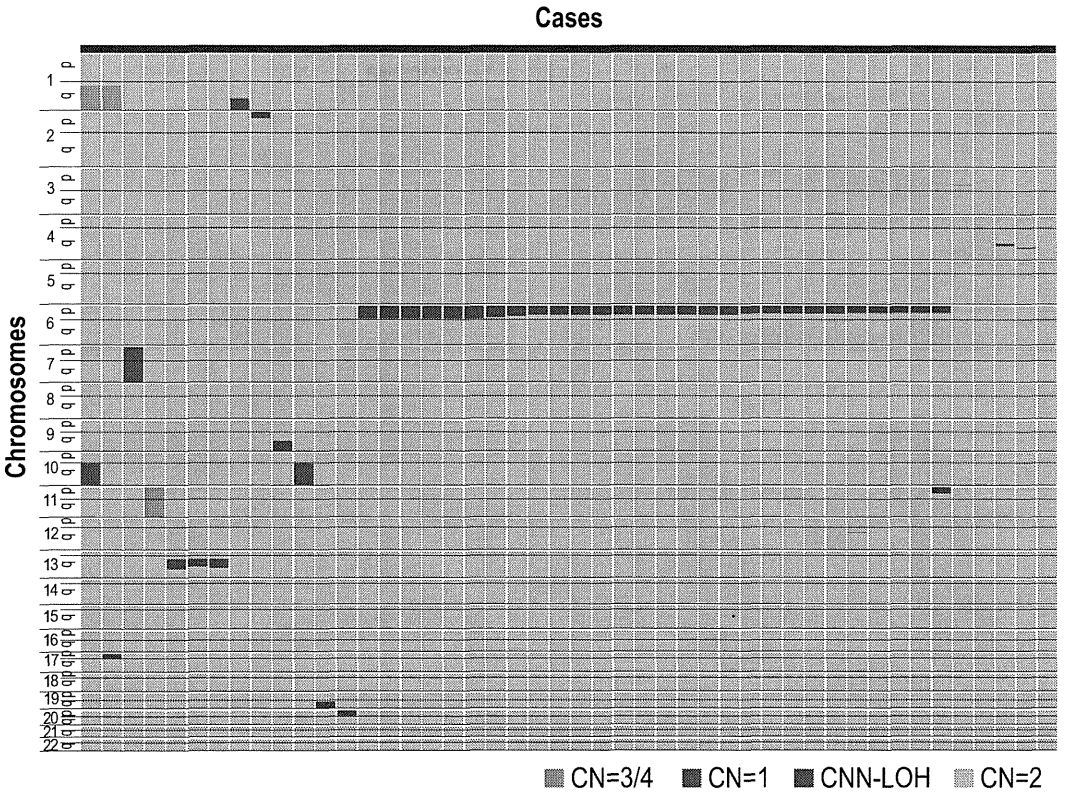

Figure 1. Copy number changes and allelic imbalances in 46 of the 306 AA cases. The copy number changes and allelic imbalances (or CNN-LOHs) in each case are summarized in the chromosomal order vertically for 46 AA cases with copy number abnormalities. Gains and losses, as well as CNN-LOHs, are shown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

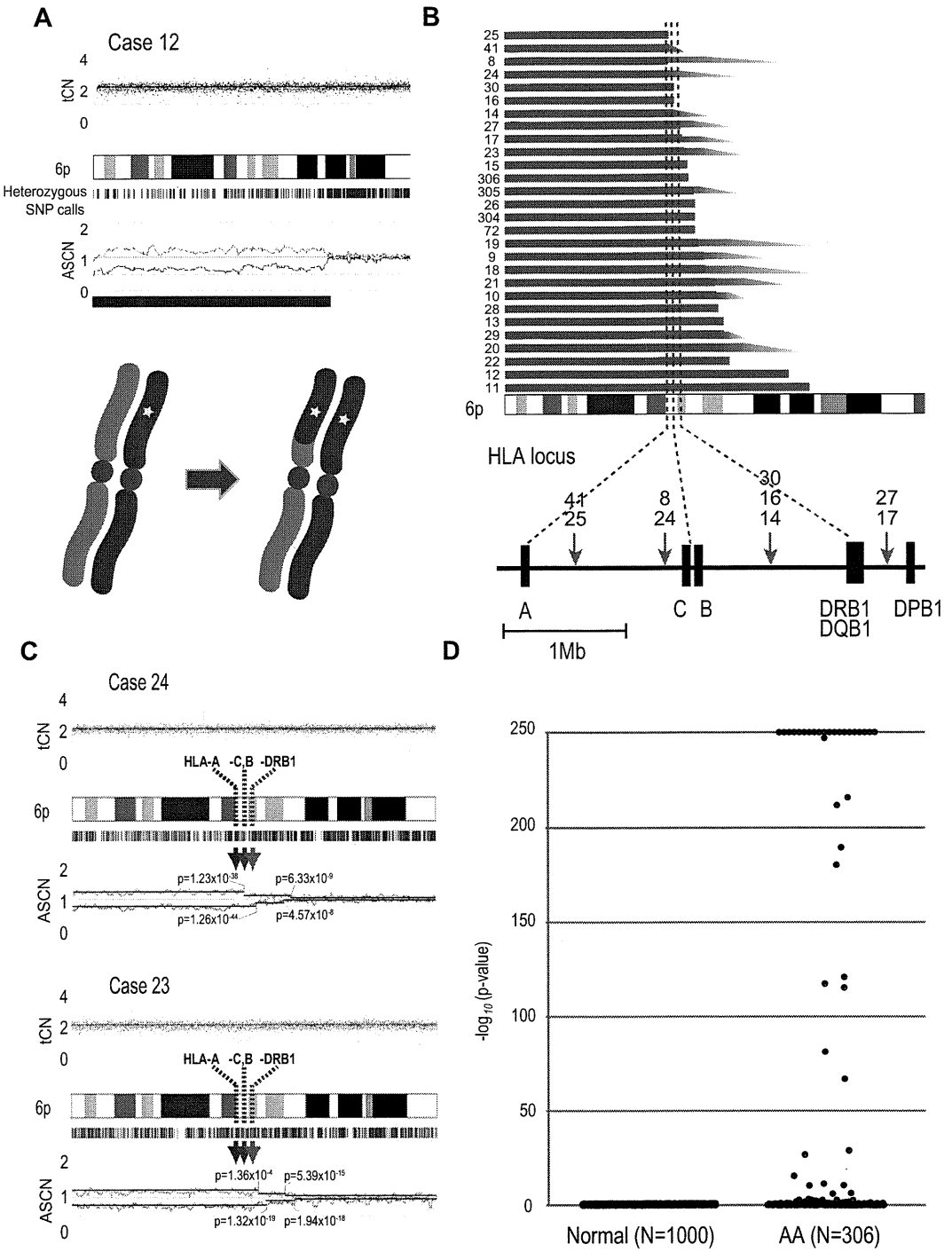

Figure 2. Acquired 6pLOHs in AA patients that target the HLA locus. (A) Typical CNAG outputs in SNP array analysis showing CNN-LOH (purple line) that appears as significant dissociation in allele-specific copy number graphs (red and green lines) from the baseline with normal total copy numbers (tCN; 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

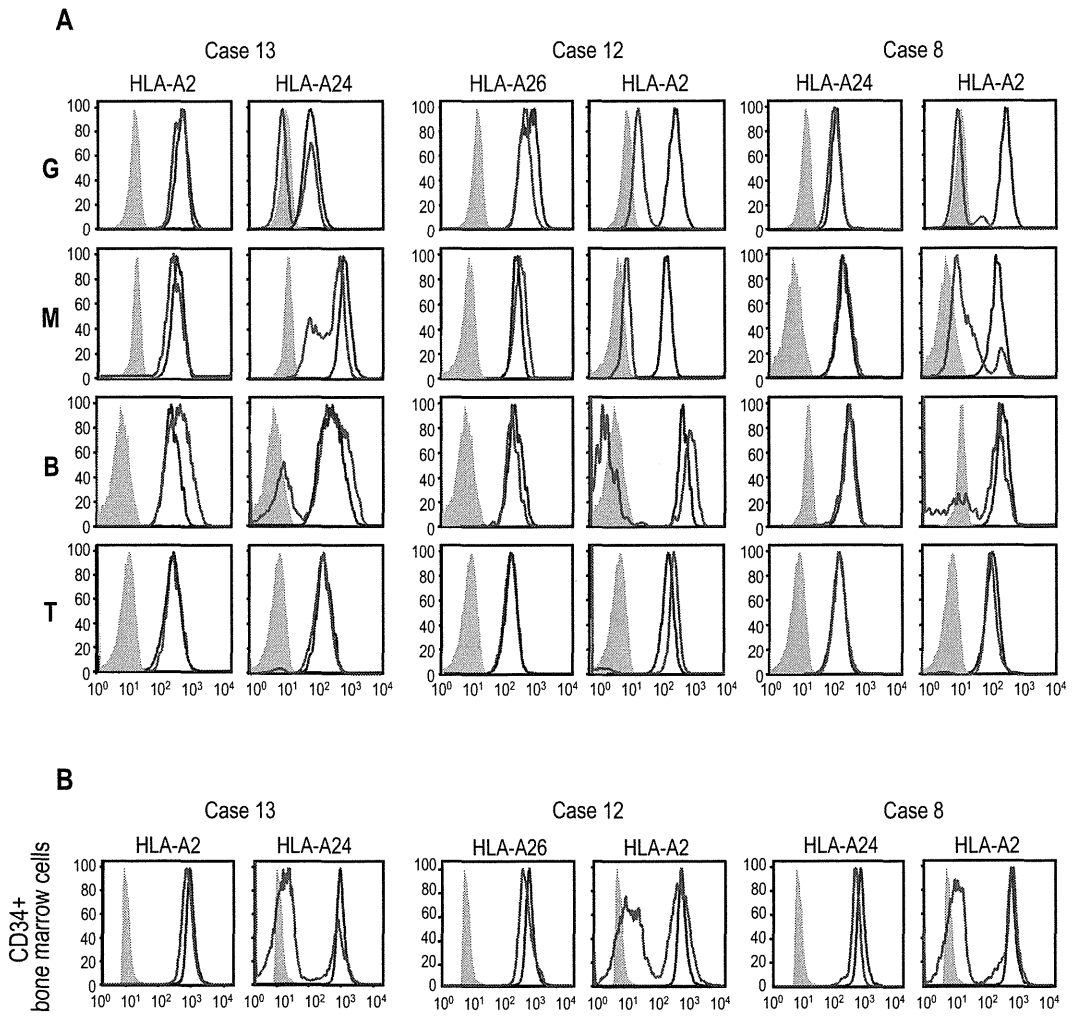

Figure 3. Uniparental expression of HLA in AA cases with CNN-LOH in 6p. Allele-specific expression of HLA-A antigens in AA specimens was examined by flow cytometry using monoclonal antibodies that specifically recognize the indicated HLA types (red lines), where leukocytes from healthy persons were used 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.

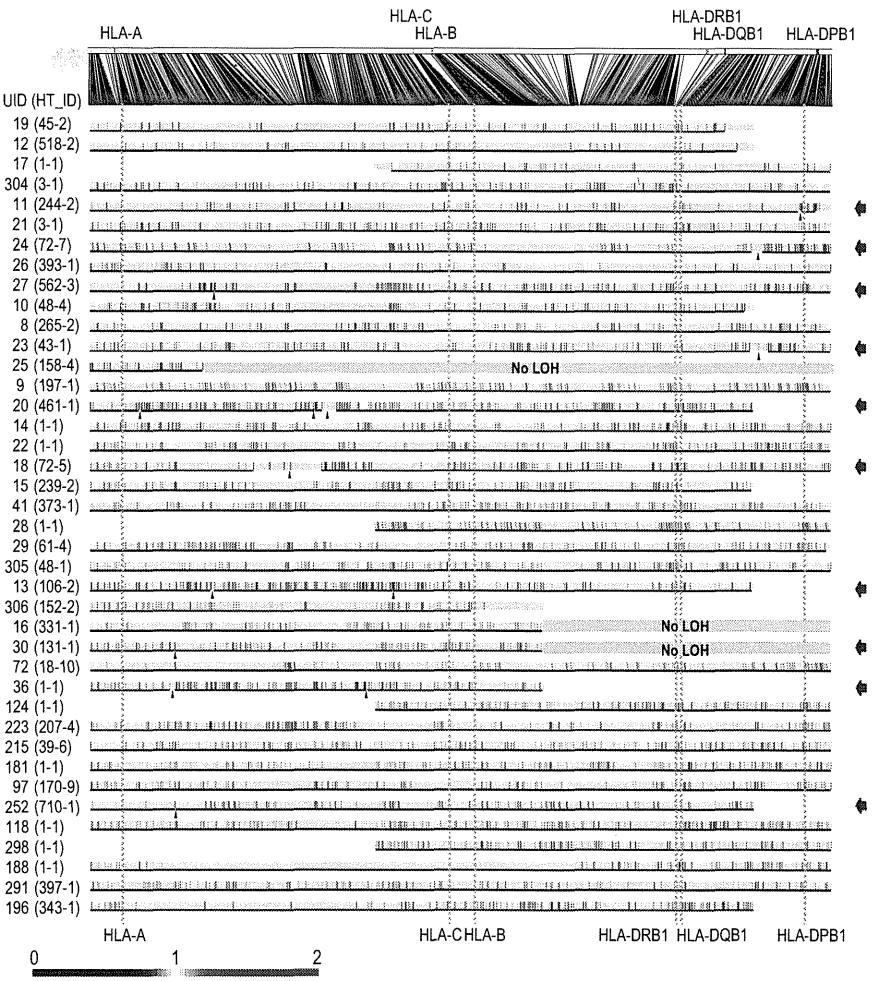

Figure 4. Imputation of missing HLA haplotypes. The observed allelic copy numbers at heterozygous SNP sites along each candidate SNP haplotype are color-coded as indicated at the bottom. Green bars showed the SNPs that are incompatible with the patient's genotype. Case IDs and haplotype ID (HT_ID) are 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 (χ^2 test)	Odds ratio (95% CI) (vs no risk alleles)
A*02:01	103	1173	1276	2.5×10^{-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	1.8×10^{-6}	1.95 (1.48–2.58)
All risk alleles	268	3250	3518	1.3×10^{-7}	1.75 (1.42–2.17)
No risk alleles	139	2956	3095	—	—

— indicates not applicable.

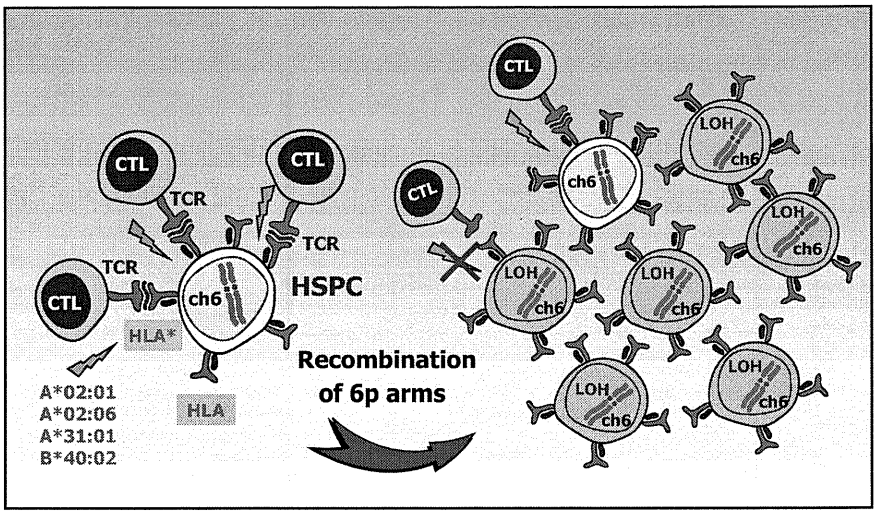

Figure 5. A proposed mechanism for escape hematopoiesis in 6pLOH(+) AA. In AA, the targets of CTLs are the HSPCs that present some auto-antigen through particular class I HLA molecules, including HLA-A*02:01, A*02:06, A*31:01, and B*40:02. In the presence of these autoimmune insults, the HSPCs that lose 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.

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 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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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 (6×10^6 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 0.5×10^9/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.

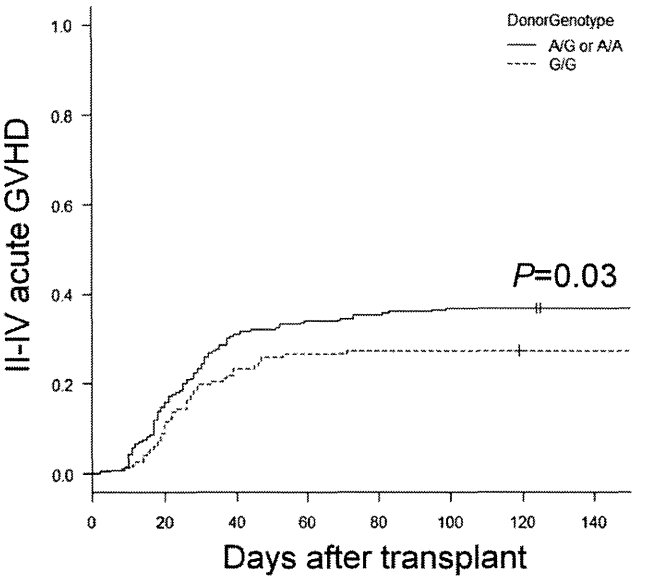

Figure 1. The estimated cumulative incidence curve of grades II–IV acute GVHD according to the donor IL-17 genotype. The solid line represents the donor 197A/G or A/A genotype, and the dashed line represents the donor 197G/G genotype.
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Table 4. The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