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BIOLOGY

Polymorphisms in Genes That Regulate Cyclosporine Metabolism Affect Cyclosporine Blood Levels and Clinical Outcomes in Patients Who Receive Allogeneic Hematopoietic Stem Cell Transplantation

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In patients who received allogeneic hematopoietic stem cell transplantation (HSCT), we investigated the correlations between single nucleotide polymorphisms (SNPs) in genes that regulate cyclosporine metabolism and clinical outcomes. All patients received sibling-matched HSCT. DNA samples of patients and donors were analyzed for 4 SNPs: MDR1 +1236C>T (rs1128503), +2677G>T>A (rs2032582), +3435C>T (rs1045642), and CYP3A5 +6986G>A (rs776746). A total of 156 patients (median age 40 years) were analyzed. Nineteen patients received HSCT for nonmalignant disease. The CYP3A5 +6986AA genotype was associated with a high cyclosporine blood level after transplantation. However, this genotype was not related to any particular clinical outcome. In contrast, the MDR1 +1236C>T SNP was correlated with specific clinical outcomes. When neither the donor nor the recipient had the CC genotype of MDR1 +1236, patients had lower creatinine levels ($P < .001$) and less transplantation-related mortality (TRM) ($P = .012$). These patients also showed longer overall survival (OS) in both univariate ($P = .003$) and multivariate ($P = .003$) analyses. Although the CYP3A5 +6986AA genotype was correlated with a high blood cyclosporine concentration, lack of the MDR1 +1236CC genotype in both the donor and recipient was correlated with less TRM and a longer OS in patients who received allogeneic HSCT.

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INTRODUCTION

Cyclosporine is 1 of the most commonly used immunosuppressants in transplantation medicine and is used in kidney [1], liver [2], and hematopoietic stem cell transplantations (HSCTs). Cyclosporine is extensively metabolized by the cytochrome P-450 3A (CYP3A) subfamily of enzymes, with <1% of the administrated dose excreted as unaltered cyclosporine [3]. At the cellular level, cyclosporine is effluxed by the P-glycoprotein, the protein product of the *MDR1* gene. P-glycoprotein, particularly in the small intestine, limits the absorption of cyclosporine by the active extrusion of cyclosporine from an enterocyte back into the gut lumen [4]. In addition, CYP3A and MDR1 in liver and biliary canaliculi can accelerate the secretion of the drug into the bile and are largely responsible for the drug clearance of cyclosporine systemically [5].

Thus, theoretically, the blood level of cyclosporine is at least partly determined by the functional activity of CYP3A and P-glycoprotein. There are genetic polymorphisms known to affect the functional activity of CYP3A and P-glycoprotein [6,7]. Several studies have investigated the association between these polymorphisms and the blood level of cyclosporine in renal transplantation [7-9]. It is well known that the blood level of cyclosporine is an important measurement in renal transplantation because cyclosporine daily doses must be adjusted

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based on whole-blood cyclosporine concentrations in order to improve the efficacy and reduce the toxicity of cyclosporine treatment [8].

Of the CYP3A family genes, CYP3A4 is the dominant enzyme and CYP3A5 the second most important in the human liver and small intestine [10]. To date, a number of single nucleotide polymorphisms (SNPs) in the CYP3A4 and CYP3A5 genes have been identified and published on the Human Cytochrome P450 Allele Nomenclature Committee home page (http://www.imm.ki.se/CYPalleles). Among these polymorphisms, only the CYP3A5 +6986G>A (rs776746) is present in more than 5% of the Asian population. There are also 3 SNPs in the MDR1 gene: +1236C>T (rs1128503), +2677G>T>A (rs2032582), and +3435C>T (rs1045642) that are present in >5% of the Asian population. These aforementioned SNPs are known to be functional and correlate with expression of CYP3A5 [11] and MDR1 [12].

Unfortunately, in contrast to renal transplantation, few are available regarding the correlation between these SNPs and the clinical outcomes of patients receiving allogeneic HSCT. In allogeneic HSCT, cyclosporine toxicity appears to be dose related [13], and there is a correlation between cyclosporine levels and clinical outcomes. In a study by Yee et al. [14], development of acute graft-versus-host disease (GVHD) was related to trough cyclosporine blood level. However, researchers also believe that there must be intricate interactions that control immune responses other than mere cyclosporine blood level that may affect development of GVHD or transplantation-related mortality (TRM). It is possible that SNPs in the MDR1 gene may affect clinical outcomes in patients who received allogeneic HSCT because the P-glycoprotein enhances the energy-dependent cellular efflux of cyclosporine in enterocytes and other cells.

To summarize, previous studies regarding SNPs in the CYP3A subfamily and MDR1 genes were performed almost exclusively in renal transplant patients and used a pharmacokinetic approach that focused primarily on the cyclosporine blood level. However, the clinical impact of these SNPs on allogeneic HSCT has yet to be studied.

In this study, we tried to define the impact of the aforementioned SNPs on allogeneic HSCT by elucidating the correlations between these SNPs and cyclosporine blood levels and clinical outcomes, including TRM and GVHD, after allogeneic HSCT. Our assumptions were as follows: (1) as CYP3A5 is related to the metabolism of cyclosporine, an SNP in this gene would affect the blood level of cyclosporine. (2) SNPs in the MDR1 gene may be associated with changes in clinical outcomes after allogeneic HSCT because P-glycoprotein is related to the cellular efflux of cyclosporine at the cellular level. Also, we performed genotyping of CYP3A5 of the recipient and MDR1 of both donor and recipient.

Design and Methods

Study population

Adult (≥15 years) patients who had received allogeneic HSCT between 2000 and 2010 and agreed to donate blood samples for genetic testing were included in this study. Data regarding patient demographics, laboratory test profiles, TRM, and overall survival (OS) were obtained by medical record review.

Patients were categorized into 3 classes (high-, intermediate-, and low-risk groups) according to the severity of their underlying disease. Diseases that led to placement in the high-risk group included leukemias, lymphomas, renal cell carcinoma, and myelodysplastic syndromes that were not in complete remission, whereas a patient with leukemia or lymphoma in complete remission was placed in the intermediate risk group. Patients with nonmalignant diseases, such as aplastic anemia or sickle cell anemia, were categorized as low risk. Assessed clinical outcomes included the baseline creatinine to peak creatinine ratio, the baseline bilirubin to peak bilirubin ratio, the peak aspartate aminotransferase, and alanine aminotransferase during 2 weeks after HSCT. The development of acute GVHD (aGVHD), the TRM, and the OS duration were also measured. OS was calculated from the time of HSCT to death from any cause.

Conditioning regimen for allogeneic HSCT varied according to the type of underlying disease and the condition of the patient. For myeloablative HSCT, combination of busulfan and cyclophosphamide was mainly used for malignant disease, wheres combination of antithymocyte globulin and cyclophosphamide (±fludarabine) was used for nonmalignant disease. For nonmyeloablative HSCT, the combination of fludarabine and melphalan was mainly used. The prophylactic regimen against GVHD used at the Seoul National University Hospital is composed of cyclosporine and methotrexate. In myeloablative HSCT, cyclosporine is administered through continuous intravenous infusion at a dose of 5 mg/kg from day −2 to day 3. The dose is then changed to 3 mg/kg (again administered via continuous intravenous infusion) from day 3 to day 14 [15]. In nonmyeloablative HSCT, cyclosporine is administered through continuous intravenous infusion at a dose of 3 mg/kg from day −1 to day 30 [16]. T cell depletion is not performed in either myeloablative or nonmyeloablative HSCT. After the infusion period, subsequent dose modification is based on blood cyclosporine levels.

The study protocol was reviewed and approved by the institutional review board of the Seoul National University Hospital, and the recommendations of the

Declaration of Helsinki for biomedical research involving human subjects were followed.

Genotyping

The genotyping for the MDR1 polymorphisms +1236C>T (rs1128503) and +3435C>T (rs1045642) and the CYP3A5 polymorphism +6986G>A (rs776746) were performed using the TaqMan fluorogenic 5′ nuclease assay (ABI, Foster City, CA). The final volume of the polymerase chain reaction (PCR) was 5 μL and contained 10 ng of genomic DNA, 2.5 μL of TaqMan Universal PCR Master Mix, and 0.13 μL of 40× Assay Mix (Assay ID C___7586662_10 for rs1128503, C__7586657_20 for rs1045642, and C__26201809_30 for rs776746). The thermal cycle conditions were as follows: 50°C for 2 minutes to activate the uracil N-glycosylase and prevent carryover contamination, then 95°C for 10 minutes to activate the DNA polymerase, followed by 45 cycles of 95°C for 15 seconds and 60°C for 1 minute. All PCRs were performed using 384-well plates and a Dual 384-Well GeneAmp PCR System 9700 (ABI), and the endpoint fluorescent readings were performed with an ABI PRISM 7900 HT Sequence Detection System (ABI). Duplicate samples and negative controls were included to ensure the accuracy of the genotyping.

The genotyping of the MDR1 polymorphism +2677G>T>A was performed via the SNaPshot method. The SNaPshot assay was performed according to the manufacturer's instructions (ABI PRISM SNaPShot Multiplex kit). Analysis was performed with Genemapper software (version 4.0; Applied Biosystems, Bedford, MA). The primer set used for the SNaPshot assay was as follows: Forward primer: TTGTTGAAATGAAAATGTTGTCTG; Reverse primer: AAAATAACACTGATTRGAATACTTTA CTCTACT. The melting temperature used for this assay was 55°C.

Statistical Analysis

Statistical analyses of 2 × 2 contingency tables of categorical variables were performed using the Pearson's chi-square test or the Fisher exact test, as appropriate. For parametric continuous variables, we performed the Student *t* test or analysis of variance (ANOVA) for comparisons. For nonparametric variables, the Kruskal-Wallis test was used. The median durations of OS were calculated using the Kaplan-Meier method, and comparisons between groups were made with the log-rank test. The impact of continuous numeric variables on clinical outcomes was calculated using logistic regression and a Cox regression model. Multivariate analysis was performed using a logistic regression model for response and Cox regression models for OS. All statistical tests were 2 sided, and significance was defined as $P < .05$. All analysis was performed using the Statistical Package for the Social Sciences for Windows Version 12.0 (IBM, Chicago, IL).

Table 1. Baseline Characteristics of 156 Patients Who Received Allogeneic HSCT

	Number of Patients (%)	Number
Age (median, range)		40.3 (16.1-71.0)
Gender		
Male	90 (57.7)	
Female	66 (42.3)	
Stem cell source		
Peripheral blood	76 (48.7)	
Bone marrow	73 (46.8)	
Both	7 (4.5)	
Risk of underlying disease		
Active malignancy*	87 (55.8)	
Malignancy in CR	50 (32.1)	
Benign disease	19 (12.2)	
Conditioning method		
Myeloablative	79 (50.6)	
Nonmyeloablative	77 (49.4)	
Cyclosporine blood level (mean, range)		
Day 1 (ng/dL)		471.6 (82-2200)
Day 3 (ng/dL)		445.6 (87-7120)
Day 7 (ng/dL)		326.7 (67-1364)
Day 30 (ng/dL)		300.3 (39-960)
Acute GVHD development		
No	109 (69.9)	
Grade 1	4 (2.6)	
Grade 2	22 (14.1)	
Grade 3	10 (6.4)	
Grade 4	11 (7.1)	
Creatinine elevation after HSCT (>2-fold)		
Yes	54 (34.6)	
No	100 (64.1)	
Unknown	2 (1.3)	
Bilirubin elevation after HSCT (>2-fold)		
Yes	116(74.4)	
No	38 (24.4)	
Unknown	2 (1.3)	

*Malignancy included acute leukemia, chronic leukemia, myelodysplastic syndrome, non-Hodgkin lymphoma, multiple myeloma, and renal cell carcinoma.

RESULTS

Patient Characteristics

A total of 156 patients were included in this study. The median age of the patients was 40.3 years (range: 16.1-71.0 years), and the male-to-female ratio was 90:66. The most common underlying disease in the patients that led to allogeneic HSCT was acute myeloid leukemia (n = 53; 34.0%). Overall, 137 patients received allogeneic HSCT for malignant disease. Among the 137 patients, 50 patients were in complete remission, whereas 87 patients were not. Seventy-seven (49.4%) patients received nonmyeloablative HSCT. The source of the stem cells used in the HSCT was peripheral blood in 76 patients, bone marrow in 73 patients, and both in 7 patients. Fifty-four

Table 2. Allele and Genotype Frequencies of the MDR1 and CYP3A5 Genes in 156 Patients

SNP	Allele Frequencies			Genotype Frequencies					
MDR1 +1236	C	T		CC	CT	TT			
Donor	38.8%	61.2%		15.4%	44.9%	37.2%			
Recipient	39.7%	60.3%		15.4%	46.2%	35.3%			
MDR1 +2677	G	T	A	GG	GT	GA	TT	AA	TA
Donor	38.4%	40.8%	20.9%	17.3%	28.8%	10.9%	16.7%	6.4%	16.7%
Recipient	46.0%	36.2%	17.8%	21.2%	30.1%	15.4%	11.5%	1.3%	16.0%
MDR1 +3435	C	T		CC	CT	TT			
Donor	60.5%	39.5%		34.0%	50.0%	13.5%			
Recipient	64.8%	35.2%		40.4%	45.5%	11.5%			
CYP3A5 +6986	G	A		GG	GA	AA			
	80.1%	19.9%		62.8%	29.5%	4.5%			

patients experienced greater than 2-fold elevations in creatinine after HSCT. GVHD that was scored at greater than grade 3 developed in 21 patients (13.5%), and the TRM rate was 30.1% During median follow-up time of 89.1 months, 99 patients died. The median OS duration of the patients was 15.9 months. The risk group a patient was placed in based on underlying disease was found to be strongly predictive of OS duration in these patients ($P < .001$), as being in the high-risk group was inversely associated with survival. These characteristics are summarized in Table 1.

Genotyping Results

Mutant allele and genotype frequencies are shown in Table 2. The genotype frequencies were not significantly different from the Hardy-Weinberg equation. A total of 36.5% (54 of 148) of the patients were in perfect linkage disequilibrium for the MDR1 +3435 and +1236 genotypes. Although strong associations were observed between MDR1 SNPs, perfect linkage disequilibrium was not observed in >50% of patients. Hence, the impact of individual SNPs on clinical outcome and cyclosporine blood level was performed without consideration of linkage disequilibrium. The MDR1 genotype and the CYP3A5 genotype did not show significant linkage disequilibrium.

Impact of Genotype Results on Cyclosporine Blood Level

Cyclosporine blood levels were measured in 89, 132, 139, and 133 patients on day 1, day 3, day 7, and day 30, respectively. The CYP3A5 +6986 AA genotype was associated with a high blood level of cyclosporine at day 1 ($P = .040$) and was marginally associated with an elevated cyclosporine blood level at day 3 ($P = .064$) (Figure 1). However, there were no significant associations found between a MDR1 genotype and the cyclosporine blood level.

Impact of Genotype Results on Clinical Outcome

The CYP3A5 +6986 genotype did not have predictive value in terms of clinical outcomes. There was no predictive value in the MDR1 genotype of either the stem cell donor or the recipient. However, when the MDR1 genotypes of the donor and the recipient were considered together, the MDR1 +1236 genotype was predictive of clinical outcomes.

When the MDR1 +1236 genotype of both the donor and the recipient were considered together, patients were able to be categorized into 3 groups: (1) both the donor and the recipient possessed the CC genotype (n = 10), (2) both the donor and the recipient possessed a non-CC genotype (n = 104), and (3) the

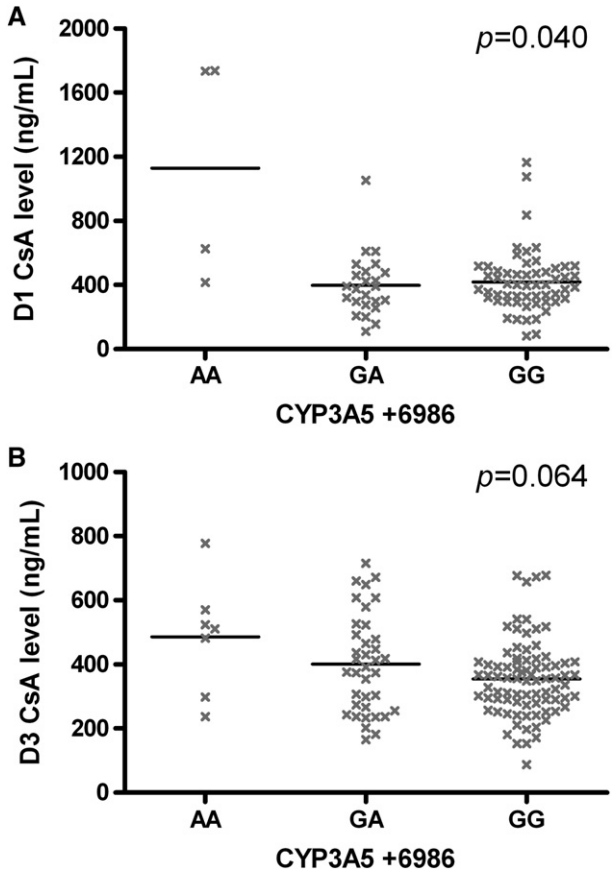

Figure 1. Associations between the CYP3A5 +6986 SNP and cyclosporine blood levels at day 1 and day 3. The CYP3A5 +6986AA genotype was associated with high cyclosporine blood levels at day 1 (A) and day 3 (B).

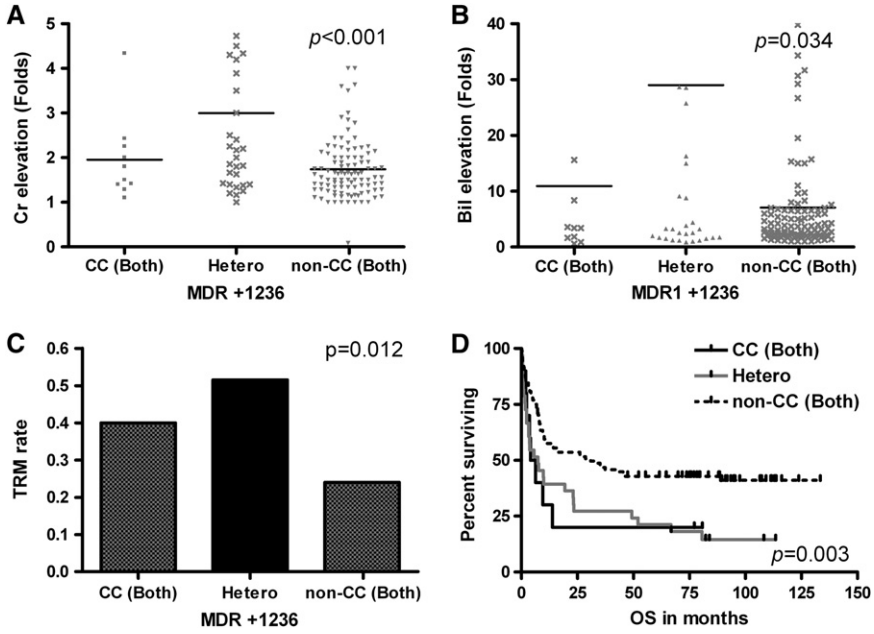

Figure 2. Impact of the MDR1 +1236 SNP on the clinical outcome of patients who received allogeneic HSCT. When both the donor and the recipient did not possess the +267 CC genotype, patients experienced lower elevations in creatinine (A) and bilirubin (B), less TRM (C), and longer OS (D). *The bar designates mean value. In panels (A) and (B), the values that exceeded the y-axis maximum are not shown.

donor and the recipient possessed different genotypes (n = 33). When both the donor and the recipient did not possess the CC genotype of MDR1 +1236, patients displayed significantly lower elevations in alanine aminotransferase ($P = .022$), aspartate aminotransferase ($P = .036$), creatinine ($P < .001$), and bilirubin ($P = .034$) after allogeneic HSCT than the other patients. This patient subset also experienced less TRM ($P = .012$) and survived longer ($P = .003$) (Figure 2). In multivariate analysis that considered a patient's age, risk group, stem cell source, conditioning method, and MDR1 +1236 genotype, the MDR1 +1236 genotype was found to be an independent prognostic factor for OS ($P = .003$) (Table 3). However, the MDR1 +1236 genotype was not correlated with the development of aGVHD ($P = .201$).

DISCUSSION

In contrast to renal transplantation, little is known about the impact of MDR1 and CYP3A subfamily SNPs on allogeneic HSCT. Recently, Japanese researchers have studied the impact of CYP3A5 and MDR1 SNPs on neurotoxicity in patients receiving allogeneic HSCT. Although performed with a small number of patients (n = 30), the study indicated that the MDR1 +1236C>T polymorphism is important in the development of neurotoxicity [17]. Likewise, although Kim et al. [18] asserted that the MDR1 +2677 SNP would have correlation with survival receiving allogeneic HSCT previously, the study focused only on OS, which depends on multiple parameters. Hence, the interpretation of the study result by Kim et al. [18] is dubious. We believe that, to specifically analyze the clinical impact of MDR1 and CYP3A subfamily SNPs on allogeneic HSCT, more precise study design focusing on specific short-term events is necessary.

In our study, a CYP3A5 polymorphism was found to be correlated with cyclosporine blood levels during the first 3 days after HCST. The CYP3A5 +6986G>A SNP was recently reported to be a frequently occurring SNP within intron 3 of CYP3A5 and the primary cause of polymorphism in the CYP3A5 protein [19]. The presence of the CYP3A5 +6986 AA genotype results in a splicing defect leading to the absence of CYP3A5 activity [20]. This finding correlates well with our result that indicated high cyclosporine blood levels in patients with the CYP3A5 AA genotype. We believe that the reason this polymorphism did not impact the day 7 and day 30 blood levels was because the attending physicians altered the dose of administered cyclosporine based on the day 1 and day 3 blood levels. Also, although there was a significant association between the CYP3A5 +6986 genotype and the blood level of cyclosporine, the genotype of CYP3A5 did not affect clinical outcome. This agrees with a previous study that failed to demonstrate a direct correlation between cyclosporine level and the occurrence of GVHD or TRM [14]. By the way, although our results are theoretically justified, because the number of patients who harbored the AA genotype was small, our results should be interpreted cautiously and we believe that further confirmatory testing is necessary.

The most interesting findings were in regard to the MDR1 +1236 polymorphism. The combination of the donor and recipient not having the MDR1 +1236 CC

Table 3. Hazard Ratio (HR) and *P* Value of Age, Stem Cell Source, Conditioning Method, Risk of Underlying Disease, and MDR1 +1236 Genotype for Death in Multivariate Analysis

	Overall Survival			
		95% Confidence Interval		
	HR	Lower	Upper	*P* Value
Age	1.018	0.998	1.039	.077
Stem cell source				.969
Peripheral blood	1			
Bone marrow	1.021	0.563	1.849	
Both	1.172	0.336	4.081	
Conditioning method				.573
Nonmyeloablative	1			
Myeloablative	1.200	0.637	2.263	
Risk of underlying disease				.008
Nonmalignant disease	1			
Malignancy in remission	2.357	0.784	7.089	
Active malignancy	4.098	1.403	11.967	
MDR1 +1236 genotype				.003
CC (both)	1			
Hetero	0.709	0.312	1.613	
Non-CC (both)	0.367	0.171	0.789	

genotype was associated with a favorable outcome after allogeneic HSCT. This association with a favorable outcome was indicated with various clinical parameters. However, the MDR1 +1236C>T polymorphism is known to be silent and is linked to the functional SNPs MDR1+3435C>T and MDR1 +2677G>T>A [21], and the MDR1 +3435TT genotype is known to express lower levels of P-glycoprotein in peripheral blood mononuclear cells [22] and duodenal cells [12]. Thus, our results indirectly suggest that the favorable outcome associated with not having the MDR1 +1236 CC genotype may be because of a low level of cellular P-glycoprotein expression. Theoretically, low P-glycoprotein expression would lead to high intracellular cyclosporine concentration in target cells, including white blood cells. We believe high intracellular cyclosporine concentration is attributable to low TRM and long OS observed in our study patients. Theoretically, one can postulate that not having the MDR1 +1236 CC genotype should lessen the probability of developing aGVHD. However, because not having the MDR1 +1236CC genotype did not have any significant relationship with the development of aGVHD, the underlying mechanism of these phenomena is not clear, and further study is necessary.

We performed genotyping on both the donors and the recipients for MDR1 SNPs because it was not certain if P-glycoprotein expression in the donor cells or the recipient would be important for the clinical outcome of the patients. Although neither the genotype of the donor nor the recipient independently influenced the clinical outcome of the patients, they had strong predictive value when considered together. We think this is because P-glycoprotein expression in both the duodenum (which consists of recipient cells) and peripheral blood cells (which are from the donor) is important for the clinical outcome.

As this study has the limitation of being a retrospective study, our results might be recognized by some as exploratory data. However, our study was not small (n = 156), and the clinical impact of MDR1 +1236 SNP was demonstrated via the measurement of multiple parameters. Hence, we hypothesize that genotyping for these SNPs may have important clinical value in the near future.

In conclusion, among patients receiving allogeneic HSCT, the CYP3A5 +6986AA genotype was associated with a high blood cyclosporine concentration, whereas the situation in which the donor and the patient did not have the MDR1 +1236 CC genotype was significantly associated with less TRM and longer OS.

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