Effect of Donor *NKG2D* Polymorphism on Relapse after Haploidentical Transplantation with Post-Transplantation Cyclophosphamide

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Highlights

- PT/Cy-haplo from rs1049174 CC donors was associated with a reduced risk of relapse

- PT/Cy-haplo from rs1049174 CC donors was associated with better OS in non-AML patients

- The GVL effect via *NKG2D* would merit further testing and validation in PT/Cy-haplo

ORIGINAL RESEARCH

Title:
Effect of donor *NKG2D* polymorphism on relapse after haploidentical transplantation with post-transplant cyclophosphamide

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Running Head:
NKG2D on relapse after PT/Cy-haplo

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ABSTRACT

The NKG2D-mediated cytotoxicity is regulated by the single nucleotide polymorphism rs1049174, and its anti-tumor effect has been observed in various clinical settings. There are no data concerning the influence of the donor rs1049174 polymorphism on *HLA*-haploidentical allogeneic hematopoietic cell transplantation using post-transplant cyclophosphamide (PT/Cy-haplo). To investigate the effect of donor NKG2D gene polymorphism on PT/Cy-haplo, we retrospectively reviewed 91 consecutive PT/Cy-haplo patients at our institute, and rs1049174 of the NKG2D gene was genotyped in both donors and patients. In the patients who received PT/Cy without anti-thymocyte globulin (ATG) as graft-versus-host disease prophylaxis, the 2-year cumulative incidence of relapse/progression (RI) of PT/Cy-haplo from rs1049174 CC donors was lower than that from rs1049174 CG/GG donors (25.0% vs. 52.4%, P = 0.041), and rs1049174 CC donors were associated with a decreased risk of relapse/progression (adjusted hazard ratio, 0.2; 95% confidence interval, 0.0–0.6; P = 0.007). Furthermore, a beneficial effect of rs1049174 CC donors on the overall survival and RI was observed in non-acute myeloid leukemia (non-AML) patients. In conclusion, this study demonstrated that PT/Cy-haplo from rs1049174 CC donors was associated with a decreased risk of

relapse/progression in the patients who received PT/Cy-haplo without ATG. In the future, large-scale validation studies will be required to test the significance of donor *NKG2D* polymorphism in the development of a new donor selection algorithm for PT/Cy-haplo.

KEYWORDS

NKG2D gene polymorphism; *HLA*-haploidentical transplantation with post-transplant cyclophosphamide; hematological malignancies; graft-versus-leukemia/tumor effect; donor selection.

Main Text

INTRODUCTION

NKG2D is a transmembrane protein belonging to the C-type lectin-like receptor family, which is encoded by *KLRK1* in chromosome 12.[1] It is expressed as a homodimer on natural killer (NK) cells, $CD8^+$ T cells, γδ T cells, most iNKT cells, some $CD4^+$ T cells and innate lymphoid cells.[1-3] It works as an activating receptor in NK cells and a co-stimulatory receptor in $CD8^+$ T cells, iNKT cells and in some cases in γδ T cells.[4-6] NKG2D ligands, including MHC class I chain-related protein A/B (MICA and MICB) and UL-16 binding protein (ULBP) 1–6, are almost absent in normal cells, although they are up-regulated by cell stress events including cellular transformation and microbial infections, e.g. MICA and MICB in leukemia and MICA and ULBP 1–6 in lymphoma.[5-7]

The *NKG2D*-mediated cytotoxic activity is regulated by the NKG2D gene single nucleotide polymorphism (SNP) rs1049174, and its anti-tumor effect has been observed in various clinical settings.[8] Specifically, it was reported that the incidences of colorectal cancer and human papillomavirus-related cancers varied by SNP rs1049174, from the viewpoint of NK cell activity.[9, 10] However, whether or

not the NKG2D gene polymorphism in cells besides NK cells, including $CD8^+$ T cells, has clinical impact remains unclear.

Only two studies have examined the influence of the donor rs1049174 polymorphism on the clinical outcomes after allogeneic hematopoietic cell transplantation (allo-HCT) for hematologic malignancies. In an unrelated *HLA*-matched allo-HCT setting, patients with standard-risk diseases who received allo-HCT from rs1049174 CG or GG donors were reported to have a higher overall survival (OS) and lower transplant-related mortality than those who received allo-HCT from rs1049174 CC donors, but CG or GG donors were not associated with relapse.[11] Another study reported that a donor rs1049174 polymorphism had no relationship with the clinical outcomes.[12] No data demonstrating the graft-versus-leukemia/tumor (GVL) effect via *NKG2D* in clinical settings have yet been collected. In addition, no study has explored the effect of the donor rs1049174 polymorphism on the outcomes of *HLA*-haploidentical allo-HCT in patients using post-transplant cyclophosphamide (PT/Cy-haplo), which is distinct from anti-thymocyte globulin (ATG)-based *HLA*-haploidentical allo-HCT with regard to the selectivity of alloreactive T cell depletion and immune reconstitution after transplantation.[13-16]

Since *NKG2D* is expressed by $CD8^+$ T cells as well as NK cells[2, 4-6], the potential *NKG2D*-mediated GVL effect may depend on not only NK cells but also T

cells or the NK cell-T cell interaction.[17] Therefore, although the NK cell-mediated GVL effect was reported to be observed in acute myeloid leukemia (AML) (although not in acute lymphoblastic leukemia)[18-20], the *NKG2D*-mediated GVL effect may also be observed in entities other than AML.

In the present study, we investigated the effect of the donor NKG2D gene polymorphism on the clinical outcomes of PT/Cy-haplo to provide a novel donor selection algorithm based on information about the NKG2D gene polymorphism status of PT/Cy-haplo donor candidates.

MATERIALS AND METHODS

Patients, Donors, and Transplantation Procedures

We retrospectively reviewed the clinical information of consecutive patients who received PT/Cy-haplo at our institute between June 2009 and December 2018 and prospectively collected blood samples from both donors and patients. Written informed consent from patients and donors was obtained in cases in which blood samples were collected prospectively from July 2013 onward. Otherwise, we

provided the opportunities to withdraw from the present study at any time for eligible living patients and donors. Moreover, the study information was officially disclosed to the public on the website of the Department of Hematology, Osaka City University Graduate School of Medicine. The study was designed in accordance with the Declaration of Helsinki and the Ethical Guidelines for Human Genome/Gene Analysis Research in Japan and approved by the Ethical Committee of Osaka City University Graduate School of Medicine (Osaka, Japan). We can provide the original data and protocols used for the work after approval of the Ethical Committee of Osaka City University Graduate School of Medicine if we receive a reasonable request (hide_koh@med.osaka-cu.ac.jp).

Patients who were enrolled in the clinical studies at our institute received the following conditioning regimen[14, 21-23]: the intravenous busulfan (Bu)-based conditioning regimen consisted of 15 mg/m^2 fludarabine and 2,000 mg/m^2 cytarabine twice a day on days -11 and -10, 30 mg/m^2 fludarabine once a day on days -6 to -3 and 0.8 mg/kg Bu 4 times a day on days -6 to -3. The melphalan (Mel)-based conditioning regimen replaced Bu with 100 mg/m^2 Mel once a day on day -2. Granulocyte colony-stimulating factor-mobilized T-cell-replete peripheral blood stem cell grafts were infused on day 0.

Graft-versus-host disease (GVHD) prophylaxis of the group with PT/Cy with

ATG consisted of 25 mg/kg PT/Cy once a day on days +3 and +4, tacrolimus that was infused continuously at a targeted blood concentration of 10–15 ng/mL from day +5, oral mycophenolate mofetil from day +5 and 2.0 mg/kg ATG (thymoglobulin) once a day on days -8 and -7. The first 6 patients received 25 mg/kg PT/Cy only on day +3. In the GVHD prophylaxis with PT/Cy without ATG, ATG was withdrawn from the GVHD prophylaxis procedure used in the patients who received PT/Cy-haplo with ATG. If GVHD did not occur, mycophenolate mofetil was discontinued at day +40, and tapering of tacrolimus was attempted in the interval between day +60 and +100 and discontinued by day +180. The other patients were treated in conformity with previously published regimens.[24-27]

Uniform supportive care was provided for all patients.[23] In brief, levofloxacin at 500 mg/day was administered as prophylaxis against bacterial infection from the start of conditioning to either neutrophil engraftment or use of other antibiotics for treatment. Fluconazole at 200 mg/day was administered as prophylaxis against fungal infection from the start of conditioning to either day +100 or use of other antifungal agents for treatment. Acyclovir at 600 mg/day was administered as prophylaxis against herpes simplex virus and varicella zoster virus infection from the start of conditioning to neutrophil engraftment and was reduced to 200 mg/day until either the discontinuation of all immunosuppressants or one year after allo-HCT.

Trimethoprim-sulfamethoxazole was administered as prophylaxis against *Pneumocystis jirovecii* infection. Cytomegalovirus antigenemia, defined as 2/50,000 of C7HRP for patients receiving any glucocorticoid treatment or 5–10/50,000 of C7HRP for other patients, was administered together with ganciclovir or valganciclovir.

NKG2D Gene Polymorphism

The NKG2D gene was genotyped using the TaqMan SNP Genotyping Assay with the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) and results were analyzed using allelic discrimination software program (Applied Biosystems). The assay ID was C___9345347_10 (Applied Biosystems) for rs1049174. The context sequence was TGTGGAGGGTGGGGTTGCACTCTCA[C/G]TGATCTGCTGGCCTTCTCTTCCTTC.

To validate this sequence determination, both this measurement and Sanger sequencing were performed for eight DNA samples (Coriell Institute, Camden, NJ, USA) independent of the study samples, and the consistency was confirmed. The primers were designed using a DNASIS Pro (Hitachi Solutions, Ltd., Tokyo, Japan) as follows: Forward primer, TCAGATATCCCCAAGGCTGCCTCTC; Reverse primer,

ACTGCCCATGCTGTGTCTCTCTGCT. The repository numbers of the eight DNA samples examined using Sanger sequencing were NA18940, NA18944, NA18952, NA18959, NA18961, NA18966, NA19207 and NA19209 (Coriell Institute).

NKG2D genotyping was performed at BML, Inc. (Saitama, Japan), independent of clinical data collection in our institution.

Reconstitution of T cells and NK cells after PT/Cy-haplo Using Flow Cytometry

Reconstitution of T cells and NK cells was examined by flow cytometry on day +30 (23–37), day + 60 (53–67), day +90 (83–97), day +180 (152–208) and day +360 (332–388) after PT/Cy-haplo: single-color $CD4^+$ T cells, $CD8^+$ T cells and $CD56^+$ NK cells were examined between June 2009 and December 2015 (n = 68). $CD4^+$ T cells were recognized by $CD45^+CD3^+CD4^+$; $CD8^+$ T cells were recognized by $CD45^+CD3^+CD8^+$; $CD56^+$ NK cells were recognized by $CD45^+CD3^-CD56^+$ using multicolor flow cytometry between January 2016 and December 2018 (n = 23).

Definitions

All patients were classified according to the hematopoietic cell transplant-

comorbidity index (HCT-CI).[28] Based on the consensus criteria, acute GVHD of grades II to IV was diagnosed and defined as clinically significant.[29]

Regarding the remission status at PT/Cy-haplo, complete remission (CR) for patients with AML was defined in the present study as both CR and CR with incomplete hematologic recovery, according to the European LeukemiaNet recommendations 2017[30]; CR for patients with myelodysplastic syndrome was defined as both CR and marrow CR with respect to the International Working Group response criteria in myelodysplasia 2006[31]; no patients with chronic myelogenous leukemia were considered to be in CR; CR in those patients with acute lymphoblastic leukemia was defined according to a previous study[32]; CR for patients with malignant lymphoma was defined according to The Lugano Classification 2014 and the Response Criteria of Adult T-cell Leukemia/Lymphoma International Consensus Meeting 2009[33, 34]. All patients deemed not to be in CR were considered NCR.

Relapse/progression was defined as the first point at which hematological or pathological evidence of relapse/progression of primary disease was detected after PT/Cy-haplo.[21]

Statistical Analyses

Accordance with Hardy-Weinberg equilibrium was assessed using the χ^2 test. The cumulative incidence of relapse/progression (RI) was estimated with non-relapse mortality (NRM) as a competing risk and compared using the Gray test. Death without acute GVHD, relapse/progression and subsequent transplantation were defined as competing risks for acute GVHD.[35] The overall survival (OS) was estimated by the Kaplan-Meier method and compared using the log-rank test. When the results of the Gray or log-rank test between three groups were significant under the null hypothesis that all of them had the same survival curves, pairwise comparisons were performed using Bonferroni's method. Subsequent transplantation was treated as a failure event.[36] In univariable and multivariable analyses of prognostic factors, the Cox proportional hazards model was used for the OS, and a cause-specific hazard model was used for relapse/progression and NRM. The Fine-Gray hazards model was used to confirm the results of cause-specific hazard model.[37] Hazard proportionality was checked using scaled Schoenfeld residuals and log-minus-log plots. The effect modification of disease was tested by the insertion of first-order interaction terms into hazards models.[38] To assess the effect of *NKG2D* by disease specificity, a subgroup analysis was conducted. T cell reconstitution at each point was compared using the Mann-Whitney U test.

All statistical tests were two-sided, and P values of less than 0.05 were

determined to be statistically significant. Confidence intervals (CIs) were 95%. Statistical analyses were performed using the R version 3.6.2 software program (The R Foundation for Statistical Computing, Vienna, Austria) with the "RcmdrPlugin.EZR"[39] and "genetics" plugins and the SPSS Statistics 24 software program (IBM, Armonk, NY, USA).

RESULTS

Characteristics of Patients and Donors

The NKG2D gene polymorphism data of 91 patients and 91 donors were available in this study. Detailed characteristics of the patients and donors are shown in Table 1. There were 32 patients who received PT/Cy-haplo with ATG (35%) and 59 patients who received PT/Cy-haplo without ATG (65%).

In the entire cohort, the frequencies of donor NKG2D gene polymorphism rs1049174 were 32% (n = 29) for CC, 49% (n = 45) for CG and 19% (n = 17) for GG, and the frequencies of patient NKG2D gene polymorphism rs1049174 were 24% (n = 22) for CC, 55% (n = 50) for CG and 21% (n = 19) for GG (Supplementary Table

1). The genotype frequencies of donors and patients were in accordance with the previously reported frequencies in the Japanese population[9-11] and did not reject the goodness of fit for Hardy-Weinberg equilibrium according to the χ^2 test (donors, $P > 0.999$; patients, $P = 0.456$). In addition, there was a hereditary correlation between the genotypes for patient rs1049174 and for related haploidentical donor rs1049174 (χ^2 test for independence, $P = 0.003$). In further analyses, therefore, the genotype of donor rs1049174 was mainly evaluated because the GVL effects after allo-HCT depend on the alloreactivity of donor cells.[23]

In the patients who received PT/Cy-haplo with ATG, the frequencies of AML and non-AML were 69% and 31%, respectively. In the patients who received PT/Cy-haplo without ATG, the frequencies of AML and non-AML were 54% and 46%, respectively. The median duration of patient follow-up among survivors was 3.5 years (range, 0.7–8.6 years).

The RI, NRM and OS Associated with Donor NKG2D Gene Polymorphism Stratified by GVHD Prophylaxis

The values of the RI, NRM, OS and acute GVHD were compared using a dominant hereditary model (CC vs. CG + GG).[8, 11] In the patients who received

PT/Cy-haplo with ATG, the 2-year RI of patients who received PT/Cy-haplo from rs1049174 CC donors was similar to that of those who received PT/Cy-haplo from rs1049174 CG/GG donors (66.7% vs. 60.0%, *P* = 0.981; Figure 1A). In the patients who received PT/Cy-haplo without ATG, however, the 2-year RI of patients who received PT/Cy-haplo from rs1049174 CC donors was significantly lower than that of those who received PT/Cy-haplo from rs1049174 CG/GG donors (25.0% vs. 52.4%, *P* = 0.041; Figure 1B). PT/Cy-haplo from rs1049174 CC donors did not statistically improve the NRM, OS or acute GVHD in either the patients who received PT/Cy-haplo with ATG or the patients who received PT/Cy-haplo without ATG (Supplementary Figure 1).

To evaluate the effect of each individual donor genotype, clinical outcomes were compared among the three donor genotypes of rs1049174 (i.e. CC vs. CG vs. GG). While no marked differences in the RI, NRM or OS were noted in the patients who received PT/Cy-haplo with ATG, a significant difference in the RI was noted in the patients who received PT/Cy-haplo without ATG (*P* = 0.029; Supplementary Figure 2B). As a post-hoc analysis, pairwise comparisons showed that the RI of PT/Cy-haplo from rs1049174 CC donors was significantly lower than that from rs1049174 GG donors (Bonferroni-adjusted *P* = 0.020).

The results of univariable analyses stratified by GVHD prophylaxis are listed

in Table 2. In the patients who received PT/Cy-haplo with ATG, no factors were associated with a reduced risk of relapse/progression. High HCT-CI scores were associated with an increased risk of NRM. PT/Cy-haplo from 2013 to 2018 was related to a decreased risk of NRM and a longer OS than PT/Cy-haplo from 2009 to 2012. In the patients who received PT/Cy-haplo without ATG, PT/Cy-haplo from rs1049174 CC donors was the only significant factor associated with a reduced risk of relapse/progression, except for remission status. Both NCR patients and those with high HCT-CI scores were associated with increased risks of NRM and a reduced OS. There were almost no differences between these results and those of the Fine-Gray hazards model (Supplementary Table 2).

Multivariable analyses were performed using the donor rs1049174 polymorphism and other previously reported donor-related risk factors (Table 3).[21, 24, 40] Several models limiting the degree of freedom were constructed to stabilize each model[38, 41, 42], although the low number of NRM events was intolerable for most of the models (23 events in the entire population). In the patients who received PT/Cy-haplo with ATG, no donor-related factors were associated with the risk of relapse/progression, NRM or the OS. In the patients who received PT/Cy-haplo without ATG, rs1049174 CC donors were significantly associated with a decreased risk of relapse/progression, independent of other donor-related factors in models 1–

4. In addition, a high infused $CD34^+$ cell dose was associated with a decreased risk of relapse/progression in model 4.

Reconstitution of $CD4^+$ T cells, $CD8^+$ T cells and $CD56^+$ NK cells did not differ to a statistically significant extent between CC and CG + GG at any point (Supplementary Figure 3).

The RI, NRM and OS Associated with Donor NKG2D Polymorphism Modified by Disease Type in the Patients Who Received PT/Cy-haplo without ATG

In the patients who received PT/Cy-haplo without ATG, the effect of the donor rs1049174 polymorphism on the survival was modified by AML or non-AML, as the product term of the interaction analysis for the OS was statistically significant, and the product terms of the interaction analyses for both relapse/progression and NRM were of borderline significance ($P_{interaction}$ for OS = 0.014; $P_{interaction}$ for relapse/progression = 0.096; $P_{interaction}$ for NRM = 0.052). In the non-AML group of the population who received PT/Cy-haplo without ATG, PT/Cy-haplo from rs1049174 CC donors significantly decreased the RI and increased the OS compared with PT/Cy-haplo from rs1049174 CG/GG donors, although the difference in the NRM was not significant (2-year RI, 19.0% vs. 66.7%, P = 0.011; 2-year OS, 75.0% vs.

8.0%, *P* = 0.005; 2-year NRM, 16.7% vs. 26.7%, *P* = 0.624; Figure 2). In contrast, in the AML group of the population who received PT/Cy-haplo without ATG, the RI, NRM and OS of PT/Cy-haplo from rs1049174 CC donors were similar to those with PT/Cy-haplo from rs1049174 CG/GG donors.

To examine the effect of the NKG2D gene polymorphism on patients with lymphoid malignancies, including acute lymphoblastic leukemia and non-Hodgkin lymphoma, a subgroup analysis was performed. When we excluded patients with myelodysplastic syndrome or chronic myelogenous leukemia from the analyses in non-AML patients in the population who received PT/Cy-haplo without ATG, we observed similar results according to the NKG2D gene polymorphism in the RI, NRM and OS of the patients with lymphoid malignancies compared with those of non-AML patients (Figure 2 and Supplementary Figure 4).

Other Additional Analyses

We performed similar analyses of patient NKG2D gene polymorphism (Supplementary Figure 5). RI, NRM and OS were not significantly influenced by the patient *NKG2D* polymorphism, regardless of the use of ATG. In addition, we performed similar analyses of donor NKG2D gene polymorphism in the periods of

2009–2012 and 2013–2018 (Supplementary Figure 6). Furthermore, we performed similar analyses on donor NKG2D gene polymorphism in the subgroup of lymphoid malignancies in CR and NCR (Supplementary Table 4 and Supplementary Figures 7 and 8), and in the subgroup of non-AML with or without central nervous system involvement (Supplementary Table 5 and Supplementary Figures 9 and 10). Based on these analyses, we found the following statistically significant findings: the RI in 2013–2018 was significantly decreased in comparison to 2009–2012 in the rs1049174 CC donors (Supplementary Figure 6); PT/Cy-haplo from rs1049174 CC donors significantly increased the OS of the subgroup of patients with lymphoid malignancies in CR who received PT/Cy-haplo without ATG (Supplementary Figure 7); and PT/Cy-haplo from rs1049174 CC donors significantly decreased the RI and increased the OS in non-AML patients without central nervous system involvement who received PT/Cy-haplo without ATG (Supplementary Figure 10).

DISCUSSION

The present study found that PT/Cy-haplo from rs1049174 CC donors was significantly associated with a decreased risk of relapse/progression in patients

without use of ATG, especially in the non-AML group.

The *NKG2D*-mediated cytotoxic activity is regulated by a haplotype block of hb-1 in the NKG2D gene. Haplotype alleles of hb-1 in the NKG2D gene consist of either high cytotoxic activity-related *HNK1* or low cytotoxic activity-related *LNK1*.[8, 10] The *HNK1* haplotype was shown to be associated with a reduced incidence of various cancers, including colorectal cancer and human papillomavirus-related cancers.[8-10] Most studies on the anti-tumor effect of haplotype alleles of the NKG2D gene hb-1 referred to CC, CG and GG of SNP rs1049174 as genotypes of *LNK1*/*LNK1*, *LNK1*/*HNK1* and *HNK1*/*HNK1*, respectively, because tight linkage disequilibrium between SNP rs1049174 and other SNPs of the NKG2D gene hb-1 was demonstrated, and the *NKG2D* expression on NK cells and $CD8^+$ T cells was reported to depend on the CC, CG or GG status of rs1049174.[8, 10, 43] However, the results in the present study were not consistent with the previously reported anti-tumor effects of the G allele of rs1049174 in the NKG2D gene, i.e. the *HNK1* haplotype. We did not identify the reason for the discrepancy in the results between the present and previous studies. Regarding the mechanism, the following hypothesis was considered: Olson et al. demonstrated that activated donor NK cells lyse alloreactive T cells (i.e., a direct interaction between NK cells and activated T cells) in an *NKG2D*-dependent manner in an *in vivo* model of T cell-mediated

GVHD.[17] Since it is reported that *NKG2D* genotype CC is associated with low NK cytotoxic activity[8,10,43], donor *NKG2D* genotype CC might contribute to less lysis of alloreactive T cells and greater preservation of the GVL effect mediated by T cells. In addition, alloreactive T cells were considerably preserved due to the relatively low doses of PT/Cy used in our protocols.[14, 21-23]. A functional study is needed to test this hypothesis and elucidate the underlying mechanisms.

To our knowledge, this is the first report investigating the influence of the donor rs1049174 polymorphism on clinical outcomes after PT/Cy-haplo. There have been two reports in allo-HCT settings aside from PT/Cy-haplo concerning the effect of the donor rs1049174 polymorphism on the clinical outcomes. Espinoza et al. suggested that patients with standard-risk diseases who received allo-HCT from rs1049174 CG or GG donors have a higher OS and lower transplant-related mortality than those who received allo-HCT from rs1049174 CC donors, but CG or GG donors were not associated with relapse or GVHD development in patients with standard-risk diseases and were not associated with any clinical outcomes in patients with high-risk diseases in an unrelated *HLA*-matched allo-HCT setting.[11] However, these findings were not observed in the present study. This discrepancy might be due to the differences in T cell alloreactivity between PT/Cy-haplo in the present study and unrelated *HLA*-matched allo-HCT in the Espinoza report. Those

authors hypothesized that *NKG2D* played a role in the protection of patients against infections through NK cell cytotoxicity, but we considered that, based on the above observations, including our own findings, not only NK cells but also T cells and the NK cell-T cell interaction might be involved in the *NKG2D*-mediated GVL effect. Another previous report found no relationship between SNP rs1049174 and the clinical outcomes because transplantation was performed in an *HLA*-matched setting, which differed from the PT/Cy-haplo conditions in the present study with regard to immune reconstitution.[12, 14, 15]

In addition, the present study suggested that the GVL effect of the donor rs1049174 polymorphism could be modified by disease type among the patients who received PT/Cy-haplo without ATG, as a beneficial effect of rs1049174 CC donors on the OS as well as the RI was observed only in the non-AML group. Evidence suggests that the GVL effect mediated by NK cells plays a more significant role in the protective on relapse in patients with AML in comparison to those with ALL.[19, 20] In contrast, the GVL effect mediated by T cells can play a more significant role in the protective effect on relapse in patients with CML and lymphoid malignancies.[44, 45] This supports the hypothesis that *NKG2D*-mediated GVL effect might be involved in alloreactive T cells. Moreover, the risk of relapse/progression was not correlated with the development of GVHD in AML patients, and this was consistent with the

above evidence that the GVL effects against AML might be responsible for non-T cell-based alloreactivity, i.e. the NK cell-mediated GVL effect.[18-20, 44, 45] Furthermore, the effect of the donor rs1049174 polymorphism on the risk of relapse/progression remained marginally significant even after adjusting by the donor *KIR2DS1* positivity[21] (Supplementary Table 3). Further studies to investigate the optimal donor selection strategy for PT/Cy-haplo according to disease type and donor *NKG2D* polymorphism would be worthwhile.

Several limitations associated with the present study warrant mention. First, the present study was a small single-center study with a retrospective design. However, almost all patients had participated in clinical trials[14, 21-23], so the heterogeneity of transplantation procedure, biased data collection and missing data were minimized. Second, relatively low doses of PT/Cy were administered to on-protocol patients[14, 21-23], as it was hypothesized that the original dose of PT/Cy[25], i.e. 50 mg/kg on days +3 and +4, could dampen the GVL effects. Our reduced dose of PT/Cy-haplo might effectively elicit the GVL effect through preserved T cell alloreactivity. Third, most of the patients in the later years of the study received PT/Cy without ATG as GVHD prophylaxis, whereas the patients in the early years were likely to receive PT/Cy with ATG as GVHD prophylaxis because of the fixed protocol.[14, 21-23] In concordance with McDonald's study[46], PT/Cy-

haplo during 2013 to 2018 was associated with an improved OS compared with that during 2009 to 2012 in the present study (hazard ratio, 0.4; 95% CI, 0.3 to 0.7; *P* = 0.001) (Supplementary Figure 6). We considered that it was difficult to analyze the whole population in itself and investigate the effect of ATG on survival because of period-related bias. To remove the bias due to the difference between these periods, the study population was stratified by PT/Cy with ATG or PT/Cy without ATG from the start of this study. Fourth, the interpretation of the reconstruction data on T cells and NK cells may be limited by the change in the flow cytometry method. However, even though the influence was taken into consideration, there did not appear to be any significant differences between CC and CG + GG (Supplementary Figure 3). Functional analyses of T cells and NK cells, rather than the numbers of reconstructed cells, would be required to elucidate the mechanism of T cell alloreactivity according to the donor *NKG2D* status.

In conclusion, the present study demonstrated that non-ATG-use PT/Cy-haplo from rs1049174 CC donors was associated with a decreased risk of relapse/progression. In the future, large-scale validation studies will be required to test the significance of donor NKG2D polymorphism in the development of a new donor selection algorithm for PT/Cy-haplo.

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AUTHORSHIP CONTRIBUTIONS

KI, HK, MH and HN contributed to the concept and design of the study. AH and MNa contributed to the acquisition of the data. TS confirmed the genetic data. KI and HK analyzed the data, interpreted the results and wrote the manuscript. AH, TS, YM, MK, TT, HO, SN, MNa, MNi, YN, MH and HN interpreted the results and critically reviewed and revised the manuscript. All authors read and approved the final version.

DISCLOSURE OF CONFLICTS OF INTEREST

The authors declare no competing financial interests.

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Table 1. Characteristics of patients and donors

Characteristic	PT/Cy with ATG (n = 32)	PT/Cy without ATG (n = 59)
Patient age, years, median (range)	47 (21–67)	49 (17–68)
Sex		
Male	19 (59)	38 (64)
Female	13 (41)	21 (36)
Disease		
AML	22 (69)	32 (54)
MDS	2 (6)	4 (7)
CML	1 (3)	1 (2)
ALL	3 (9)	10 (17)
NHL	4 (13)	12 (20)
Disease status		
CR	12 (38)	22 (37)
NCR	20 (63)	37 (63)
HCT-CI score		
0	5 (16)	33 (56)
1–2	16 (50)	13 (22)
≥ 3	11 (34)	13 (22)
Conditioning regimen		
Bu-based	18 (56)	2 (3)
Mel-based	14 (44)	54 (92)
Others	0 (0)	3 (5)
TBI		
No	32 (100)	52 (88)
Yes	0 (0)	7 (12)
PT/Cy		
25-0	6 (19)	0 (0)
25-25	26 (81)	52 (88)
50-50	0 (0)	7 (12)
GVHD prophylaxis		
Tac + MMF	31 (97)	57 (97)
Others	1 (3)	2 (3)
Number of allo-HCT		
1	15 (47)	39 (66)
2 or 3	17 (53)	20 (34)

Donor relationship		
Parent	5 (16)	11 (19)
Sibling	10 (31)	19 (32)
Child	17 (53)	29 (49)
Donor-patient sex		
Female to male	6 (19)	17 (29)
Others	26 (81)	42 (71)
Infused $CD34^+$ cell dose*, $\times 10^6$/kg, median (range)	4.5 (2.1–12.8)	4.6 (2.2–37.8)
HLA mismatch** (GVH)		
HLA-A	17 (61)	43 (74)
HLA-B	25 (89)	52 (90)
HLA-C	22 (79)	45 (78)
HLA-DRB1	25 (89)	45 (78)
CMV serostatus		
D+/R+	23 (72)	41 (69)
D+/R-	1 (3)	2 (3)
D-/R+	7 (22)	13 (22)
D-/R-	0 (0)	3 (5)
unknown	1 (3)	0 (0)
Year at transplant		
2009–2012	22 (69)	1 (2)
2013–2018	10 (31)	58 (98)
Donor *KIR2DS1*		
Negative	22 (69)	34 (58)
Positive	10 (31)	25 (42)
Donor rs1049174		
CC	12 (38)	17 (29)
CG	14 (44)	31 (53)
GG	6 (19)	11 (19)

ALL, acute lymphoblastic leukemia; allo-HCT, allogeneic hematopoietic cell transplantation; AML, acute myeloid leukemia; ATG, anti-thymocyte globulin; Bu, busulfan; CML, chronic myelogenous leukemia; CMV, cytomegalovirus; CR, complete remission; D/R, donor/recipient; GVH, graft-versus-host; GVHD, graft-versus-host disease; HCT-CI, hematopoietic cell transplantation comorbidity index; MDS, myelodysplastic syndrome; Mel, melphalan; NCR, non-complete remission; NHL, non-Hodgkin lymphoma; PT/Cy, post-transplantation cyclophosphamide; TBI, total body irradiation.

*The infused $CD34^+$ cell dose was analyzed in 32 patients in the GVHD prophylaxis with

PT/Cy with ATG and in 52 patients in the GVHD prophyalxis with PT/Cy without ATG.
****HLA** mismatch was analyzed in 28 patients in the GVHD prophylaxis with PT/Cy with ATG and in 58 patients in the GVHD prophylaxis with PT/Cy without ATG.

Table 2. Univariable analyses of relapse/progression, non-relapse mortality and overall survival stratified by GVHD prophylaxis

	Relapse/progression		Non-relapse mortality		Overall survival	
	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*
(A) PT/Cy with ATG (n = 32)						
Patient age (per 10 years)	0.9 (0.7–1.4)	0.779	1.4 (0.8–2.5)	0.303	1.0 (0.8–1.4)	0.785
Sex, male (vs. female)	1.5 (0.6–3.6)	0.414	2.0 (0.5–8.2)	0.332	1.4 (0.7–3.0)	0.380
Disease						
AML	1.0 (Ref)	-	1.0 (Ref)	-	1.0 (Ref)	-
Non-AML	0.3 (0.1–1.1)	0.065	2.1 (0.5–7.9)	0.292	0.6 (0.3–1.5)	0.280
Disease status						
CR	1.0 (Ref)	-	1.0 (Ref)	-	1.0 (Ref)	-
NCR	2.2 (0.8–5.7)	0.119	1.0 (0.3–4.0)	0.944	1.7 (0.8–3.8)	0.199
HCT-CI						
0–2	1.0 (Ref)	-	1.0 (Ref)	-	1.0 (Ref)	-
≥ 3	0.7 (0.3–2.0)	0.517	4.1 (1.0–16.6)	0.048	1.4 (0.7–3.1)	0.367
Conditioning regimen						
Bu-based	0.8 (0.3–1.8)	0.524	1.0 (0.3–3.6)	0.954	1.1 (0.5–2.2)	0.886
Mel-based or others	1.0 (Ref)	-	1.0 (Ref)	-	1.0 (Ref)	-
TBI						
No	1.0 (Ref)	-	1.0 (Ref)	-	1.0 (Ref)	-
Yes	NE	NE	NE	NE	NE	NE
PT/Cy						
25-0 or 25-25	1.0 (Ref)	-	1.0 (Ref)	-	1.0 (Ref)	-
50-50	NE	NE	NE	NE	NE	NE

Number of allo-HCT						
1	1.0 (Ref)	-	1.0 (Ref)	-	1.0 (Ref)	-
2 or 3	1.2 (0.5–3.0)	0.631	1.9 (0.5–7.5)	0.380	1.3 (0.6–2.8)	0.470
Donor relationship						
Parent	0.8 (0.2–2.6)	0.677	1.0 (0.2–5.1)	0.972	0.8 (0.3–2.3)	0.659
Sibling or child	1.0 (Ref)	-	1.0 (Ref)	-	1.0 (Ref)	-
Donor-patient sex						
Female to male	1.9 (0.7–5.4)	0.209	0.7 (0.1–5.5)	0.726	0.9 (0.4–2.5)	0.917
Others	1.0 (Ref)	-	1.0 (Ref)	-	1.0 (Ref)	-
Infused $CD34^+$ cell dose						
Tertile 1	1.0 (Ref)	-	1.0 (Ref)	-	1.0 (Ref)	-
Tertile 2	0.6 (0.2–1.6)	0.282	1.8 (0.3–10.1)	0.487	0.7 (0.3–1.7)	0.437
Tertile 3	0.5 (0.2–1.7)	0.268	2.1 (0.3–12.9)	0.426	0.9 (0.3–2.3)	0.827
HLA-A mm GVH, yes (vs. no or unknown)	0.9 (0.4–2.3)	0.901	1.2 (0.3–4.6)	0.755	1.0 (0.4–2.0)	0.894
HLA-B mm GVH, yes (vs. no or unknown)	0.7 (0.3–2.0)	0.550	2.0 (0.2–15.9)	0.520	0.8 (0.3–2.0)	0.653
HLA-C mm GVH, yes (vs. no or unknown)	1.0 (0.4–2.7)	0.944	1.0 (0.2–4.0)	0.996	0.8 (0.4–1.8)	0.627
HLA-DRB1 mm GVH, yes (vs. no or unknown)	1.0 (0.3–3.0)	0.988	0.5 (0.1–2.2)	0.380	0.7 (0.3–1.6)	0.375
CMV serostatus						
D-/R+	1.2 (0.4–3.6)	0.761	3.1 (0.7–14.0)	0.140	1.9 (0.8–4.7)	0.141
Others	1.0 (Ref)	-	1.0 (Ref)	-	1.0 (Ref)	-

Year at transplant						
2009–2012	1.0 (Ref)	-	1.0 (Ref)	-	1.0 (Ref)	-
2013–2018	0.6 (0.2–1.5)	0.285	0.1 (0.0–1.0)	0.045	0.3 (0.1–0.7)	0.009
Donor *KIR2DS1*						
Negative	1.0 (Ref)	-	1.0 (Ref)	-	1.0 (Ref)	-
Positive	0.5 (0.2–1.4)	0.169	0.6 (0.2–2.7)	0.547	0.5 (0.2–1.2)	0.136
Donor rs1049174						
CC	1.2 (0.2–5.6)	0.830	0.3 (0.1–1.7)	0.198	0.6 (0.2–1.6)	0.289
CG	2.1 (0.5–9.8)	0.333	0.5 (0.1–2.7)	0.438	0.9 (0.3–2.5)	0.790
GG	1.0 (Ref)	-	1.0 (Ref)	-	1.0 (Ref)	-

(B) PT/Cy without ATG (n = 59)						
Patient age (per 10 years)	0.9 (0.7–1.2)	0.631	1.3 (0.8–1.9)	0.252	1.1 (0.9–1.3)	0.530
Sex, male (vs. female)	1.4 (0.6–3.1)	0.458	2.9 (0.8–10.5)	0.102	1.7 (0.8–3.3)	0.146
Disease						
AML	1.0 (Ref)	-	1.0 (Ref)		1.0 (Ref)	
Non-AML	0.9 (0.4–2.0)	0.805	0.9 (0.3–2.6)	0.818	0.8 (0.4–1.5)	0.522
Disease status						
CR	1.0 (Ref)	-	1.0 (Ref)	-	1.0 (Ref)	-
NCR	5.7 (2.0–16.8)	0.001	3.5 (1.1–11.3)	0.039	4.3 (1.9–9.4)	< 0.001
HCT-CI						
0–2	1.0 (Ref)	-	1.0 (Ref)	-	1.0 (Ref)	-
≥ 3	1.9 (0.8–4.5)	0.161	3.1 (1.0–9.3)	0.044	2.4 (1.2–4.7)	0.013

Conditioning regimen						
Bu-based	NE	NE	2.8 (0.3–22.3)	0.336	0.8 (0.1–6.2)	0.871
Mel-based or others	1.0 (Ref)	-	1.0 (Ref)	-	1.0 (Ref)	-
TBI						
No	1.0 (Ref)	-	1.0 (Ref)	-	1.0 (Ref)	-
Yes	0.6 (0.1–2.4)	0.439	1.3 (0.3–6.1)	0.712	0.8 (0.3–2.3)	0.711
PT/Cy						
25-0 or 25-25	1.0 (Ref)	-	1.0 (Ref)	-	1.0 (Ref)	-
50-50	0.6 (0.1–2.4)	0.439	1.3 (0.3–6.1)	0.712	0.8 (0.3–2.3)	0.711
Number of allo-HCT						
1	1.0 (Ref)	-	1.0 (Ref)	-	1.0 (Ref)	-
2 or 3	1.6 (0.7–3.5)	0.236	2.2 (0.7–6.4)	0.161	1.8 (1.0–3.4)	0.067
Donor relationship						
Parent	1.7 (0.7–4.1)	0.228	0.8 (0.2–3.6)	0.768	1.3 (0.6–2.7)	0.488
Sibling or child	1.0 (Ref)	-	1.0 (Ref)	-	1.0 (Ref)	-
Donor-patient sex						
Female to male	2.1 (0.9–4.6)	0.068	1.4 (0.4–4.5)	0.573	1.8 (0.9–3.4)	0.085
Others	1.0 (Ref)	-	1.0 (Ref)	-	1.0 (Ref)	-
Infused $CD34^+$ cell dose						
Tertile 1	1.0 (Ref)	-	1.0 (Ref)	-	1.0 (Ref)	-
Tertile 2	0.4 (0.1–1.0)	0.057	0.6 (0.1–2.9)	0.500	0.5 (0.2–1.1)	0.087
Tertile 3	0.4 (0.2–1.1)	0.089	0.7 (0.2–3.2)	0.646	0.6 (0.2–1.2)	0.155
HLA-A mm GVH, yes (vs. no or unknown)	0.6 (0.3–1.3)	0.184	0.7 (0.2–2.3)	0.563	0.7 (0.4–1.4)	0.298

HLA-B mm GVH, yes (vs. no or unknown)	0.8 (0.3–2.4)	0.713	1.9 (0.3–14.8)	0.524	1.1 (0.4–2.7)	0.909
HLA-C mm GVH, yes (vs. no or unknown)	1.6 (0.6–4.3)	0.336	1.7 (0.5–6.0)	0.437	1.5 (0.7–3.3)	0.316
HLA-DRB1 mm GVH, yes (vs. no or unknown)	1.0 (0.4–2.4)	0.993	4.8 (0.6–36.6)	0.132	1.5 (0.7–3.2)	0.324
CMV serostatus						
D-/R+	0.4 (0.1–1.4)	0.142	1.6 (0.5–4.8)	0.404	0.8 (0.4–1.7)	0.568
Others	1.0 (Ref)	-	1.0 (Ref)	-	1.0 (Ref)	-
Year at transplant						
2009–2012	1.0 (Ref)	-	1.0 (Ref)	-	1.0 (Ref)	-
2013–2018	NE	NE	NE	NE	NE	NE
Donor *KIR2DS1*						
Negative	1.0 (Ref)	-	1.0 (Ref)	-	1.0 (Ref)	-
Positive	0.9 (0.4–2.0)	0.776	1.9 (0.7–5.5)	0.237	1.1 (0.6–2.0)	0.835
Donor rs1049174						
CC	0.2 (0.1–0.8)	0.019	NE	NE	0.6 (0.2–1.7)	0.329
CG	0.6 (0.2–1.3)	0.195	NE	NE	1.2 (0.5–2.8)	0.681
GG	1.0 (Ref)	-	1.0 (Ref)	-	1.0 (Ref)	-

allo-HCT, allogeneic hematopoietic cell transplantation; AML, acute myeloid leukemia; ATG, anti-thymocyte globulin; CI, confidence interval; CMV, cytomegalovirus; CR, complete remission; D/R, donor/recipient; GVH, graft-versus-host; GVHD, graft-versus-host disease; HCT-CI, hematopoietic cell transplantation comorbidity index; HR, hazard ratio; mm, mismatch; NCR, non-complete remission; NE, not evaluable; PT/Cy, post-transplantation cyclophosphamide; Ref, reference; TBI, total body irradiation.

*Because infused $CD34^+$ cell dose showed nonlinear association with risk of relapse/progression, we fitted a model using categorization into tertiles (2.1 to 3.7, 3.8 to 5.6 and 5.7 to 37.8, $\times 10^6$/kg).

Table 3. Multivariable analyses of relapse/progression, non-relapse mortality and overall survival stratified by GVHD prophylaxis

	Relapse/progression		Non-relapse mortality		Overall survival	
	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*
(A) PT/Cy with ATG (n = 32)						
Model 1						
Donor rs1049174, CC (vs. GG)	1.2 (0.2–5.8)	0.826	0.3 (0.1–1.7)	0.179	0.6 (0.2–1.7)	0.308
Donor rs1049174, CG (vs. GG)	2.1 (0.5–9.8)	0.336	0.5 (0.1–2.7)	0.460	0.9 (0.3–2.5)	0.790
Donor relationship, parent (vs. sibling or child)	1.0 (0.3–3.7)	0.963	1.5 (0.3–9.2)	0.646	1.0 (0.3–3.2)	0.994
Model 2						
Donor rs1049174, CC (vs. GG)	0.8 (0.2–4.4)	0.843	0.4 (0.1–1.9)	0.224	0.5 (0.2–1.7)	0.275
Donor rs1049174, CG (vs. GG)	2.0 (0.4–9.1)	0.388	0.5 (0.1–2.7)	0.446	0.9 (0.3–2.4)	0.763
Female to male (vs. others)	2.5 (0.8–8.0)	0.112	1.0 (0.1–8.5)	0.966	1.2 (0.4–3.3)	0.775
Model 3						
Donor rs1049174, CC (vs. GG)	1.2 (0.2–5.6)	0.830	0.3 (0.1–1.7)	0.175	0.6 (0.2–1.7)	0.293
Donor rs1049174, CG (vs. GG)	2.1 (0.5–9.8)	0.333	0.5 (0.1–2.6)	0.426	0.9 (0.3–2.5)	0.795
HLA-DRB1 mm GVH, yes (vs. no or unknown)	1.0 (0.3–3.0)	1.000	0.5 (0.1–2.0)	0.325	0.7 (0.3–1.6)	0.382
Model 4						
Donor rs1049174, CC (vs. GG)	1.4 (0.3–6.9)	0.666	0.3 (0.1–1.5)	0.138	0.6 (0.2–1.8)	0.354
Donor rs1049174, CG (vs. GG)	2.5 (0.5–12.2)	0.241	0.4 (0.1–2.2)	0.296	0.9 (0.3–2.7)	0.874
Infused $CD34^+$ cell dose, Tertile 2–3 (vs. Tertile 1)	0.5 (0.2–1.3)	0.149	2.5 (0.5–13.3)	0.290	0.8 (0.4–1.8)	0.639

(B) PT/Cy without ATG (n=59)						
Model 1						
Donor rs1049174, CC (vs. GG)	0.2 (0.0–0.6)	0.007	NE	NE	0.5 (0.2–1.5)	0.251
Donor rs1049174, CG (vs. GG)	0.4 (0.2–1.1)	0.085	NE	NE	1.1 (0.5–2.6)	0.826
Donor relationship, parent (vs. sibling or child)	2.5 (1.0–6.5)	0.057	NE	NE	1.4 (0.7–3.1)	0.369
Model 2						
Donor rs1049174, CC (vs. GG)	0.2 (0.1–0.8)	0.018	NE	NE	0.6 (0.2–1.7)	0.356
Donor rs1049174, CG (vs. GG)	0.4 (0.2–1.1)	0.083	NE	NE	1.1 (0.5–2.6)	0.858
Female to male (vs. others)	2.2 (0.9–5.2)	0.072	NE	NE	1.6 (0.8–3.1)	0.194
Model 3						
Donor rs1049174, CC (vs. GG)	0.2 (0.1–0.8)	0.019	NE	NE	0.6 (0.2–1.8)	0.392
Donor rs1049174, CG (vs. GG)	0.6 (0.2–1.3)	0.190	NE	NE	1.3 (0.5–2.9)	0.594
HLA-DRB1 mm GVH, yes (vs. no or unknown)	0.9 (0.4–2.2)	0.830	NE	NE	1.5 (0.7–3.2)	0.337
Model 4*						
Donor rs1049174, CC (vs. GG)	0.3 (0.1–1.0)	0.044	NE	NE	0.7 (0.2–2.1)	0.467
Donor rs1049174, CG (vs. GG)	0.6 (0.2–1.7)	0.373	NE	NE	1.3 (0.5–3.5)	0.600
Infused $CD34^+$ cell dose, Tertile 2–3 (vs. Tertile 1)	0.4 (0.2–0.9)	0.028	NE	NE	0.6 (0.3–1.1)	0.107

AML, acute myeloid leukemia; ATG, anti-thymocyte globulin; CI, confidence interval; D/R, donor/recipient; GVH, graft-versus-host; GVHD, graft-versus-host disease; HR, hazard ratio; mm, mismatch; NE, not evaluable; PT/Cy, post-transplantation cyclophosphamide; Ref, reference.

*This multivariable analysis was performed in 52 patients with the infused $CD34^+$ cell dose data.

FIGURE LEGENDS

Figure 1. Cumulative incidence of relapse/progression (RI) stratified by graft-versus-host disease (GVHD) prophylaxis, according to the donor NKG2D gene polymorphism rs1049174. (A) RI in the GVHD prophylaxis with post-transplant cyclophosphamide (PT/Cy) with anti-thymocyte globulin (ATG). (B) RI in GVHD prophylaxis with PT/Cy without ATG.

Figure 2. Cumulative incidence of relapse/progression (RI) and non-relapse mortality (NRM) and the Kaplan-Meier estimate of the overall survival (OS) in the graft-versus-host disease prophylaxis with post-transplant cyclophosphamide without anti-thymocyte globulin stratified by disease type, according to the donor NKG2D gene polymorphism rs1049174. (A) RI in acute myeloid leukemia (AML) patients. (B) RI in non-AML patients. (C) NRM in AML patients. (D) NRM in non-AML patients. (E) The OS in AML patients. (F) The OS in non-AML patients.

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Figure 1

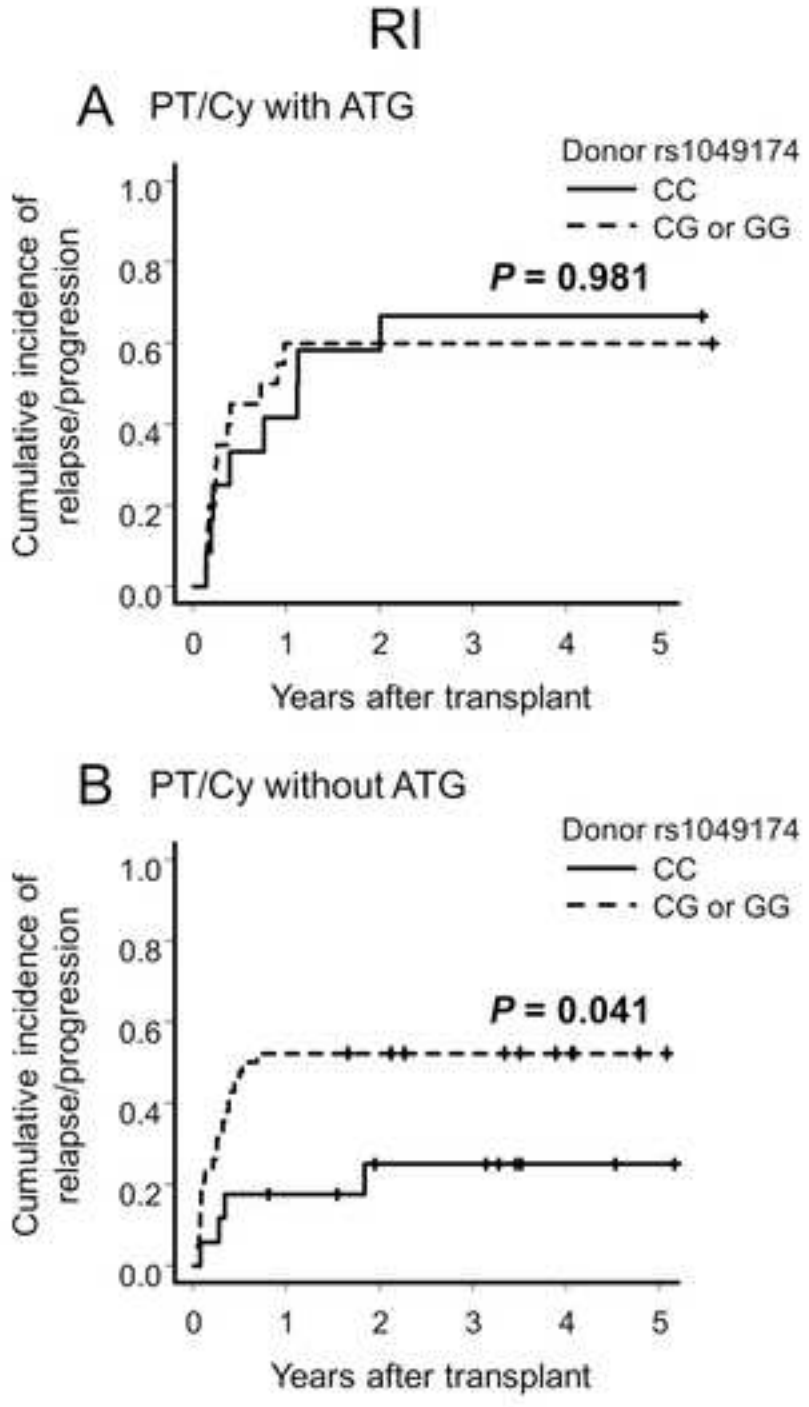

Figure 2

Fig2_20210709.tif