의학박사학위논문

# Association of *FOXP3* polymorphisms with clinical outcomes after allogenic hematopoietic stem cell transplantation

*FOXP3* 단일염기다형성과 동종 조혈모세포이식 성적과의 연관성

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서울대학교 대학원

의학과 검사의학 전공

남 민 정

A thesis of the Degree of Doctor of Philosophy

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August 2018

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# Association of *FOXP3* polymorphisms with clinical outcomes after allogenic hematopoietic stem cell transplantation

by

Minjeong Nam

A thesis submitted to the Department of Laboratory Medicine in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Medicine at Seoul National University College of Medicine, Seoul, Korea

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# ABSTRACT

## Association of *FOXP3* polymorphisms with clinical outcomes after allogenic hematopoietic stem cell transplantation

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**Background:** Forkhead box P3 (FOXP3) is an important marker of regulatory T cells. *FOXP3* polymorphisms are associated with autoimmune diseases, cancers, and allograft outcome. We examined whether single nucleotide polymorphisms (SNPs) at the *FOXP3* locus are associated with clinical outcomes after allogenic hematopoietic stem cell transplantation (HSCT).

**Methods:** Five *FOXP3* SNPs (rs5902434, rs3761549, rs3761548, rs2232365, and rs2280883) were analyzed by PCR-sequencing of 172 DNA samples from allogenic HSCT patients from April 2006 to August 2014 at Seoul National University Hospital.

We examined the relationship between each SNP and the occurrence of graft-versus-host disease (GVHD), post-HSCT infection, relapse, and patient survival.

**Results:** Patients with acute GVHD (grade II-IV) showed higher frequencies of the rs3761549 T/T genotype, rs5902434 ATT/ATT genotype, and rs2232365 G/G genotype than did patients without acute GVHD [$P$ = 0.017, odds ratio (OR) = 5.3; $P$ = 0.031, OR = 2.4; $P$ = 0.023, OR = 2.6, respectively). Multivariate analysis showed that the T/T genotype of rs3761549 was an independent risk factor for occurrence of acute GVHD ($P$ = 0.032, hazard ratio = 5.6). In contrast, the genotype frequencies of rs3761549 T/T, rs5902434 ATT/ATT, and rs2232365 G/G were lower in patients with post-HSCT infection than in patients without infection ($P$ = 0.026, $P$ = 0.046, and $P$ = 0.031, respectively).

**Conclusions:** This study suggests that rs3761549, rs5902434, and rs2232365 are correlated with an increased risk of acute GVHD and a decreased risk of post-HSCT infection.

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**Keywords:** allogenic hematopoietic stem cell transplantation, *FOXP3,* graft-versus-host disease, infection, polymorphism

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| ABL | Acute biphenotypic leukemia |
| ALL | Acute lymphoblastic leukemia |
| AML | Acute myeloid leukemia |
| AT | Annealing temperature |
| Bu | Busulfan |
| CI | Confidence interval |
| CML | Chronic myelogenous leukemia |
| CMV | Cytomegalovirus |
| CNI | Calcineurin-inhibitor |
| DEL | Deletion |
| DLBL | Diffuse large B cell lymphoma |
| EBMT | European Society for Blood and Marrow Transplantation |
| EBV | Epstein-barr virus |
| EMR | Electronic medical records |
| eQTL | expression Quantitative Trait Loci |
| F | Forward primer |
| Flu | Fludarabine |
| FOXP3 | Forkhead box P3 |
| GVHD | Graft-versus-host disease |
| HLA | Human leukocyte antigen |
| HSCT | Hematopoietic stem cell transplantation |
| IPEX syndrome | Immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome |

| | |
|---|---|
| Ig | Immunoglobulin |
| MPD | Myeloproliferative disease |
| NK cell | Natural killer cell |
| R | Reverse primer |
| SAA | Severe aplastic anemia |
| SLE | Systemic lupus erythematosus |
| SNP | Single nucleotide polymorphism |
| TB | Tuberculosis |
| TBI | Total body irradiation |
| Treg | Regulatory T cells |
| TWAS | Genome-wide association studies |

# 1. INTRODUCTION

Allogenic hematopoietic stem cell transplantation (HSCT) is a curative treatment for patients with hematologic malignancies, bone marrow failure or congenital immunologic diseases. Conceptually, the allogenic HSCT recipient is treated with a conditioning regimen (combinations of radiation and/or chemotherapy) to deplete residual tumor, immunity, and remaining bone marrow cells, followed by infusion of progenitor cells capable of reconstituting hematopoiesis [1]. The rates for 1-year and disease-free survival associated with allogenic HSCT have significantly improved over the last few years [2]. However, despite the increasing use of allogenic HSCT, it still has severe complications with high morbidity and mortality, such as acute and chronic graft-versus-host disease (GVHD) and post-HSCT infections [3].

GVHD is caused by allo-reactive donor T cells that are activated by host antigen presenting cells (APCs) and causes a severe inflammatory disorder affecting major organs [4]. In the past, acute and chronic GVHD were distinguished strictly, with a threshold of 100 days after transplantation. However, acute and chronic GVHD are now considered to have distinct pathogenesis, and may have overlapping time courses. Acute GVHD involves a cascade of tissue damage amplified by immune and cytokine activation. The pre-allogenic HSCT conditioning regimen may result in: i) damage

to host cells and tissues and ii) cytokine secretion from macrophages. This process contributes to activation and co-stimulation of host antigen-reactive donor T cells leading to host tissue damage and recruitment of other immune cells. The remaining pathophysiology of chronic GVHD is poorly understood, but current theories suggest that T cells interact with macrophages and B cells, resulting in the formation of auto-antibodies [5].

Prevention strategies for GVHD have been focused almost exclusively on reducing acute GVHD, which is the most important risk factor for chronic GVHD. Currently, the most widely used regimen are based on calcineurin-inhibitor (CNI), however, detailed regimens vary by medical center and/or patients' condition. Based on an improved biological understanding of immune cells, new approaches that target different cells of the immune system (eg, T and B cells), are garnering greater attention [6]. These new regimens include the removal of donor T cells to prevent GVHD, but may contribute to delayed immune reconstitution, thus increasing the risk of opportunistic infections [7.8].

T cell-mediated immunoregulation is one of the main mechanism to control immune homeostasis and maintain tolerance after transplantation [9,10]. In cases of tolerance (including deletion, anergy, ignorance, and clonal exhaustion), major and minor histocompatibility antigens of an organ or cell donor do not cause an immune reaction in the transplant recipient. It is now understood that regulatory T cells (Tregs) play a critical role in T

cell-mediated regulation of transplantation tolerance [11]. Recent studies have shown that Tregs are involved in the development of GVHD and suggest a potential therapeutic role for these cells in reducing the incidence and/or severity of GVHD based on their immunosuppressive potential [12]. Thus, interest in Tregs relating to transplantation has grown; many researchers have attempted to find new Tregs-related markers and develop therapeutic strategies to improve graft survival and prevent post-transplant complications [13,14].

Forkhead/winged helix box P3 (FOXP3) is a master regulator of Tregs development and function, changing expression of various regulators of gene expression [15]. Mutations in the *FOXP3* gene are associated with the development of a fatal multisystem autoimmune disorder known as immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome [16]. Recently, many studies have shown that the regulatory mechanism of gene expression is controlled by genomic polymorphisms. Single nucleotide polymorphisms (SNPs) in *FOXP3* have been associated with various diseases [17] such as asthma [18], preeclampsia [19], systemic lupus erythematosus (SLE) [20], autoimmune thyroid disease [21], lung cancer [22], breast cancer [23], and colorectal cancer [24]. Recently, several studies reported that *FOXP3* SNPs are associated with allograft outcomes after renal transplantation, but there is ongoing debate over whether *FOXP3* SNPs have a positive or negative association with allograft outcomes [25-28].

Moreover, few studies have investigated whether there is an association between SNPs in *FOXP3* and clinical outcomes after allogenic HSCT [29]. We examined the association between five SNPs (rs5902434, rs3761549, rs3761548, and rs2232365 located in the promoter region and rs2280883 located in the intronic region) in *FOXP3* and different clinical outcomes after allogenic HSCT: the occurrence of GVHD, post-HSCT infection, relapse, and patient survival. These five SNPs were selected among those directly or potentially associated with diseases by a literature search and analysis using the HaploReg v. 4.1 database.

# 2. MATERIALS AND METHODS

## 2.1. Subjects

Our retrospective study included 172 patients with hematologic malignancy or bone marrow failure who received allogenic HSCT between April 2006 and August 2014 at Seoul National University Hospital, Seoul, Korea. Baseline clinical characteristics, including age and gender of patients and donors, underlying diagnosis, stem cell source, the number of HLA mismatches, cytomegalovirus (CMV) IgG seropositivity, conditioning regimens, and European Society for Blood and Marrow Transplantation (EBMT) risk score [30] were obtained from medical records, and were summarized in Table 1. Conditioning chemotherapy was performed before transplantation for patients who received HSCT from an unrelated donor. The regimen varied according to the type of underlying disease and the condition of the patient, but included the following: busulfan plus (cyclophosphamide or anti-thymocyte globulin plus fludarabine), fludarabine plus (cyclophosphamide, melphalan, cyclophosphamide plus anti-thymocyte globulin, or melphalan plus anti-thymocyte globulin), total body irradiation plus cyclophosphamide, and total lymphocyte irradiation plus anti-thymocyte globulin. Patients were treated with cyclosporine or tacrolimus with or without a short course of methotrexate (1, 3, 6, and 10 days) as GVHD

prophylaxis and treated with ciprofloxacin, itraconazole, acyclovir, sulfamethoxazole/trimethoprim, or intravenous immune globulins as infection prophylaxis. Our study was approved by the Institutional Review Board for Human Research of Seoul National University (IRB No. 1702-024-829).

Acute (grade II-IV) and chronic GVHD were diagnosed based on published criteria [31,32]. To reduce potential bias of the acute GVHD group, 13 patients who died within 28 days after allogenic HSCT were excluded from our analysis of acute GVHD, but they were included in our analysis of infection. Infection was defined as the isolation of a certain pathogen, such as virus, bacteria, fungus, and tuberculosis (TB), from microbial cultures or positive results from nucleic acid amplification or antigen tests. Disease relapse was determined based on the bone marrow examination of patients. Overall survival was defined as the time from graft infusion to death from any cause at time of analysis (March 1, 2017). For event-free survival, death or relapse was considered events.

**Table 1.** The characteristics of the study population

| Characteristics | Recipient n (%) |
|---|---|
| Age (median, range), year | 37 (17-67) |
| Gender | |
| Male | 104 (60.5) |
| Female | 68 (39.5) |
| HLA matches | |
| 10/10 | 68 (39.5) |
| 9/10 | 61 (35.5) |
| 8/10 | 37 (21.5) |
| ≤7/10 | 6 (3.5) |
| Disease at transplantation | |
| ALL | 31 (18.0) |
| AML | 73 (42.4) |
| ABL | 5 (2.9) |
| CML | 2 (1.2) |
| MPD | 10 (5.8) |
| SAA | 14 (8.1) |
| MDS | 19 (11.0) |
| DLBL | 11 (6.4) |
| Others* | 7 (4.1) |
| Conditioning regimen | |
| Bu-based | 132 (76.7) |
| Flu-based | 35 (20.3) |
| TBI-based | 5 (2.9) |
| Type of stem cell source | |
| Bone marrow | 13 (7.6) |
| Peripheral blood | 159 (92.4) |
| EBMT risk score | |
| 1 | 4 (2.3) |
| 2 | 44 (25.6) |
| 3 | 51 (29.7) |
| 4 | 51 (29.7) |

| | |
|---|---|
| 5 | 19 (11.0) |
| 6 | 3 (1.7) |
| Unrelated donor age (median, range), year | 38 (17-67) |
| Unrelated donor Gender | |
| Male | 141 (82.0) |
| Female | 31 (18.0) |

*Others include NK cell lymphoma (n = 5), Hodgkin lymphoma (n = 1), and Blastic plasmacytoid dendritic cell neoplasm (n = 1).

Abbreviations: HLA, human leukocyte antigen; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; ABL, acute biphenotypic leukemia; CML, chronic myelogenous leukemia; MPD, myeloproliferative disease; SAA, severe aplastic anemia; DLBL, diffuse large B cell lymphoma; NK cell, natural killer cell; Bu, busulfan; Flu, fludarabine; TBI, total body irradiation; EBMT, European Group for Blood and Marrow Transplantation.

## 2.2. SNP selection

Regulatory element such as enhancers and transcription factor binding sites are essential to interpreting genome-wide association studies (GWAS), developing mechanistic hypothesis, and ultimately understanding the genetic characteristics of complex trait and disease. HaploReg Version 4.1 defined a core set of 52,054,804 variants, consisting of single nucleotide polymorphisms (SNPs) using dbSNP release b141 with other data set such as GWAS, expression quantitative trait loci (eQTL), and 1000 Genomes data [33]. The HaploReg integrates regulatory genomic maps together in the context of haplotype blocks, allowing researchers to intersect regulatory elements with genetic variants to quickly make functional hypothesis and wide analysis of a set of associated loci. In addition, using data from ENCODE, it can annotate non-coding regions of the genome and predict the function of disease associated non-coding variants. The predicted functional effect of a variant falls into following categories: coding, splice site, 5'-UTR, 3'-UTR, intronic, 5'-upstream, and 3'-downstream [34]. Therefore, HaploReg annotation of GWAS has been applied for protein-binding sites from a variety of cell types, as well as a library of position weight matrices from commercial, literature, and motif finding analysis of the ENCODE, using the score of the effect of variants on regulatory motif [35]. As a result, five *FOXP3*

SNPs (rs5902434, rs3761549, rs3761548, rs2232365, and rs2280883) are selected (Figure 1).

Query SNP: rs5902434 and variants with $r^2$ >= 1

| chr | pos (hg38) | LD ($r^2$) | LD (D') | variant | Ref | Alt | AFR freq | AMR freq | ASN freq | EUR freq | SiPhy cons | Promoter histone marks | Enhancer histone marks | DNAse | Proteins bound | Motifs changed | NHGRI/EBI GWAS hits | GRASP QTL hits | Selected eQTL hits | GENCODE genes | dbSNP func annot |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| X | 49264508 | 1 | 1 | rs5902434 | TA | T,TATT | | | | | | BLD | BLD, THYM | | | | | | | FOXP3 | intronic |

Query SNP: rs3761549 and variants with $r^2$ >= 1

| chr | pos (hg38) | LD ($r^2$) | LD (D') | variant | Ref | Alt | AFR freq | AMR freq | ASN freq | EUR freq | SiPhy cons | Promoter histone marks | Enhancer histone marks | DNAse | Proteins bound | Motifs changed | NHGRI/EBI GWAS hits | GRASP QTL hits | Selected eQTL hits | GENCODE genes | dbSNP func annot |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| X | 49260888 | 1 | 1 | rs3761549 | G | A | 0.03 | 0.08 | 0.22 | 0.12 | | BLD | THYM | THYM | | GR | | | | FOXP3 | intronic |

Query SNP: rs3761548 and variants with $r^2$ >= 1

| chr | pos (hg38) | LD ($r^2$) | LD (D') | variant | Ref | Alt | AFR freq | AMR freq | ASN freq | EUR freq | SiPhy cons | Promoter histone marks | Enhancer histone marks | DNAse | Proteins bound | Motifs changed | NHGRI/EBI GWAS hits | GRASP QTL hits | Selected eQTL hits | GENCODE genes | dbSNP func annot |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| X | 49261784 | 1 | 1 | rs3761548 | T | G | 0.94 | 0.69 | 0.82 | 0.54 | | | THYM | | | DEC,Egr-1,Myb | | | | FOXP3 | intronic |

Query SNP: rs2232365 and variants with $r^2$ >= 1

| chr | pos (hg38) | LD ($r^2$) | LD (D') | variant | Ref | Alt | AFR freq | AMR freq | ASN freq | EUR freq | SiPhy cons | Promoter histone marks | Enhancer histone marks | DNAse | Proteins bound | Motifs changed | NHGRI/EBI GWAS hits | GRASP QTL hits | Selected eQTL hits | GENCODE genes | dbSNP func annot |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| X | 49259429 | 1 | 1 | rs2232365 | T | C | 0.74 | 0.48 | 0.40 | 0.58 | | BLD | BLD, THYM | | | | | | | FOXP3 | intronic |

Query SNP: rs2280883 and variants with $r^2$ >= 1

| chr | pos (hg38) | LD ($r^2$) | LD (D') | variant | Ref | Alt | AFR freq | AMR freq | ASN freq | EUR freq | SiPhy cons | Promoter histone marks | Enhancer histone marks | DNAse | Proteins bound | Motifs changed | NHGRI/EBI GWAS hits | GRASP QTL hits | Selected eQTL hits | GENCODE genes | dbSNP func annot |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| X | 49252667 | 1 | 1 | rs2280883 | T | C | 0.04 | 0.30 | 0.18 | 0.44 | | | BLD | | | Rad21 | | 2 hits | | FOXP3 | intronic |

Figure 1. Five *FOXP3* SNPs (rs5902434, rs3761549, rs3761548, rs2232365, and rs2280883) selected by using HaploReg annotation of GWAS. rs5902434, rs3761549, rs3761548, rs2232365 are located in a functional promoter region among intronic region.

Abbreviations: FOXP3, Forkhead box P3; SNP, single nucleotide polymorphism; GWAS, genome-wide association study

## 2.3. DNA preparation and *FOXP3* SNP genotyping

We collected DNA samples from 172 patients whose pre-transplant HLA typing test had been requested. DNA was extracted from peripheral blood or bone marrow using the QuickGene-Mini80 DNA isolation system (Fujifilm, Tokyo, Japan) when pre-transplant HLA typing was done. After HLA typing, DNA stored at −80 ℃ were genotyped for the selected five *FOXP3* SNPs (rs5902434, rs3761549, rs3761548, and rs2232365 in promoter region, and rs2280883 in intronic region) by PCR sequencing. Among our cohort of 172 patients, we excluded two to five cases depending on the SNP, because they could not be accurately genotyped due to poor DNA sample quality. PCR was performed with 40 μL reaction mixtures containing 40 ng DNA, 0.8 μL dNTP mix (10 mM of each dNTP), 2 μL of each primer at a concentration of 10 pmol/μL, 2.0 mM $MgCl_2$, 1.0 U TaqDNA polymerase (Roche, Basel, Switzerland), and 4 μL of 10X reaction buffer. Five SNPs were analyzed following the identical PCR protocol. Initial denaturation was performed at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at the respective annealing temperature for 30 sec, and extension at 72°C for 30 sec with a final extension at 72°C for 5 min. Every genomic sequence data of primers was provided from the National Center for Biotechnology Information

(NCBI) Reference Sequence (RefSeq) database (http://www.ncbi.nlm.nih.gov/RefSeq) (Table 2). Next, 2 μL ExoSAP-IT PCR Clean Up (Affymetrix, Santa Clara, CA, USA) was added to 5 μL of PCR product, followed by incubation at 37°C for 15 min and at 80°C for 15 min. We then added 1 μL of 5 pmol/μL sequencing primer, 4 μL of deionized water, and 4 μL of BigDye Terminator Ready Reaction Mix (Life Technologies, Grand Island, NY, USA) to 1 μL of purified PCR product. Following 30 thermal cycles of 96°C for 10 sec, 50°C for 5 sec, and 60°C for four min, 25 μL of absolute ethanol (EtOH) and 2 μL of 3 M sodium acetate/EDTA buffer (pH 4.6) were added. After vortexing and centrifugation at 2,000*g* for 30 min, the supernatant was discarded, following by the addition of 50 μL of 80% EtOH and centrifugation at 2,000*g* for 5 min. Finally, 15 μL of Hi-Di Formamide (Life Technologies) was added, and the sample was heated at 95°C for 4 min. Samples were then analyzed on an ABI 3730XL DNA analyzer (Applied Biosystems, Foster City, CA, USA), and electropherograms were analyzed using Chromas Lite 2.1.1 (Technelysium, South Brisbane, Australia).

**Table 2.** Primers for PCR-sequencing

| Polymorphism | | AT (℃) | | Sequence (5' → 3') |
|---|---|---|---|---|
| rs5902434 | del/ATT | 56 | F | 5'-CTGCTCTCCCCTACCAGATG-3' |
| | | | R | 5'-CCCTGCCCATGCATTAAGTA-3' |
| rs3761549 | C/T | 60 | F | 5'-GTCCTCTCCACAACCCAAGA-3' |
| | | | R | 5'-CAGATTTTTCCGCCATTGAC-3' |
| rs3761548 | C/A | 60 | F | 5'-TTGTCTACTCCACGCCTCTCC-3' |
| | | | R | 5'-TGCCTCCATCATCACCACG-3' |
| rs2232365 | A/G | 60 | F | 5'-GAGGGCTTTCAGGTGAGGA-3' |
| | | | R | 5'-GGGAGTTGGATTGGGTGCA-3' |
| rs2280883 | C/T | 60 | F | 5'-TCAGGGTTTCAGTTCAGAGACAGT-3' |
| | | | R | 5'-CCCTTTCCAGATGTCCACCTCAG-3' |
| | | | Inner F | 5'-TGGCGCTAGGATGAAGGTTC-3' |

Abbreviations: PCR, polymerase chain reaction; AT, annealing temperature; del, deletion; F, forward primer; R, reverse primer.

## 2.4. Luciferase gene reporter assays

A luciferase gene reporter assay was performed to determine whether rs3761549 SNP located in the promoter regions of the *FOXP3* gene might affect FOXP3 expression. The genomic DNA extracted from HSCT recipients with each genotype T/T and C/C was utilized as template. The human *FOXP3* gene fragments were amplified by PCR from genomic DNA position 49008107 to 49008707 (containing core promoter) [36] and 49260488 to 49261288 (partial sequence around rs376549). PCR was performed using gDNA, a forward primer (5'-ccggCTCGAGccaccatttcccatccac acataga-3', upper letter indicate the XhoI linker) and a reverser primer (5'-ccgggAAGCTTtagctgggtacatcccactg-3', upper letter indicate the HindIII linker), and a forward primer (5'-gaaGCTAGC gccaccggtaggcaagaggccctatg-3', upper letter indicate the NheI linker) and a reverser primer (5'-cccttGAGCTCcgacggacctgtgaaccggt -3', upper letter indicate the XhoI linker). The PCR amplicons were joined and cloned into the pGL3-basic vector (Promega, Madison, USA). The sequence and orientation of the insert were confirmed by sequencing.

For cell culture, the human embryonic kidney 293 T cell line was maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, UK) with 10% fetal calf serum (FCS; Sigma-Aldrich, UK) supplemented with 2 mM L-glutamine and 100

units/mL penicillin and 100 ㎍/mL streptomycin. Using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA), $2\times10^4$ HEK 293 T cells were plated in 24-well plates with 1.6 ㎍ of the *FOXP3* promoter-reporter vector. After 24 hours of transfection, luciferase activity was measured with the Dual-Glo luciferase system (Promega, Madison, USA). The assay was performed independently three times.

## 2.5. Statistical analysis

Differences in genotype distributions between each group were calculated using Pearson's chi-squared test or Fisher's exact test. Univariate and multivariate analysis were performed using logistic regression. The following variables were included in our analysis: recipient age and gender, the number of HLA allelic matches for HLA-A, B, Cw, DR, and DQ locus, the type of disease at transplantation, the conditioning regimens, stem cell source, the EBMT risk score, and donor age and gender. Estimates of acute GVHD and relapse were calculated using cumulative incidence rates. Overall survival and event-free survival were calculated using the Kaplan-Meier method and compared using the log-rank test. Odds ratios (ORs) and 95% confidence intervals (CIs) were estimated for genotypes showing a significant $P$ value. $P < 0.05$ was considered statistically significant. Statistical analysis was performed using SPSS Statistics for Windows Version 23.0 (IBM Corp., Armonk, NY, USA).

# 3. RESULTS

## 3.1. Demographic profile and clinical characteristics of patients and donors

The characteristics of 172 patients who received allogenic HSCT are shown in Table 1. The majority of patients were male (60.5%) and the median patient age was 37 years (range 17 - 67 years). All but six patients presented with two or fewer HLA alleles mismatching (96.5%); the remaining six presented with three or more HLA alleles mismatching (3.5%). Most patients were diagnosed with acute leukemia before allogenic HSCT (42.4%), and peripheral blood was the dominant source of stem cells (92.4%).

## 3.2. Association of *FOXP3* SNPs and acute GVHD (grade II–IV)

For rs3761549, patients with acute GVHD showed a significantly higher frequency of the T/T genotype compared with patients without acute GVHD (15.5% vs. 3.3%, OR = 5.3, $P$ = 0.017). The frequency of the ATT/ATT genotype of rs5902434 and the G/G genotype of rs2232365 were also significantly higher in patients with acute GVHD compared with patients without acute GVHD (32.3% vs. 16.7%, OR = 2.4, $P$ = 0.031, and 31.2% vs. 15.0%, OR = 2.6, $P$ = 0.023, respectively) (Table 3).

The cumulative incidence of acute GVHD is depicted according to genotypes of five *FOXP3* SNPs. Patients with rs3761549 T/T genotype had a higher cumulative incidence of acute GVHD compared with patients with C/C or C/T genotypes ($P$ = 0.023). Patients with rs5902434 ATT/ATT or rs2232365 G/G genotype showed a tendency of higher cumulative incidence of acute GVHD, although the differences were not statistically significant ($P$ = 0.155 and $P$ = 0.123, respectively) (Figure 2).

In multivariate analysis, *FOXP3* rs3761549 T/T genotype was an independent risk factor for the occurrence of acute GVHD ($P$ = 0.032, hazard ratio = 5.584, 95% confidence interval 1.160–26.882) (Table 4). However, difference in rs5902434 ATT/ATT and rs2232365 G/G were not statistically significant in multivariate

analysis, and their variance inflation factor (VIF) in a multicolinearity test was greater than 10. Therefore, rs5902434 and rs2232365 were correlated with rs3761549, so we presented the result of multivariate regression analysis without them.

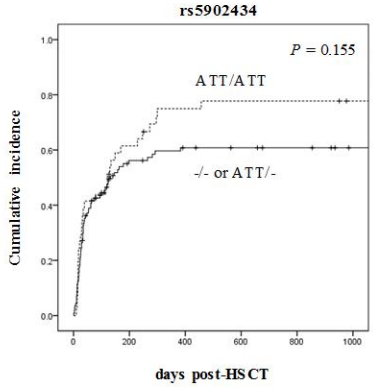
**Table 3.** Association between genotype of *FOXP3* SNPs and the incidence of acute GVHD (grade II–IV)

| Polymorphism | aGVHD (−) n (%) | aGVHD (+) n (%) | *P*-value | Odds ratio (95% CI) |
|---|---|---|---|---|
| rs5902434 | | | | |
| Genotype | | | | |
| del/del + del/ATT | 50 (83.3) | 65 (67.7) | | |
| ATT/ATT | 10 (16.7) | 31 (32.3) | 0.031 | 2.385 (1.069–5.320) |
| rs3761549 | | | | |
| Genotype | | | | |
| C/C + C/T | 58 (96.7) | 82 (84.5) | | |
| T/T | 2 (3.3) | 15 (15.5) | 0.017 | 5.305 (1.168–24.092) |
| rs3761548 | | | | |
| Genotype | | | | |
| C/C + A/C | 54 (90.0) | 84 (87.5) | | |
| A/A | 6 (10.0) | 12 (12.5) | 0.634 | |
| rs2232365 | | | | |
| Genotype | | | | |
| A/A + A/G | 51 (85.0) | 66 (68.8) | | |
| G/G | 9 (15.0) | 30 (31.2) | 0.023 | 2.576 (1.123–5.905) |
| rs2280883 | | | | |
| Genotype | | | | |
| T/T + T/C | 54 (90.0) | 83 (86.5) | | |
| C/C | 6 (10.0) | 13 (13.5) | 0.511 | |

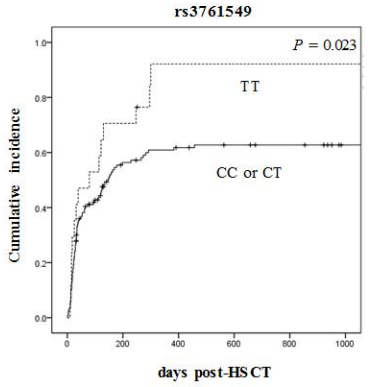
*Among a cohort of 172 patients, of which 13 patients who died within 28 days after allogenic HSCT were excluded and two to five cases, depending on the SNP, failed to provide genotypes because of unavailable DNA samples after quality control.

Abbreviations: FOXP3, Forkhead box P3; SNP, single nucleotide polymorphism; aGVHD, acute graft-versus-host disease; CI, confidence interval; del, deletion.

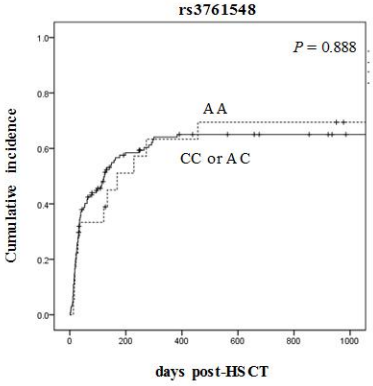
(A)


rs5902434
P = 0.155
ATT/ATT
-/- or ATT/-
Cumulative incidence
days post-HSCT

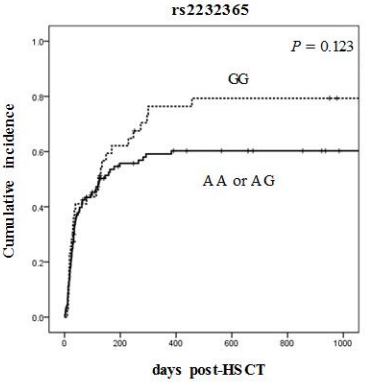
(B)


rs3761549
P = 0.023
TT
CC or CT
Cumulative incidence
days post-HSCT

(C)


rs3761548
P = 0.888
AA
CC or AC
Cumulative incidence
days post-HSCT

(D)


rs2232365
P = 0.123
GG
AA or AG
Cumulative incidence
days post-HSCT

(E)

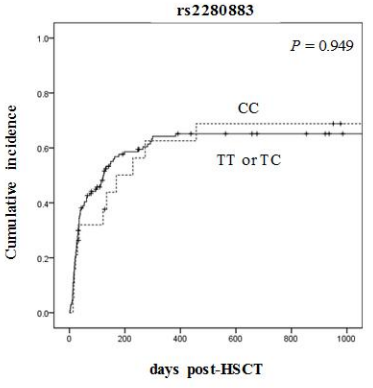

rs2280883
P = 0.949
CC
TT or TC
Cumulative incidence
days post-HSCT

**Figure 2.** Impact of the *FOXP3* polymorphism on acute GVHD occurrence after allogenic HSCT. (A) rs5902434, (B) rs3761549, (C) rs3761548, (D) rs2232365, and (E) rs2280883. Patients with rs3761549 T/T genotype showed higher cumulative incidence of acute GVHD than patients with rs3761549 C/C or C/T genotype ($P$ = 0.023).

Abbreviation: FOXP3, Forkhead box P3; GVHD, graft-versus-host disease; HSCT, hematopoietic stem cell transplantation.

**Table 4.** Multivariate analysis of risk factors for acute GVHD (grade II-IV)

| Risk factors | N (%) | *P*-value | HR (95% CI) |
|---|---|---|---|
| Recipient age (range), year | 16-67 | 0.579 | |
| Recipient gender, female | 66 (41.5) | 0.917 | |
| HLA allelic matches, < 9/10 | 40 (25.2) | 0.490 | |
| Conditioning regimen | | | |
| Bu-based | 125 (78.6) | 0.056 | |
| Flu-based | 30 (18.9) | 0.604 | |
| Graft type, peripheral blood | 147 (92.5) | 0.716 | |
| EBMT risk score, ≥ 4 | 66 (41.5) | 0.811 | |
| CMV IgG titer | 8.2-250 | 0.562 | |
| rs3761549, T/T genotype | 17 (10.7) | 0.032 | 5.584 (1.160-26.882) |
| Donor age (range), year | 18-47 | 0.915 | |
| Donor gender, female | 29 (18.2) | 0.994 | |

Abbreviations: GVHD, graft-versus-host disease; HR, hazard ratio; CI, confidence interval; HLA, human leukocyte antigen; BU, busulfan; Flu, fludarabine; EBMT, European Group for Blood and Marrow Transplantation; CMV, cytomegalovirus; IgG, immunoglobulin G

## 3.3. Association of *FOXP3* SNPs and post-HSCT infection

Among the five *FOXP3* SNPs evaluated, the frequency of the rs3761549 T/T genotype was significantly lower in patients with post-HSCT infection compared with patients without infection (8.9% vs. 33.3%, OR = 0.2, $P$ = 0.026). The frequency of the rs5902434 ATT/ATT and the rs2232365 G/G genotype were also significantly lower in patients with post-HSCT infection than in patients without infection (23.9% vs. 50.0%, OR = 0.3, $P$ = 0.046, and 22.3% vs. 50.0%, OR = 0.3, $P$ = 0.031, respectively) (Table 5). To more precisely detail the impact of each pathogen such as bacteria, cytomegalovirus (CMV), Epstein-Barr Virus (EBV), fungus, and tuberculosis (TB), we tested whether the presence of a specific *FOXP3* SNP genotype prevented infections caused by each pathogen. However, no association was observed between genotype frequency of these five *FOXP3* SNPs and infection of a given pathogen (Table 6, Table 7, Table 8, Table 9, Table 10).

**Table 5.** Association of *FOXP3* SNPs with occurrence of infection after allogenic HSCT

| Polymorphism | Infection (−) n (%) | Infection (+) n (%) | *P*−value | Odds ratio (95% CI) |
|---|---|---|---|---|
| rs5902434 | | | | |
| Genotype | | | | |
| del/del + del/ATT | 6 (50.0) | 118 (76.1) | | |
| ATT/ATT | 6 (50.0) | 37 (23.9) | 0.046 | 0.314 (0.095−1.031) |
| rs3761549 | | | | |
| Genotype | | | | |
| C/C + C/T | 8 (66.7) | 144 (91.1) | | |
| T/T | 4 (33.3) | 14 (8.9) | 0.026 | 0.194 (0.052−0.728) |
| rs3761548 | | | | |
| Genotype | | | | |
| C/C + A/C | 11 (91.7) | 139 (88.5) | | |
| A/A | 1 (8.3) | 18 (11.5) | 1.000 | |
| rs2232365 | | | | |
| Genotype | | | | |
| A/A + A/G | 6 (50.0) | 122 (77.7) | | |
| G/G | 6 (50.0) | 35 (22.3) | 0.031 | 0.287 (0.087−0.945) |
| rs2280883 | | | | |
| Genotype | | | | |
| T/T + T/C | 11 (91.7) | 137 (87.3) | | |
| C/C | 1 (8.3) | 20 (12.7) | 1.000 | |

*Among a cohort of 172 total patients, two to five cases failed to provide genotypes because of unavailable DNA samples after quality control.

Abbreviations: FOXP3, Forkhead box P3; SNP, single nucleotide polymorphism; HSCT, hematopoietic stem cell transplantation; CI, confidence interval; del, deletion.

**Table 6.** Association between genotype of *FOXP3* SNPs and bacterial infection

| Polymorphism | Bacterial infection (−) n (%) | Bacterial infection (+) n (%) | *P*−value |
|---|---|---|---|
| rs5902434 | | | |
| Genotype | | | |
| del/del + del/ATT | 35 (76.1) | 89 (73.6) | |
| ATT/ATT | 11 (23.9) | 32 (26.4) | 0.738 |
| rs3761549 | | | |
| Genotype | | | |
| C/C + C/T | 41 (89.1) | 111 (89.5) | |
| T/T | 5 (10.9) | 13 (10.5) | 0.942 |
| rs3761548 | | | |
| Genotype | | | |
| C/C + A/C | 42 (91.3) | 108 (87.8) | |
| A/A | 4 (8.7) | 15 (12.2) | 0.522 |
| rs2232365 | | | |
| Genotype | | | |
| A/A + A/G | 35 (76.1) | 93 (75.6) | |
| G/G | 11 (23.9) | 30 (24.4) | 0.949 |
| rs2280883 | | | |
| Genotype | | | |
| T/T + T/C | 42 (91.3) | 106 (86.2) | |
| C/C | 4 (8.7) | 17 (13.8) | 0.369 |

*Among a cohort of 172 total patients, two to five cases failed to provide genotypes because of unavailable DNA samples after quality control.

Abbreviations: FOXP3, Forkhead box P3; SNP, single nucleotide polymorphism; del, deletion.

**Table 7.** Association between genotype of *FOXP3* SNPs and CMV infection

| Polymorphism | CMV infection (−) n (%) | CMV infection (+) n (%) | *P*−value |
|---|---|---|---|
| rs5902434 | | | |
| Genotype | | | |
| del/del + del/ATT | 44 (74.6) | 80 (74.1) | |
| ATT/ATT | 15 (25.4) | 28 (25.9) | 0.943 |
| rs3761549 | | | |
| Genotype | | | |
| C/C + C/T | 54 (90.0) | 98 (89.1) | |
| T/T | 6 (10.0) | 12 (10.9) | 0.854 |
| rs3761548 | | | |
| Genotype | | | |
| C/C + A/C | 53 (88.3) | 97 (89.0) | |
| A/A | 7 (11.7) | 12 (11.0) | 0.897 |
| rs2232365 | | | |
| Genotype | | | |
| A/A + A/G | 45 (76.3) | 83 (75.5) | |
| G/G | 14 (23.7) | 27 (24.5) | 0.906 |
| rs2280883 | | | |
| Genotype | | | |
| T/T + T/C | 53 (86.9) | 95 (88.0) | |
| C/C | 8 (13.1) | 13 (12.0) | 0.838 |

*Among a cohort of 172 total patients, two to five cases failed to provide genotypes because of unavailable DNA samples after quality control.

Abbreviations: FOXP3, Forkhead box P3; SNP, single nucleotide polymorphism; CMV, cytomegalovirus; del, deletion.

**Table 8.** Association between genotype of *FOXP3* SNPs and EBV infection

| Polymorphism | EBV infection (−) n (%) | EBV infection (+) n (%) | *P*−value |
|---|---|---|---|
| rs5902434 | | | |
| Genotype | | | |
| del/del + del/ATT | 121 (74.2) | 3 (75.0) | |
| ATT/ATT | 42 (25.8) | 1 (25.0) | 0.972 |
| rs3761549 | | | |
| Genotype | | | |
| C/C + C/T | 149 (89.8) | 3 (75.0) | |
| T/T | 17 (10.2) | 1 (25.0) | 0.343 |
| rs3761548 | | | |
| Genotype | | | |
| C/C + A/C | 146 (88.5) | 4 (100) | |
| A/A | 19 (11.5) | 0 (0) | 0.471 |
| rs2232365 | | | |
| Genotype | | | |
| A/A + A/G | 125 (75.8) | 3 (75.0) | |
| G/G | 40 (24.2) | 1 (25.0) | 0.972 |
| rs2280883 | | | |
| Genotype | | | |
| T/T + T/C | 144 (87.3) | 4 (100) | |
| C/C | 21 (12.7) | 0 (0) | 0.446 |

*Among a cohort of 172 total patients, two to five cases failed to provide genotypes because of unavailable DNA samples after quality control.

Abbreviations: FOXP3, Forkhead box P3; SNP, single nucleotide polymorphism; EBV, Epstein−Barr Virus; del, deletion.

**Table 9.** Association between genotype of *FOXP3* SNPs and fungal infection

| Polymorphism | Fungal infection (−) n (%) | Fungal infection (+) n (%) | *P*−value |
|---|---|---|---|
| rs5902434 | | | |
| Genotype | | | |
| del/del + del/ATT | 66 (75.0) | 58 (73.4) | |
| ATT/ATT | 22 (25.0) | 21 (26.6) | 0.815 |
| rs3761549 | | | |
| Genotype | | | |
| C/C + C/T | 77 (87.5) | 75 (91.5) | |
| T/T | 11 (12.5) | 7 (8.5) | 0.401 |
| rs3761548 | | | |
| Genotype | | | |
| C/C + A/C | 80 (90.9) | 70 (86.4) | |
| A/A | 8 (9.1) | 11 (13.6) | 0.356 |
| rs2232365 | | | |
| Genotype | | | |
| A/A + A/G | 66 (75.0) | 62 (76.5) | |
| G/G | 22 (25.0) | 19 (23.5) | 0.815 |
| rs2280883 | | | |
| Genotype | | | |
| T/T + T/C | 80 (89.9) | 68 (85.0) | |
| C/C | 9 (10.1) | 12 (15.0) | 0.336 |

*Among a cohort of 172 total patients, two to five cases failed to provide genotypes because of unavailable DNA samples after quality control.

Abbreviations: FOXP3, Forkhead box P3; SNP, single nucleotide polymorphism; del, deletion.

**Table 10.** Association between genotype of *FOXP3* SNPs and Tuberculosis infection

| Polymorphism | TB infection (−) n (%) | TB infection (+) n (%) | *P*−value |
|---|---|---|---|
| rs5902434 | | | |
| Genotype | | | |
| del/del + del/ATT | 120 (74.1) | 4 (80.0) | |
| ATT/ATT | 42 (25.9) | 1 (20.0) | 0.765 |
| rs3761549 | | | |
| Genotype | | | |
| C/C + C/T | 147 (89.1) | 5 (100) | |
| T/T | 18 (10.9) | 0 (0) | 0.435 |
| rs3761548 | | | |
| Genotype | | | |
| C/C + A/C | 146 (89.0) | 4 (80.0) | |
| A/A | 18 (11.0) | 1 (20.0) | 0.529 |
| rs2232365 | | | |
| Genotype | | | |
| A/A + A/G | 124 (75.6) | 4 (80.0) | |
| G/G | 40 (24.4) | 1 (20.0) | 0.822 |
| rs2280883 | | | |
| Genotype | | | |
| T/T + T/C | 144 (88.3) | 4 (66.7) | |
| C/C | 19 (11.7) | 2 (33.3) | 0.114 |

*Among a cohort of 172 total patients, two to five cases failed to provide genotypes because of unavailable DNA samples after quality control.

Abbreviations: FOXP3, Forkhead box P3; SNP, single nucleotide polymorphism; TB, tuberculosis; del, deletion.

## 3.4. Association of *FOXP3* SNPs and relapse and patient survival

Using univariate Cox regression and Kaplan–Meier analysis, we determined whether there was an association between five *FOXP3* SNPs and either disease relapse or patient survival. Based on the 50 patients in our cohort who relapsed, we found no evidence of an effect of *FOXP3* SNP genotype on the incidence of relapse (Table 11, Figure 3). Similarly, the genotypes at these five *FOXP3* SNPs did not influence their survival (Table 12, Figure 4).

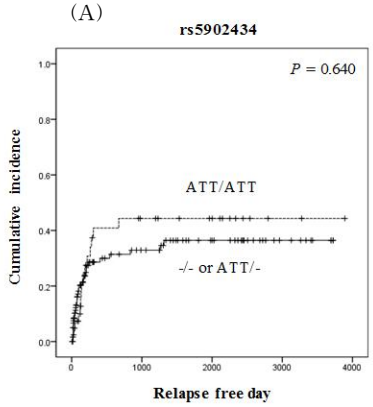
**Table 11.** Association between genotype of *FOXP3* SNPs and relapse

| Polymorphism | Relapse (−) n (%) | Relapse (+) n (%) | *P*−value |
|---|---|---|---|
| rs5902434 | | | |
| Genotype | | | |
| del/del + del/ATT | 90 (76.3) | 34 (69.4) | |
| ATT/ATT | 28 (23.7) | 15 (30.6) | 0.354 |
| rs3761549 | | | |
| Genotype | | | |
| C/C + C/T | 108 (89.3) | 44 (89.8) | |
| T/T | 13 (10.7) | 5 (10.2) | 0.917 |
| rs3761548 | | | |
| Genotype | | | |
| C/C + A/C | 107 (89.2) | 43 (87.8) | |
| A/A | 13 (10.8) | 6 (12.2) | 0.792 |
| rs2232365 | | | |
| Genotype | | | |
| A/A + A/G | 93 (76.9) | 35 (72.9) | |
| G/G | 28 (23.1) | 13 (27.1) | 0.590 |
| rs2280883 | | | |
| Genotype | | | |
| T/T + T/C | 105 (88.2) | 43 (86.0) | |
| C/C | 14 (11.8) | 7 (14.0) | 0.688 |

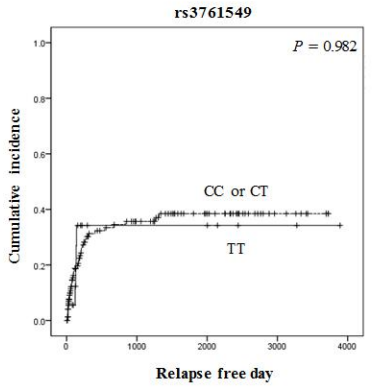
*Among a cohort of 172 patients, two to five cases failed to provide genotypes because of unavailable DNA samples after quality control.

Abbreviations: FOXP3, Forkhead box P3; SNP, single nucleotide polymorphism; del, deletion.

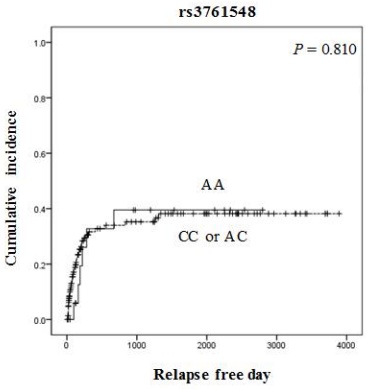
(A)


rs5902434
P = 0.640
ATT/ATT
-/- or ATT/-
Cumulative incidence
Relapse free day

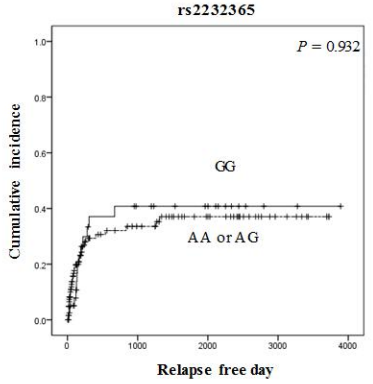
(B)


rs3761549
P = 0.982
CC or CT
TT
Cumulative incidence
Relapse free day

(C)


rs3761548
P = 0.810
AA
CC or AC
Cumulative incidence
Relapse free day

(D)


rs2232365
P = 0.932
GG
AA or AG
Cumulative incidence
Relapse free day

(E)

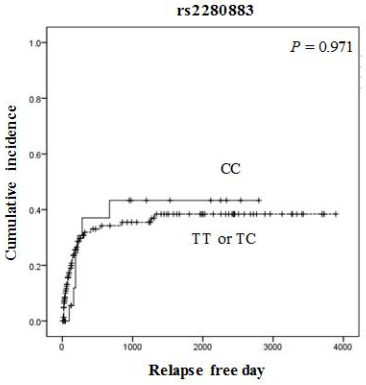

rs2280883
P = 0.971
CC
TT or TC
Cumulative incidence
Relapse free day

**Figure 3.** Impact of the *FOXP3* polymorphism on relapse after allogenic HSCT. (A) rs5902434, (B) rs3761549, (C) rs3761548, (D) rs2232365, and (E) rs2280883. Five *FOXP3* SNPs did not show statistically significant difference.

Abbreviations: FOXP3, Forkhead box P3; HSCT, hematopoietic stem cell transplantation; SNP, single nucleotide polymorphism.

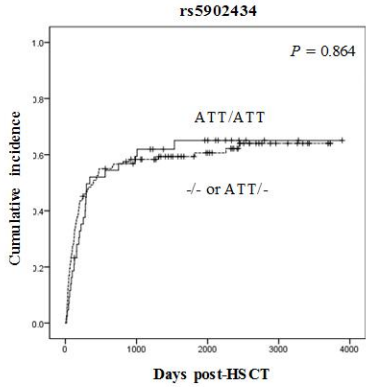
**Table 12.** Association between genotype of *FOXP3* SNPs and survival

| Polymorphism | Survival n (%) | Death n (%) | *P*-value |
|---|---|---|---|
| rs5902434 | | | |
| Genotype | | | |
| del/del + del/ATT | 48 (75.0) | 76 (73.8) | |
| ATT/ATT | 16 (25.0) | 27 (26.2) | 0.862 |
| rs3761549 | | | |
| Genotype | | | |
| C/C + C/T | 59 (92.2) | 93 (87.7) | |
| T/T | 5 (7.8) | 13 (12.3) | 0.361 |
| rs3761548 | | | |
| Genotype | | | |
| C/C + A/C | 56 (87.5) | 94 (89.5) | |
| A/A | 8 (12.5) | 11 (10.5) | 0.686 |
| rs2232365 | | | |
| Genotype | | | |
| A/A + A/G | 49 (76.6) | 79 (75.2) | |
| G/G | 15 (23.4) | 26 (24.8) | 0.846 |
| rs2280883 | | | |
| Genotype | | | |
| T/T + T/C | 56 (87.5) | 92 (87.6) | |
| C/C | 8 (12.5) | 13 (12.4) | 0.982 |

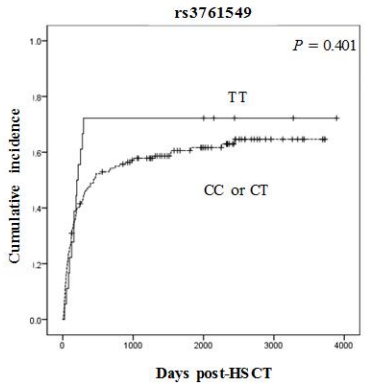
*Among a cohort of 172 patients, two to five cases failed to provide genotypes because of unavailable DNA samples after quality control.

Abbreviations: FOXP3, Forkhead box P3; SNP, single nucleotide polymorphism; del, deletion.

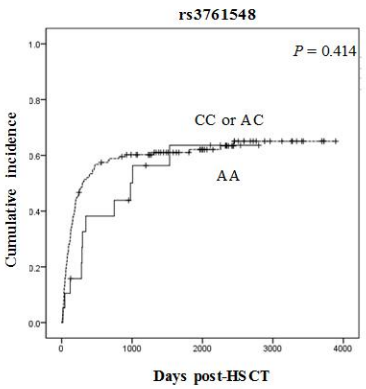
(A)


rs5902434
P = 0.864
ATT/ATT
-/- or ATT/-
Cumulative incidence
Days post-HSCT

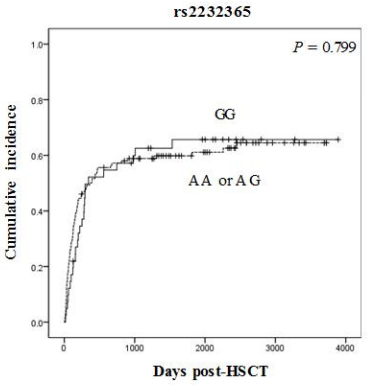
(B)


rs3761549
P = 0.401
TT
CC or CT
Cumulative incidence
Days post-HSCT

(C)


rs3761548
P = 0.414
CC or AC
AA
Cumulative incidence
Days post-HSCT

(D)


rs2232365
P = 0.799
GG
AA or AG
Cumulative incidence
Days post-HSCT

(E)

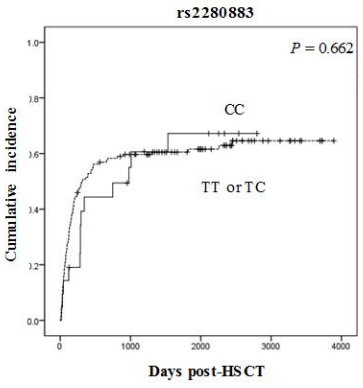

rs2280883
P = 0.662
CC
TT or TC
Cumulative incidence
Days post-HSCT

**Figure 4.** Impact of the *FOXP3* polymorphism on overall survival after allogenic HSCT. (A) rs5902434, (B) rs3761549, (C) rs3761548, (D) rs2232365, and (E) rs2280883. Statistically significant difference was not observed in five *FOXP3* SNPs.

Abbreviations: FOXP3, Forkhead box P3; HSCT, hematopoietic stem cell transplantation; SNP, single nucleotide polymorphism.

## 3.5. Luciferase gene reporter activity at SNP rs3761549

To verify the influence of SNP rs3761549 on the transcription levels of *FOXP3,* HEK 293 T cells were transfected with a promoter construct containing T/T or C/C genotype of the *FOXP3* gene. The relative luciferase activity was lower for the T/T genotype compared with the C/C genotype, with a difference of approximately 0.65 fold (P = 0.001) (Figure 5).

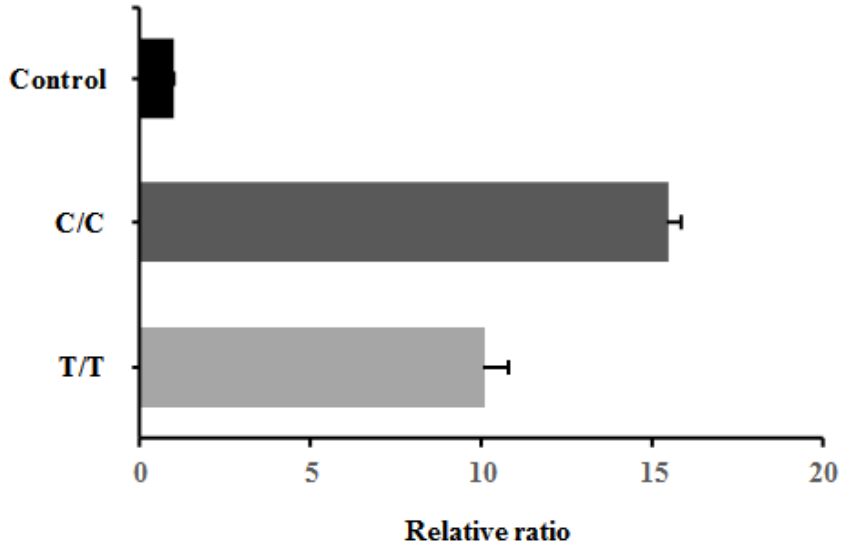



**Figure 5.** T/T genotype at rs3761549 site reduced reporter activity. Results were the mean ± SD of luciferase light units normalized for *Renilla* luciferase of the same sample (n = 3). T/T genotype at 3761549 site dramatically reduced by 0.65 fold activity. All experiments were repeated three times.

Abbreviations: SD, standard deviation.

# 4. DISCUSSION

The present study was performed to evaluate the clinical impacts of five different *FOXP3* SNPs on the outcomes of allogenic HSCT. Our results reveal that the rs3761549 T/T genotype, the rs5902434 ATT/ATT genotype, and the rs2232365 G/G genotype were associated with higher incidence of acute GVHD and lower risk of post-HSCT infection.

Regarding the impact of *FOXP3* SNP on clinical outcomes of allogenic HSCT, only one study analyzed the rs3761548 SNP. This study reported that patients with the rs3761548 C/C genotype showed higher incidence of hepatic veno-occlusive disease and CMV infections, but there was no association with GVHD or relapse [29]. For other cases, *FOXP3* the rs3761548 A/C genotype and A/C + A/A genotypes were not only associated with an increased risk for psoriasis [17,37], but the A/A genotype was also associated with greater risk for rejection in renal transplant, compared with the C/C genotype [25,26]. In our study, no significant association between the rs3761548 SNP and clinical outcomes in allogenic HSCT were observed, a result consistent with a previous report [29].

Previous studies have reported that the rs3761549 T/T genotype was correlated with the development and progression of endometriosis [38], endometriosis-related infertility [39] and Graves'

disease [21], suggesting that this genotype might be involved in the regulation of inflammatory or autoimmune responses. However, no study has characterized the potential association between rs3761549 and clinical outcomes in organ transplantation or HSCT.

Wu *et al.* [40] reported an association between the rs5902434 del genotype and an increased risk of unexplained recurrent spontaneous abortion. However, there was no significant association with renal allograft outcome [27] or psoriasis susceptibility [37]. Wu *et al.* [40] also reported an association between the rs2232365 G/G genotype and an increased risk of unexplained recurrent spontaneous abortion. Misra *et al.* [26] reported that the rs2232365 G/G genotype in renal allograft recipients had lower overall survival and 5-year survival. However, Qui *et al.* [25] reported no association between rs2232365 and renal allograft rejection.

Because of these conflicting findings, the impact of rs3761548, rs3761549, rs5902434, and rs2232365 at the *FOXP3* locus on regulatory immune responses, which are related to the development of acute GVHD after allogenic HSCT, remains unclear. These four SNPs are all located in the *FOXP3* promoter region, which contains DNA binding sites for transcription factors (TFs). It can be hypothesized that depending on the alleles of these SNPs in *FOXP3*, FOXP3 expression are modulated by altering the binding affinity of transcription factors to their binding element and by modifying the kinetics of transcription regulation. A variant(s) is likely to contribute to a decrease in the quantity or quality of

FOXP3, resulting in a sequential decrease of Tregs, which play a critical role in suppressing auto-reactive lymphocytes and regulating hyperactive immune responses.

Furthermore, the pathophysiological link between GVHD and Tregs remains poorly understood. Tregs can exert suppressive function in a contact-dependent or contact-independent manner by directly targeting the function of effector T cells and APCs. The suppression mechanisms deployed by Tregs can be grouped into four types, suppression by: i) inhibitory cytokines, ii) killing of target cells, iii) metabolic disruption, and iv) modulation of APCs maturation or function [41]. In our model, donor Tregs which are characterized by high level expression of the high affinity IL-2R, may compete with other effector T cells for local IL-2 consumption in a contact-independent manner. Consequently, active effector T cells can be deprived of IL-2 and undergo apoptosis [42]. Another potential mechanism of Tregs is delivered via the surface expression of lymphocytes activation gene 3 (LAG-3), preventing the maturation and the ability of APCs to activate effector T cells [43]. Therefore, Tregs may play a critical role in the suppression of auto-reactive lymphocytes and regulation of a hyperactive immune response.

To confirm a relationship between SNP genotypes and FOXP3 expression, *in vitro* functional evaluation of *FOXP3* rs3761549 that is located in promoter region was done using a luciferase gene reporter assay, which compared transcriptional activity between the

T/T and C/C genotypes. HSCT patients with rs3761549 T/T genotype showed lower FOXP3 transcription level compared with the C/C genotype. These results strengthened the relationship among *FOXP3* rs3761549, FOXP3 transcription level, and acute GVHD susceptibility. To better characterize differences in the binding of specific transcription factors to variants, several computer tools can be utilized [44]. In this study, we identified specific interaction of TF-binding site using a P-match program (http://gene-regulation.com/cgi-bin/pub/programs/pmatch/bin/p-match.cgi) [45]. Interestingly, this TF search analysis revealed that the C/C genotype of *FOXP3* rs3761549 is situated in a DNA binding site for activating enhancer binding protein 4 (AP4) and that AP4 is not able to bind to the rs3761549 T/T genotype. AP4 is a TF regulated by IL-2R signaling involved in a Myc-dependent gene expression that sustains the rapid clonal expansion of antigen-specific CD8+ T cells by encoding components of glycolysis pathways [46]. Therefore, although our understanding of the molecular mechanism of AP4 and *FOXP3* in Tregs is limited, the modification of AP4 binding affinity in rs3761549 T/T genotype might induce less clonal expansion of Tregs and the relative proliferation of effector T lymphocytes. It might cause the destruction of tissues and organs, therefore leading to an increase in the incidence and/or severity of acute GVHD [47].

In our study, associations of the rs3761549 T/T genotype, the 5902434 ATT/ATT genotype, and the rs2232365 G/G genotype

with decreased risk of post-HSCT infection were observed. Tregs cells have been reported to relate with the immune responses for pathogens and the outcomes of some infectious diseases. In detail, Tregs cells inhibited the proliferation of effector cells (eg, CD4+ helper T cells, CD8+ cytotoxic T cells), the production of cytokines (eg, interferon-γ), and directly secreted immunosuppressive cytokines (eg, IL-10 and TGF-β) [48,49]. Researchers have also demonstrated that a decrease in the number of Tregs increases resistance to viral infections [50]. Therefore, in our study, the association of *FOXP3* SNPs with a lower infection rate after HSCT may reflect down-regulation of Tregs, which inhibit helper T cell, cytotoxic T cells, and cytokine production, and may contribute in preventing pathogen proliferation by effector T cells and cytokine release.

Several studies have focused on the association of *FOXP3* SNPs and CMV infection after organ or HSCT. Piao *et al.* [29] reported an association between rs3761548 and CMV infection after allogenic HSCT. SNPs of other immune-regulating genes, such as *IL28B, TLR9*, *DC-SIGN* (also known as CD209), and *IFNL3/4* (interferon lambda 3/interferon lambda 4), have been associated with CMV infection in solid organ transplantation [51,52]. In our study, although specific *FOXP3* SNPs were related with post-HSCT infection, the associations of *FOXP3* SNPs with post-HSCT infection in subgroup analysis including CMV infection did not show statistical significance, possibly due to the relatively small

number of cases. Therefore, additional large, long-term studies are needed to confirm our hypothesis.

Nevertheless, our findings provide the first evidence that allogenic HSCT patients harboring the rs3761549 T/T genotype, the rs5902434 ATT/ATT genotype, and the rs2232365 G/G genotype have an increased risk of acute GVHD and a decreased risk of post-HSCT infection. These SNPs may be used as potential markers to predict clinical outcomes of allogenic HSCT and provide personalized care for high-risk patients. Furthermore, these findings may also contribute toward the development of new treatment modalities targeting immune reconstitution or enhancing Tregs population in allogenic HSCT.

# REFERENCES


1. Kambham N, Higgins JP, Sundram U, Troxell ML. Hematopoietic stem cell transplantation: graft versus host disease and pathology of gastrointestinal tract, liver, and lung. Adv Anat Pathol 2014;21(5):301-20.
2. Krenger W, Blazar BR, Hollander GA. Thymic T-cell development in allogeneic stem cell transplantation. Blood 2011;117(25):6768-76.
3. Arnaout K, Patel N, Jain M, El-Amm J, Amro F, Tabbara IA, et al. Complications of allogenic hematopoietic stem cell transplantation. Cancer Invest 2014;32(7):349-62.
4. Beres AJ and Drobyski WR. The role of regulatory T cells in the biology of graft versus host disease. Front Immunol 2013;4:163.
5. Min CK. The pathophysiology of chronic graft-versus-host disease: the unveiling of an enigma. Korean J Hematol 2011;46:80-7.
6. Choi SW, Reddy P. Current and emerging strategies for the prevention of graft-versus-host disease. Nat Rev Clin Oncol 2014;11(9):536-47.
7. Jan WG, Joost WJ, Van E, Cor HJ, Claire T, Bob LW, et al. Tetramer-based quantification of cytomegalovirus (CMV)-specific CD81 T lymphocytes in T-cell-depleted stem cell

grafts and after transplantation may identify patients at risk for progressive CMV infection. Blood 2001;98(5):1358-64.

8. Joost WJ, Van E, Bronno H, Ellen M, Hubert GMN, Rudolf T, et al. Epstein-Barr virus (EBV) reactivation is a frequent event after allogenic stem cell transplantation (SCT) and quantitatively predicts EBV-lymphoproliferative disease following T-cell-depleted SCT. Blood 2001;98(4):972-8.
9. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. Cell 2008;133(5):775-87.
10. Von Herrath MG and Harrison LC. Antigen-induced regulatory T cells in autoimmunity. Nat Rev Immunol 2003;3(3):223-32.
11. Wood KJ, Sakaguchi S. Regulatory T cells in transplantation tolerance. Nat Rev Immunol 2003;3(3):199-210.
12. Le NT and Chao N. Regulating regulatory T cells. Bone Marrow Transplant 2007;39(1):1-9.
13. Elbadry MI, Noreldin AK, Hassanein HA. After moving of regulatory T-cell therapy to the clinic: Will we need a new Tregs source? Hematol Transfus Int J 2017;5(2):00117.
14. Singer BD, King LS, D'Alessio FR. Regulatory T cells as immunotherapy. Front Immunol 2014;5(46):1-10.
15. Curiel TJ. Regulatory T-cell development: is FOXP3 the decider? Nat Med 2007;13(3):250-3.
16. Bennett CL CJ, Ramsdell F, Brunkow ME, Ferguson PJ, Whitesell L, Kelly TE, et al. The immune dysregulation,

polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. Nat Genet 2001;27(1):20-1.
17. Oda JM, Hiraata BK, Guembarovski RL, Watanabe MA. Genetic polymorphism in FOXP3 gene: imbalance in regulatory T-cell role and development of human diseases. J Genet 2013; 92(1):163-71.
18. Marques CR, Costa RS, Costa GNO, da Silva TM, Teixeira TO, de Andrade EMM, et al. Genetic and epigenetic studies of FOXP3 in asthma and allergy. Asthma Res Pract 2015;1:10.
19. Chen X, Gan T, Liao Z, Chen S, Xiao J. FOXP3 (-/ATT) polymorphism contributes to the susceptibility of preeclampsia. PLoS One 2013;8(4):e59696.
20. Lin YC, Lee JH, Wu AS, Tsai CY, Yu HH, Wang LC, et al. Association of single-nucleotide polymorphisms in FOXP3 gene with systemic lupus erythematosus susceptibility: a case-control study. Lupus 2011;20(2):137-43.
21. Bossowski A, Borysewicz-Sanczyk H, Wawrusiewicz-Kurylonek N, Zasim A, Szalecki M, Wikiera B, et al. Analysis of chosen polymorphisms in FOXP3 gene in children and adolescents with autoimmune thyroid diseases. Autoimmunity 2014;47(6):395-400.
22. Fazelzadeh Haghighi M, Ali Ghayumi M, Behzadnia F, Erfani N. Investigation of FOXP3 genetic variations at positions -2383 C/T and IVS9+459 T/C in southern Iranian patients with lung carcinoma. Iran J Basic Med Sci 2015;18(5):465-71.

23. Jiang LL and Ruan LW. Association between FOXP3 promoter polymorphisms and cancer risk: A meta-analysis. Oncol Lett 2014;8(6):2795-9.
24. Mojtahedi Z, Erfani N, Haghshenas MR, Hosseini SV, Ghaderi A. Association of FOXP3/Scurfin germline polymorphism (C-2383T/rs3761549) with colorectal cancer. Ann Colorectal Res 2013;1(1):12-6.
25. Qiu XY, Jiao Z, Zhang M, Chen JP, Shi XJ, Zhong MK, et al. Genetic association of FOXP3 gene polymorphisms with allograft rejection in renal transplant patients. Nephrology 2012;17(4):423-30.
26. Misra MK, Mishra A, Pandey SK, Kapoor R, Sharma RK, Agrawal S, et al. Association of functional genetic variants of transcription factor Forkhead Box P3 and Nuclear Factor-kappaB with end-stage renal disease and renal allograft outcome. Gene 2016;581(1):57-65.
27. Engela AU, Boer K, Roodnat JI, Peeters AM, Eilers PH, Kal-van Gestel JA, et al. Genetic variants of FOXP3 influence graft survival in kidney transplant patients. Hum Immunol 2013;74(6):751-7.
28. Song EY, Park MH, Kang SJ, Park HJ, Kim BC, Tokunaga K, et al. HLA class II allele and haplotype frequencies in Koreans based on 107 families. Tissue Antigens 2002;59(6):475-86.
29. Piao Z, Kim HJ, Choi JY, Hong CR, Lee JW, Kang HJ, et al. Effect of FOXP3 polymorphism on the clinical outcomes after

allogeneic hematopoietic stem cell transplantation in pediatric acute leukemia patients. Int Immunopharmacol 2016;31:132-9.
30. Gratwohl A. The EBMT risk score. Bone Marrow Transplant 2012;47(6):749-56.
31. Przepiorka D, Weisdorf D, Martin P, Klingemann HG, Beatty P, Hows J, et al. 1994 Consensus conference on acute GVHD grading. Bone Marrow Transplant 1995;15(6):825-8.
32. Filipovich AH, Weisdorf D, Pavletic S, Socie G, Wingard JR, Lee SJ, et al. National Institutes of Health consensus development project on criteria for clinical trials in chronic graft-versus-host disease: I. Diagnosis and staging working group report. Biol Blood Marrow Transplant 2005;11(12):945-56.
33. Ward LD, Kellis M. HaploReg v4: systematic mining of putative causal variants, cell types, regulators and target genes for human complex traits and disease. Nucleic Acids Res 2016;44:D877-81.
34. Dayem Ullah AZ, Lemoine NR, CChelala C. A practical guide for the functional annotation of genetic variations using SNPnexus. Brief Bioinform 2013;14(4):437-47.
35. Nishizaki SS, Boyle AP. Mining the unknown: Assigning function to noncoding single nucleotide polymorphisms. Trends Genet 2017;33(1):34-45.

36. Mantel PY, Ouaked N, Ruckert B, Karagiannidis C, Welz R, Blaser K, et al. Molecular mechanisms underlying FOXP3 induction in human T cells. J Immunol 2006;176(6):3593-602.

37. Gao L, Li K, Li F, Li H, Liu L, Wang L, et al. Polymorphisms in the FOXP3 gene in Han Chinese psoriasis patients. J Dermatol Sci 2010;57(1):51-6.

38 Barbosa CP, Teles JS, Lerner TG, Peluso C, Mafra FA, Vilarino FL, et al. Genetic association study of polymorphisms FOXP3 and FCRL3 in women with endometriosis. Fertil Steril 2012;97(5):1124-8.

39. Andre GM, Barbosa CP, Teles JS, Vilarino FL, Christofolini DM, Bianco B, et al. Analysis of FOXP3 polymorphisms in infertile women with and without endometriosis. Fertil Steril 2011;95(7):2223-7.

40. Wu Z, You Z, Zhang C, Li Z, Su X, Zhang X, et al. Association between functional polymorphisms of FOXP3 gene and the occurrence of unexplained recurrent spontaneous abortion in a Chinese Han population. Clin Dev Immunol 2012;2012:896458.

41. Vignali DA, Collison LW, Workman CJ. How regulatory T cells work. Nat Rev Immunol. 2008;8(7):523-32

42. Arce-Sillas A, Alvarez-Luquin DD, Tamaya-Dominguez B, Gomez-Fuentes S, Trejo-Garcia A, Melo-Salas M, et al.

Regulatory T cells: Molecular actions on effector cells in immune regulation. J Immunol Res 2016;2016:1720827

43. Beres AJ, Drobyski WR. The role of regulatory T cells in the biology of graft versus host disease. Front Immunol 2013;24(4):163

44. Hitomi Y, Tokunaga K. Significance of functional disease-causal/susceptible variants identified by whole-genome analyses for the understanding of human diseases. Proc Jpn Acad Ser B Phys Biol Sci 2017;93(9):657-76.

45. Chekmenev DS, Haid C, Kel AE. P-Match: transcription factor binding site search by combining pattern and weight matrices. Nucleic Acids Res 2005;1(33):432-7.

46. Chou C, Pinto AK, Curtis JD, Persaud SP, Cella M, Lin CC, et al. c-Myc-induced transcription factor AP4 is required for host protection mediated by CD8+ T cells. Nat Immunol 2014;15(9):884-93.

47. Zorn E, Kim HT, Lee SJ, Floyd BH, Litsa D, Arumugarajah S, et al. Reduced frequency of FOXP3+CD4+CD25+ regulatory T cells in patients with chronic graft-versus-host disease. Blood 2005;106(8):2903-11.

48. Mills KH. Regulatory T cells: friend or foe in immunity to infection? Nat Rev Immunol 2004;4(11):841-55.

49. Belkaid Y and Rouse BT. Natural regulatory T cells in infectious disease. Nat Immunol 2005;6(4):353-60.

50. Aandahl EM, Michaelsson J, Moretto WJ, Hecht FM, Nixon DF. Human CD4+CD25+ regulatory T cells control T-cell responses to human immunodeficiency virus and cytomegalovirus antigens. J Virol 2004;78(5):2454-9.
51. Fernandez-Ruiz M, Corrales I, Arias M, Campistol JM, Gimenez E, Crespo J, et al. Association between individual and combined SNPs in genes related to innate immunity and incidence of CMV infection in seropositive kidney transplant recipients. Am J Transplant 2015;15(5):1323-35.
52. Manuel O, Wojtowicz A, Bibert S, Mueller NJ, van Delden C, Hirsch HH, et al. Influence of IFNL3/4 polymorphisms on the incidence of cytomegalovirus infection after solid-organ transplantation. J Infect Dis 2015;211(6):906-14.

# 국문 초록

**서론**: Forkhead box P3 (FOXP3)는 조절 T 세포의 중요한 표지자이다. *FOXP3* 단일염기다형성 (SNP)은 자가면역질환, 암 및 동종장기이식의 성적과 관련이 있다. 본 연구에서는 *FOXP3* 단일염기다형성 (SNP)과 동종조혈모세포이식 (HSCT) 성적과의 연관성에 대해 분석하고자 한다.

**방법**: 2006 년 4 월부터 2014 년 8 월까지 서울대학교병원에서 동종조혈모세포이식을 받은 172 명의 DNA 샘플을 대상으로 *FOXP3* SNP (rs5902434, rs3761549, rs3761548, rs2232365 및 rs2280883)를 PCR-sequencing 방법으로 분석 하였다. 수혜자의 각 SNP 와 이식편대숙주병 (GVHD), 이식후감염증, 재발 및 환자의 생존률 등을 분석하였다.

**결과**: 급성이식편대숙주병 (GVHD) 환자 (grade II-IV)는 급성이식편대숙주병이 없는 환자들보다 rs3761549 T/T, rs5902434의 ATT/ATT 및 rs2232365 G/G 유전자형의 비율이 더 높았다 ($P$ = 0.017, OR = 5.3, $P$ = 0.031, OR = 2.4, $P$ = 0.023, OR = 2.6). 다변량 분석에 따르면 rs3761549의 T/T 유전자형은 급성이식편대숙주병 발병의 독립적 위험 인자로 작용한다 ($P$ = 0.032, HR = 5.6). 그와 반대로, 이식후감염증 환자들은 감염증이 없는 환자들보다

rs3761549 T/T, rs5902434의 ATT/ATT 및 rs2232365 G/G 유전자형의 비율이 더 낮았다 ($P$ = 0.026, $P$ = 0.046, $P$ = 0.031).

**결론**: 본 연구에서 rs3761549 T/T, rs5902434 ATT/ATT 및 rs2232365 G/G 유전자형은 높은 급성이식편대숙주병 발병과 낮은 이식후감염증과 상관관계가 있었다.

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**주요어: 동종조혈모세포이식, *FOXP3*, 이식편대숙주병, 감염, 단열염기다형성**

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